Characterization of (per)chlorate-reducing bacteria

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Characterization of (per)chlorate-reducing bacteria

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Chapter 1

Introduction

Bacteria that are dependent on respiration for their energy supply may use several types of electron acceptors, like oxygen, nitrate, sulfate manganese (IV), iron (III). About one decade ago, two new compounds could be added to this list, viz. perchlorate and chlorate. Although the compounds themselves were not novel, their use as terminal electron acceptor for growth had not been reported before. Ever since, the process of (per)chlorate reduction received a lot of attention, resulting in the isolation of various (per)chlorate-reducing bacteria and the characterization of the key enzymes. Compared to the other respiratory reactions, (per)chlorate reduction is unique because it involves the formation of oxygen. This aspect made (per)chlorate reduction of special interest, because the process could be used to provide oxygen at anoxic sites for certain bioremediation strategies. For example, benzene-oxidizing bacteria can use the oxygen to degrade the aromatic ring by means of oxygenases.

In this chapter background information is given about the compounds perchlorate and chlorate (characteristics, pollution problems), the bacteria and enzymes involved in (per)chlorate-reduction and the potential of (per)chlorate-reducing bacteria in bioremediation strategies.

1.1 Characteristics of chlorate and perchlorate

1.1.1 Production of chlorate and perchlorate

The oxyanions chlorate and perchlorate can be found in the environment at sites where these compounds are produced and used by man. Because the only known natural source of (per)chlorate is from mineral deposits in Chile, the presence of these oxyanions in other places must be a result of anthropogenic contamination (Achenbach *et al*., 2001). In Table 1, some other chlorine compounds are given including their molecular structure formula.

Table 1 Chlorine compounds

ClO_4^-	:	perchlorate	Cl^-	:	chloride
ClO_3^-	:	chlorate	Cl_2	:	chlorine
ClO_2^-	:	chlorite	ClO_2	:	chlorine dioxide
ClO^-	:	hypochlorite	HClO	:	hypochlorous acid

The sodium salt, $NaClO_3$, is made in an electrolytic cell. During the electrolysis of a solution of sodium chloride (NaCl), a number of simultaneous reactions take place. At the

anode chloride ions are discharged to form chlorine (reaction 1). At the cathode, hydroxide ions (OH^-) are formed and hydrogen (H_2) is liberated (reaction 2).

$$2Cl^- \rightarrow Cl_2 + 2e^- \qquad \text{reaction 1}$$

$$2H_2O + 2e^- \rightarrow H_2 + 2\ OH^- \qquad \text{reaction 2}$$

The chlorine can react in two ways; with water to form hypochlorous (HClO) and hydrochloric acid (HCl) (reaction 3) or with a hydroxide ion to form hypochlorite and chloride ions (reaction 4).

$$Cl_2 + H_2O \rightarrow HClO + HCl \qquad \text{reaction 3}$$

$$Cl_2 + 2OH^- \rightarrow ClO^- + Cl^- + H_2O \qquad \text{reaction 4}$$

Sodium chlorate is then formed chemically by the oxidation of the alkali hypochlorite by the hypochlorous acid (reaction 5).

$$NaOCl + 2HOCl \rightarrow NaClO_3 + 2\ HCl \qquad \text{reaction 5}$$

The overall cell reaction for the electrolysis of a salt solution to form sodium chlorate can be expressed by the following equation (reaction 6);

$$NaCl + 3H_2O + \text{electricity (6 Faradays)} \rightarrow NaClO_3 + 3\ H_2 \qquad \text{reaction 6}$$

(Mellor, 1946; http://people.delphi.com). Chlorates can also be formed as a result of ozonation of drinking waters that are treated with chlorine for disinfection (Siddiqui, 1996). Perchlorates are prepared by thermal decomposition and by electrolysis of chlorates (Mellor, 1946).

1.1.2 Application of chlorate and perchlorate

Chlorate is applied in agriculture as weed controller (herbicide) and as defoliant (Logan, 1998). Chlorate is also manufactured for the production of chlorine dioxide which is used as bleaching agent in the pulp and paper industry. After use this chlorine dioxide is disposed off as chlorate. When hypochlorite (ClO^-) is utilized as disinfectant, hypochlorite decomposes to form chloride and chlorate (van Ginkel *et al*., 1995). Chlorate is also a component in matches. Recently, it has been reported that sodium chlorate, fed in low doses to cows and pigs prior to slaughter, selectively kills *Salmonella typhimurium* and *Escherichia coli* (USDA, 2001). Annually, 1.4 million cases of salmonellosis and 73,000 cases of diarrheal illness are caused by these two pathogens. Perchlorate has been manufactured for use as ammonium perchlorate, which is an energetic compound utilized as an oxidizer in solid rocket propellant. Perchlorate is also a component in fire-works and in explosives.

1.1.3 Toxicity of chlorate and perchlorate

In plants and microorganisms, chlorate may compete with nitrate in the nitrate reductase system (van Wijk and Hutchinson, 1995). When fed to rats and mice in their drinking water, the effect of chlorate and chlorite causes oxidative damage to red blood cells, resulting in hemolytic anemia and methemoglobin formation (Condie, 1986; Gonce and Voudrias, 1994; Siddiqui, 1996; USEPA, 1999). Toxicological studies have demonstrated that perchlorate has a direct effect on iodide uptake by the thyroid gland and can result in fatal bone marrow disease (Stanbury and Wyngaarden, 1952; Achenbach *et al*., 2001). Therefore, the U.S. Environmental Protection Agency has put (per)chlorate on the drinking water candidate contaminant list (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid-23292). To assure well-being of men, recent epidemiological studies recommended a maximum perchlorate contaminant level, that could result in a drinking water standard of 0.01 μM (Anonymous, 2002).

1.1.4 Removal of chlorate and perchlorate from polluted sites

By the use of (per)chlorate, these compounds often end up in the environment. Surface water concentrations of chlorate in the Netherlands have been reported to contain 0.38 mM in the river Meuse and 0.19 mM in the Rhine and IJssel rivers (Rosemarin *et al*., 1994; Versteegh *et al*., 1993). Chlorate and perchlorate have been detected as groundwater contaminants in the United States (USEPA, 1999). Concentrations of perchlorate in groundwater in Nevada have reached $3.7 * 10^6$ μg/l, and as much as 165 μg/l has been found in water samples from Lake Mead (Gullick *et al*., 2001). A new ion chromatography procedure lowered the detection level to 0.04 μM which has led to the detection of perchlorate in a large number of ground water and surface water supplies (Jackson *et al*., 2000; Logan *et al.*, 2001).

Chlorate and perchlorate are highly soluble and chemically stable under environmental conditions (Urbansky, 1998). Removal of (per)chlorate from waste water or polluted sites is possible using different techniques. Chlorate removal from bleached effluents is possible using sulfur dioxide, but this results in an incomplete removal (Malmqvist and Welander, 1992). For removing perchlorate from drinking water, conventional water treatment technologies, such as ion exchange, carbon adsorption, have not been proven to be cost-efficient (Logan, 1998). Besides, these techniques produce a concentrated (per)chlorate waste that must be treated. For treatment of (per)chlorate contaminated sites and water sources, biotreatment may be recommended. Recently, removal of (per)chlorate by means of microbial

reduction was identified as the most feasible method of remediation of contaminated environments (Urbansky, 1998). The high oxidation states of perchlorate and chlorate makes them ideal electron acceptors for microbial metabolism. When this reduction is coupled to electron-transport phosphorylation, the microbes are able to respire on perchlorate and chlorate. The following pathway was proposed for the biological reduction of (per)chlorate into chlorite and the conversion of chlorite into chloride and oxygen (Rikken *et al*., 1996) :

$$ClO_4^- \xrightarrow{1} ClO_3^- \xrightarrow{2} ClO_2^- \xrightarrow{3} Cl^- + O_2 \qquad \text{reaction 7}$$

In reaction 1, perchlorate is reduced to chlorate and this compound is reduced further to chlorite in reaction 2. In strain GR-1 one enzyme, the (per)chlorate reductase, showed both activities (Kengen *et al*., 1999). These reactions are assumed to be part of the electron transport chain. Rikken *et al*. (1996) proposed that chlorite is disproportionated into chloride and oxygen by a chlorite dismutase (reaction 3).

1.2 Historical overview of research in microbial (per)chlorate reduction

Already in 1928, Aslander proved that chlorate was a suitable electron acceptor for microbial metabolism (Aslander, 1928). Species of *Penicillium*, *Aspergillus*, and *Fusarium* reduced chlorate when growing on the surface of hay. Research of Bryan and Rohlich (1954) and Bryan (1966) showed that microorganisms easily reduce chlorate and that during this reduction chloride production occurred proportional to the amount of organic matter degraded. Until 1996, very little was known about the biochemical pathway of (per)chlorate reduction. Many denitrifying bacteria showed to be able of chlorate reduction, but in general, this reduction was not coupled to growth (De Groot and Stouthamer, 1969; Oltmann *et al*., 1976[a]). Denitrifying bacteria likely cannot grow on chlorate because of the accumulation of toxic chlorite after reduction of chlorate by the nitrate reductase which prevents growth.

The chlorate reductase activity was ascribed to the action of the nitrate reductase. However, besides a nitrate reductase A, a chlorate reductase C was purified from the denitrifying strain *Proteus mirabilis*, which did not show nitrate reductase activity (Oltmann *et al*., 1976[a,b]). However, for this strain dissimilatory chlorate reduction was not reported. In Table 2, an overview is given of bacteria which were isolated and showed to be able of (per)chlorate respiration.

Table 2 Dissimilatory (per)chlorate-reducing bacteria

Strain	electron acceptor	Reference
Vibrio dechloraticans Cuznesove B-1168	ClO_3^-, ClO_4^-, NO_3^-	Korenkov *et al.* (1976)
Acinetobacter thermotoleranticus	ClO_3^-, SO_4^{2-}	Stepanyuk *et al.* (1993)
Ideonella dechloratans	ClO_3^-, O_2, NO_3^-	Malmqvist *et al.* (1994)
AB-1	ClO_3^-, NO_3^-	Bliven *et al.* (1996)
GR-1	ClO_3^-, ClO_4^-, O_2, NO_3^-, Mn (IV)	Rikken *et al.* (1996)
Wolinella succinogenes HAP-1	ClO_3^-, ClO_4^-, NO_3^-	Wallace *et al.* (1996)
Dechloromonas agitata	ClO_3^-, ClO_4^-, O_2	Achenbach *et al.* (2001)
Dechlorosoma suillum	ClO_3^-, ClO_4^-, O_2, NO_3^-	Achenbach *et al.* (2001)
Dechloromonas sp. strain HZ	ClO_3^-, ClO_4^-, O_2, NO_3^-	Zhang *et al.* (2002)

Vibrio dechloraticans Cuznesove B-1168 is a non-spore-forming, motile Gram-negative bacterium, that can grow with acetate or ethanol as electron donor and chlorate as electron acceptor (Korenkov *et al.*,1976). *Acinetobacter thermotoleranticus* can grow at elevated temperatures (up to 47ºC). This Gram-negative bacterium can assimilate xylose, ethanol and n-alkanes (C_9-C_{18}) (Stepanyuk *et al.,* 1993). *Ideonella dechloratans* was isolated from a laboratory wastewater treatment reactor. It is a Gram-negative, motile bacterium (Malmqvist *et al.,* 1994). In 1996, *Wolinella succinogenes* HAP-1 was isolated from an anaerobic sewage enrichment culture (Wallace *et al*., 1996). In the same year, strain GR-1 was isolated and studies with this strain revealed insight into the biochemical reduction pathway of (per)chlorate reduction. Strain GR-1 is able to couple the reduction of nitrate, chlorate, perchlorate and oxygen to growth. Purification of the enzymes involved in (per)chlorate reduction demonstrated that one enzyme was responsible for both chlorate and perchlorate reduction (Kengen *et al*., 1999). Substrate specificity tests indicated that the enzyme also exhibits bromate, iodate and nitrate reduction activity. It is not known if a distinct nitrate reductase is present in strain GR-1 when nitrate is the sole electron acceptor in batch cultures. Not all (per)chlorate-reducing bacteria can use nitrate as electron acceptor (Table 2). This supports the hypothesis that nitrate and chlorate reduction are separate pathways (Malmqvist *et al*., 1994; Rikken *et al*., 1996; Bruce *et al*., 1999).

In addition to the (per)chlorate reductase, the chlorite dismutase was also purified from strain GR-1. This enzyme catalyses the dismutation of chlorite yielding chloride and molecular oxygen (van Ginkel *et al.*, 1996). This means that oxygen production during (per)chlorate reduction and subsequently chlorite dismutation is, besides photosynthesis and the detoxification of H_2O_2 by catalases, the only known biological oxygen generating

pathway. A logical consequence of this oxygen production is, that (per)chlorate-reducing bacteria are not strictly anaerobic microorganisms. The strains isolated so far (Table 2) showed to be facultative anaerobic bacteria. An exception is *Wolinella succinogenes* which was originally described as a strict anaerobe (Wallace *et al*., 1996), but a recent study indicated that this bacterium is microaerophilic (Wallace *et al*., 1998). In washed cell suspensions of perchlorate-grown strain GR-1, oxygen and perchlorate reduction occurred simultaneously. Washed cell suspensions of nitrate-grown cells of strain GR-1 do not respire with oxygen, which indicates that enzymes involved in oxygen reduction are induced in perchlorate-grown cells (Rikken *et al*., 1996). Attaway and Smith (1993) showed that oxygen inhibited perchlorate and chlorate reduction by both pure culture and mixed cultures. The simultaneous (per)chlorate and oxygen reduction ensures anaerobic conditions, needed for a proper activity of the (per)chlorate reductase.

Acetate is often used as electron donor (and carbon (C-)source) to enrich and isolate (per)chlorate-reducing bacteria. Therefore most (per)chlorate-reducing bacteria are heterotrophic microorganisms. However, a perchlorate-respiring, hydrogen-oxidizing bacterium, *Dechloromonas* sp. strain HZ, grows with carbon dioxide as sole carbon source (Zhang *et al*., 2002). The ability of this strain to grow autotrophically was confirmed by measuring the incorporation of labeled CO_2 into cellular material. Strain HZ was also able to utilize acetate as electron donor. Because on plates no colonies developed, purity of the culture was checked by microscopic examination and analysis of the variable 16S-23S ribosomal RNA spacer region of independently cloned PCR products (Zhang *et al*., 2002). Despite the difference in heterotrophic or autotrophic growth, all these bacteria are capable of dissimilatory perchlorate and/or chlorate reduction. Achenbach *et al*. (2001) isolated strains, which were classified as two novel genera, *Dechlorosoma* and *Dechloromonas*. The perchlorate and chlorate reduction pathway was the same as was proposed for strain GR-1 (reaction 7).

1.3 Enzymes involved in dissimilatory perchlorate and chlorate reduction

1.3.1 Respiration

The fundament of respiration are the oxidation-reduction (redox) reactions in which electrons flow from an electron donor (or reductant) to an electron acceptor (or oxidant). The equilibrium constant for the reaction is directly related to the standard midpoint redox potential (E^0, based on the hydrogen electrode scale at pH 0 and 25ºC ; $E^{0'}$, based on the hydrogen electrode scale at pH 7 and 25ºC), and is a measure of the tendency of the reductant to lose electrons (Table 3). Electrons have the tendency to move from reductants at the top of the list of Table 3 to oxidants with more positive potentials at the bottom of the list.

H_2 is energetically a good electron donor for chlorate reduction ClO_3^- :

H_2	$\rightarrow$	$2H^+ + 2\ e^-$	$E^{0'} = -0.414$
$ClO_3^- + 2H^+ + 2\ e^-$	$\rightarrow$	$ClO_2^- + H_2O$	$E^{0'} = 0.709$
$ClO_3^- + H_2$	$\rightarrow$	$ClO_2^- + H_2O$	$\Delta E^{0'} = 1.123$

When electrons flow from an electron donor to an electron acceptor with a more positive redox potential, free energy is released. The larger the difference between the reduction potentials of the two redox couples ($\Delta E^{0'}$) the higher the free energy change. This can also be expressed as the Gibbs free energy ($\Delta G^{0'}$, at pH 7 and 25ºC). The relation between $\Delta E^{0'}$ and $\Delta G^{0'}$ is written in the following equation:

$\Delta G^{0'} = - n * F * \Delta E^{0'}$

n = the number of electrons transferred

F = Faraday constant (96.5 kJ * mol^{-1} * $volt^{-1}$)

Table 3 Biologically important redox couples

			$E^{0'}$ (Volts)
$SO_4^{2-} + 3\ H^+ + 2\ e^-$	→	$HSO_3^- + H_2O$	-0.516
$2\ H^+ + 2\ e^-$	→	H_2	-0.414
Ferredoxin (Fe^{3+}) + e^-	→	Ferredoxin (Fe^{2+})	-0.420
$NAD(P)^+ + H^+ + e^-$	→	NAD(P)H	-0.320
$2\ CO_2 + 7\ H^+ + 6\ e^-$	→	$CH_3COO^- + 2\ H_2O$	-0.290
$S + 2\ H^+ + 2\ e^-$	→	H_2S	-0.274
$Pyruvate^- + 2\ H^+ + 2\ e^-$	→	$Lactate^-$	-0.185
$FAD + 2\ H^+ + 2\ e^-$	→	$FADH_2$	-0.180
$HSO_3^- + 6\ H^+ + 6\ e^-$	→	$HS^- + 3\ H_2O$	-0.110
Cytochrome b (Fe^{3+}) + e^-	→	Cytochrome c (Fe^{2+})	0.075
Ubiquinone + $2\ H^+ + 2\ e^-$	→	Ubiquinone H_2	0.100
Cytochrome c (Fe^{3+}) + e^-	→	Cytochrome c (Fe^{2+})	0.254
$MnO_2 + 4\ H^+ + 2\ e^-$	→	$Mn^{2+} + 2\ H_2O$	0.380
$NO_3^- + 2\ H^+ + 2\ e^-$	→	$NO_2^- + H_2O$	0.430
$NO_2^- + 8\ H^+ + 6\ e^-$	→	$NH_4^+ + 2\ H_2O$	0.440
$ClO_3^- + 2\ H^+ + 2\ e^-$	→	$ClO_2^- + H_2O$	0.709
$Fe^{3+} + e^-$	→	Fe^{2+}	0.771
$ClO_4^- + 2\ H^+ + 2\ e^-$	→	$ClO_3^- + H_2O$	0.788
$O_2 + 4\ H^+ + 4\ e^-$	→	$2\ H_2O$	0.815
$2\ NO + 2\ H^+ + 2\ e^-$	→	$N_2O + H_2O$	1.175
$ClO_2^- + 4\ H^+ + 4\ e^-$	→	$Cl^- + 2\ H_2O$	1.199
$N_2O + 2\ H^+ + 2\ e^-$	→	$N_2 + H_2O$	1.355

$E^{0'}$ = standard reduction potential at pH 7.0

1.3.2 Dissimilatory chlorate reduction

The ability of bacteria to grow with perchlorate and/or chlorate is possible when: 1) these oxyanions are used as an electron acceptor and 2) the toxic intermediate compound chlorite is converted into chloride and oxygen. For chlorate reduction (acetate used as electron donor) the following reaction stoichiometry can be written, when the dismutation of chlorite is taken into account (Rikken *et al*., 1996):

	$\Delta G^{o'}$
$1/3\ CH_3COO^- + 4/3\ ClO_3^- \rightarrow 2/3\ HCO_3^- + 1/3\ H^+ + 4/3\ ClO_2^-$	-262 kJ (1/3 mol acetate)$^{-1}$
$4/3\ ClO_2^- \rightarrow 4/3\ O_2 + 4/3\ Cl^-$	*
$2/3\ CH_3COO^- + 4/3\ O_2 \rightarrow 4/3\ HCO_3^- + 2/3\ H^+$	-563 kJ (2/3 mol acetate)$^{-1}$
$CH_3COO^- + 4/3\ ClO_3^- \rightarrow 2\ HCO_3^- + H^+ + 4/3\ Cl^-$	-825 kJ (mol acetate)$^{-1}$

* The disproportionation of ClO_2^- does not require the oxidation of acetate and therefore the $\Delta G^{o'}$ is not included.

For strain GR-1 it was shown that the transformation of chlorite is not dependent on the presence of acetate. Washed cell suspensions were shown to liberate molecular oxygen from chlorite without the addition of any reductive substrates. Rikken *et al*. (1996) speculated that strain GR-1 was unable to grow with chlorite because the conversion of chlorite into chloride and oxygen is probably a detoxification which enables strain GR-1 to grow with perchlorate and chlorate. Similar to the calculations made for chlorate, calculations can be made for perchlorate (Rikken *et al*., 1996) and oxygen:

$CH_3COO^- + ClO_4^- \rightarrow 2HCO_3^- + H^+ + Cl^-$	-957 kJ (mol acetate)$^{-1}$
$CH_3COO^- + 2\ O_2 \rightarrow 2\ HCO_3^- + H^+$	-884 kJ (mol acetate)$^{-1}$

These Gibbs free-energy values are in the same range and explain the comparable growth yield values for O_2, ClO_3^- and ClO_4^- in different bacteria (Table 4). Therefore, (per)chlorate reductases are involved in energy yielding reactions.

Table 4 Comparison of cell yields of perchlorate reducing bacteria using different electron acceptors

Strain	Cell yield (g [DW]/g of acetate) with following electron acceptors			Reference
	O_2	ClO_3^-	ClO_4^-	
Dechlorosoma sp. strain KJ	0.46 ± 0.07	0.44 ± 0.05	0.50 ± 0.08	Logan *et al*., 2001
Dechlorosoma sp. strain GR-1	0.27 ± 0.01	0.28 ± 0.01	0.24 ± 0.01	Rikken *et al*., 1996

1.3.3 Electron-transport phosphorylation in denitrifiers

Transfer of electrons derived from organic- (acetate) or inorganic substrates (hydrogen) to oxyanions, such as perchlorate, chlorate or nitrate, allows electron transport phosphorylation. Nothing is known about the components, their organization and function in electron transfer phosphorylation in (per)chlorate reducers. The nitrate reductase found in denitrifyers showed besides nitrate reductase activity also chlorate reduction activity. Therefore the electron transport chains may be similar for both reduction pathways. Fig. 1 gives an overview of the organization of the cytoplasmic nitrate reductase found in *E. coli*, *Pseudomonas aeroginosa* and *Bacillus subtilis* (Richardson *et al*., 2001). Fig. 1 B shows the organization of a periplasmic nitrate reductase of *Paracoccus denitrificans* (Richardson *et al*., 2001; Zumft, 1997).

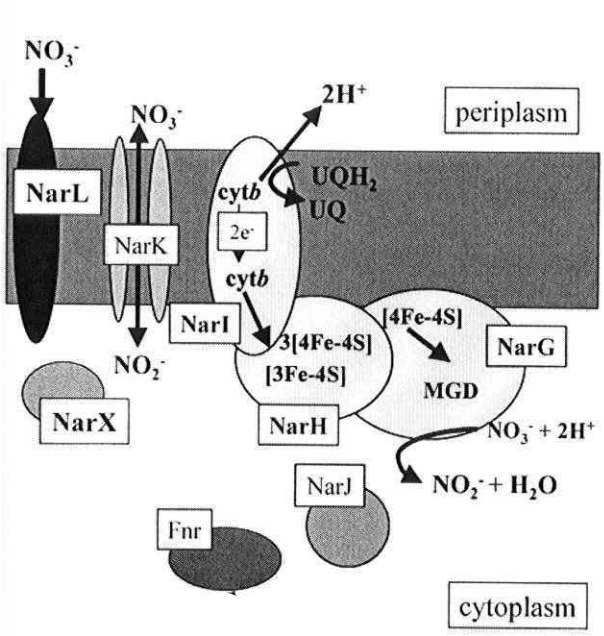

Fig. 1A Organization of the membrane-bound nitrate reductase (NAR) (Richardson *et al*., 2001).

NarG: bis-molybdopterin guanine dinucleotide cofactor (MGD) catalytic subunit (~140-kDa)
NarH: electron transfer subunit (~60-kDa)
NarI: di-*b*-heme integral membrane quinol dehydrogenase subunit
NarJ: chaperone
NarK: Nitrogen oxyanion transporter
NarXL: two-component nitrate-responsive sensor-regulator system
Fnr: transcriptional activator required for activation of narK operon

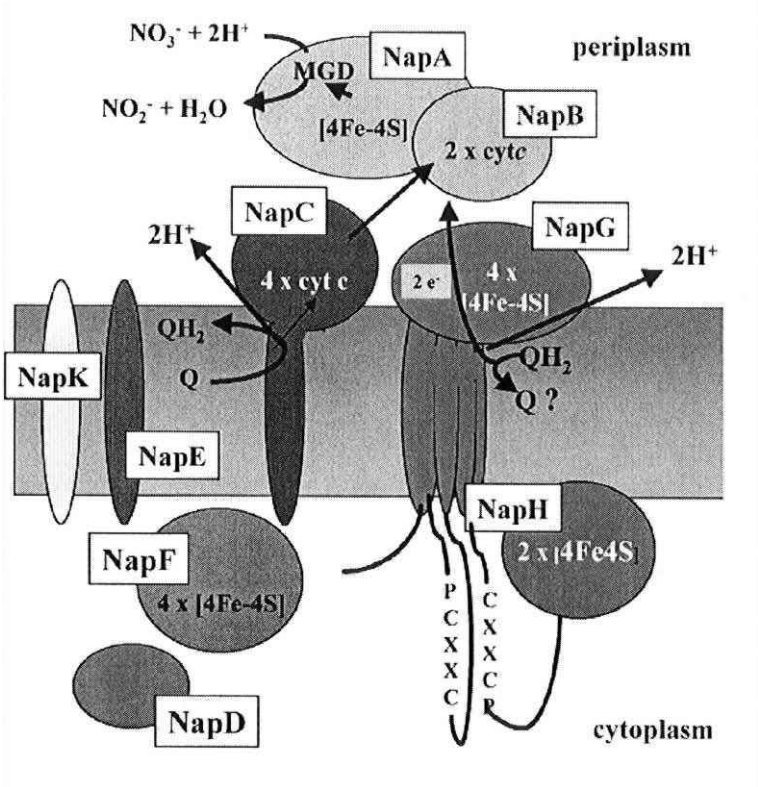

Fig. 1B Organization of the periplasmic nitrate reductase (NAP) (Richardson *et al*., 2001).

NapA: catalytic subunit, which binds MGD cofactor and a [4Fe4S] cluster (~90-kDa)
NapB: electron transfer subunit which binds two c-type hemes (~16-kDa)
NapC: tetraheme components of electron transfer system
NapD: chaperone involved in maturation of NapA
NapE: monotopic integral membraneproteins of unknown function
NapF: cytoplasmic protein which binds four [4Fe-4S] clusters (20-kDa)
NapG: protein which binds four [4Fe-4S] clusters (20-kDa)
NapH: integral membrane protein with four transmembrane helices (32-kDa)
NapK: monotopic integral membrane proteins of unknown function

Reducing equivalents ([H] or electrons) derived from substrates (acetate, hydrogen, NADH) enter the respiratory chain at the innerside of the cytoplasmic membrane and are transferred across the membrane in such a way that electrons are finally transferred to a terminal electron acceptor ((per)chlorate, oxygen, nitrate) and protons (H^+) are transported to the outside of the membrane (proton pump). In this way an electrochemical gradient is generated. As a consequence of the backflow of protons, a membrane associated ATP synthase synthesizes ATP from ADP and P_i. The energy conservation by electron-transport phosphorylation under anaerobic conditions is also referred as anaerobic respiration.

1.3.4 (Per)chlorate reductase

Sofar, two enzymes have been purified and characterized that can reduce chlorate and/or perchlorate. From the denitrifying strain *Proteus mirabilis*, chlorate reductase C has been purified, besides two nitrate reductases (Oltmann *et al*., 1976[a]). This enzyme had a molecular mass of approximately 180 kDa and consisted of three subunits with molecular masses of 75, 63 and 56 kDa. The latter two subunits were most probably similar to nitrate reductase A. The only known substrate of chlorate reductase C was chlorate, which was reduced to chlorite, and the activity was not inhibited by azide. The chlorate reductase C was purified from the membrane. A different localization was found for the purified (per)chlorate reductase from strain GR-1. This enzyme had a periplasmic localization (Kengen *et al*. 1999). The apparent molecular mass (420 kDa) and subunit sizes (95 and 40 kDa) were also different. One enzyme was responsible for both chlorate and perchlorate reduction. Besides (per)chlorate, nitrate, iodate or bromate were also reduced by the (per)chlorate reductase of strain GR-1. Perchlorate-grown cells were unable to convert nitrate or nitrite, indicating that another nitrate reductase may be involved in nitrate-grown cells (Rikken *et al*., 1996).

When the localization and subunit composition of these two enzymes are compared to nitrate reductases, chlorate reductase C resembles the membrane attached nitrate reductase (NAR, Fig. 1A) and the (per)chlorate reductase of strain GR-1 is comparable to the periplasmic nitrate reductase (NAP, Fig. 1B). The species *Alcaligenes eutrophus* (Siddiqui *et al*., 1993), *Rhodobacter sphaeroides* (Castillo *et al*., 1996), *Thiosphaera pantotropha* (Bell *et al*., 1993) and *Pseudomonas stutzeri* (Zumft, 1997) contain a periplasmic nitrate reductase in addition to a typical respiratory membrane-bound nitrate reductase. Whereas the periplasmic enzyme is synthesized and active in the presence of oxygen, the membrane-bound nitrate reductase is expressed only under anaerobic growth conditions. Washed cells of strain GR-1 of oxygen-grown cells were not able to reduce perchlorate and chlorate, indicating that the periplasmic (per)chlorate reductase of strain GR-1 was not expressed under aerobic conditions (Rikken *et al*, 1996). Similar results were obtained with *Dechlorosoma suillum* where the reduction of perchlorate was immediately inhibited when air was introduced into the headspace of an anaerobic culture growing on perchlorate (Chaudhuri *et al*., 2002).

Metal analysis showed the presence of iron, molybdenum and selenium in the (per)chlorate reductase of strain GR-1 (Kengen *et al*, 1999). Nutritional requirement for molybdenum was demonstrated with strain *D. aromatica*. Growth and perchlorate stopped when an active perchlorate-reducing culture of *D. aromatica* was transferred into medium from which

molybdenum was omitted (Chaudhuri *et al*., 2002). Molybdenum is also present in periplasmic and membrane-bound nitrate reductases.

N-terminal sequencing of the α- and β-subunit of the (per)chlorate reductase of strain GR-1 was only successful for the β-subunit. A BLAST-search did not give any similar sequence. However, in the database the β-subunit of the selenate reductase of *Thauera selenatis* did reveal several identical amino acids. This enzyme had an apparent molecular mass of 180 kDa and consisted of three subunits (96 kDa (α), 40 kDa (β) and 23 kD (γ)). The selenate reductase contained molybdenum and iron and was specific for the reduction of selenate. Nitrate and chlorate were not reduced at detectable rates (Schröder *et al*., 1997).

1.3.5 Chlorite dismutase

In 1996, van Ginkel *et al*. purified an enzyme from strain GR-1 which catalyzed the disproportionation of chlorite into chloride and oxygen. The stoichiometry of this dismutation was 1 mol of chloride and 1 mol of oxygen out of 1 mol of chlorite. Sofar, two other types of enzymes, horseradish peroxidase and chloroperoxidase were reported to catalyze the conversion of chlorite (Hewson and Hager, 1979; Shahangian and Hager, 1981). These peroxidases did not produce chloride and oxygen in equimolar amounts. Chlorine dioxide and chloride were the products of horseradish peroxidase (Hewson and Hager, 1979). Chloroperoxidase generated from 1 mol of chlorite, chlorate, chloride and oxygen in a stoichiometry of 0.6:0.4:0.13 (Shahangian and Hager, 1981). Besides the chlorite dismutase of strain GR-1, chlorite dismutases have been purified from *Ideonella dechloratans* (Stenklo *et al*., 2001) and *Dechloromonas agitata* (Coates *et al*., 1999)[a]. The three purified chlorite dismutases showed similar characteristics. The data suggest that the enzymes are homotetramers with a molecular mass of approximately 120 kDa, 140 kDa and 115 kDa for *D. agitata*, strain GR-1 and *I. dechloratans*, respectively. Subunit sizes were 32 kDa for strain GR-1 and *D. agitata* and 25 kDa for *I. dechloratans*. The EPR spectroscopic properties of the chlorite dismutase of *I. dechloratans* and strain GR-1 were similar, showing two different high-spin ferric heme species (Hagedoorn *et al*., 2002; Stenklo *et al*., 2001). Heme analysis of the purified enzymes of strain GR-1 and *I. dechloratans* gave a pyridine hemochrome spectrum with peaks at 419 and 556.5 nm suggesting that the heme group in chlorite dismutase is Fe-protoporphyrin IX (Falk, 1964). The spectral characteristics of chlorite dismutase resemble those of other heme iron enzymes like catalases and peroxidases (Brown-Peterson and Salin, 1993; Kim *et al*., 1994). In the chlorite dismutase reaction, four electrons

have to be transferred when the valence of Cl is reduced from +3 in ClO_2^- to -1 in Cl^-. The valence of oxygen changes from -2 in ClO_2^- to 0 in O_2. The question arises if the chlorite dismutation is properly named. The reduction of Cl and oxidation of O in ClO_2^- entails an intramolecular redox reaction. The systematic name for this enzyme is chloride:oxygen oxido-reductase or chlorite O_2-lyase (EC 1.13.11.49). Another question is whether or not H_2O is one of the substrates of the enzyme. Hagedoorn *et al*. (2002) doubted if both oxygen atoms in the dioxygen product of chlorite dismutase come from chlorite.

Inhibition studies with chlorite dismutase of strain GR-1 showed that dismutase activity was inhibited by hydroxylamine and cyanide, which are both potent inhibitors of heme-protein-catalyzed reactions. The typical catalase inhibitor 3-amino-1,2,4-triazole, did not inhibit the chlorite dismutase. Inhibition studies with azide (20 mM) did not show any inhibition of chlorite dismutase of strain GR-1 (van Ginkel *et al*., 1996). Catalases and peroxidases differ from chlorite dismutase because those enzymes are inhibited by 0.1 mM azide (Tuisel *et al*., 1990; Kim *et al*., 1994).

Initial studies suggested that chlorite dismutase is highly conserved among (per)chlorate-reducing bacteria. An immunoprobe with high affinity for chlorite dismutase was specific to (per)chlorate-reducing bacteria and cross-reactivity with non-chlorate-reducing bacteria, which were phylogenetic related (16S rDNA similarity > 97%), was not observed (O'Conner and Coates, 2002). Besides the gene sequence of the chlorite dismutase of *D. agitata* (Bender *et al*., 2002), also the gene sequence of the chlorite dismutase of *I. dechloratans* was determined (Danielsson Thorell *et al*., 2002). An alignment indicated that the *D. agitata* chlorite dismutase sequence was 71% similar to *I. dechloratans* at the amino acid level. No other proteins in the Genbank database were more than 24% similar, emphasizing the unique nature of the chlorite dismutase gene. In addition to an immunoprobe against the purified chlorite dismutase of *Dechloromonas agitata*, a chlorite dismutase gene probe was developed based on the gene sequence of the same enzyme (Bender *et al*., 2002). The chlorite dismutase gene probe hybridized to all the tested dissimilatory perchlorate-reducing bacteria used in that study. The gene probe was specific for dissimilatory perchlorate-reducing bacteria, and cross hybridization with non-chlorate-reducing bacteria was not observed. However, *Magnetospirillum magnetotacticum*, a non-chlorate-reducing bacterium, did hybridize to the gene probe and a genome sequence analysis revealed the presence of a putative chlorite dismutase gene.

1.4 Ubiquity of (per)chlorate reducing bacteria

Aslander (1928), Bryan and Rohlich (1954) and Malmqvist (1991) investigated the occurrence of biological chlorate reduction in natural environments. In the Netherlands, van Ginkel *et al*. (1995) found chlorate reduction in IJssel river samples, anoxic sediments from a ditch and surface soils from a public garden. Most probable number (MPN) countings done by Coates *et al*. (1999)[a] and Wu *et al.* (2001) gave 10^3 to 10^6 chlorate-reducing cells per gram of sample from diverse environments (Table 5).

Table 5 Chlorate reducer abundance in different environments (acetate used as electron donor)

Sample type	Source	Amount (cells per g)	Reference
Waste water	Swine waste lagoon	$2.4 * 10^6$	Coates *et al.* (1999)[a]
	Raw wastewater	$0.2\text{-}1.7 * 10^5$	Wu *et al*. (2001)
	Treated wastewater	$0.3\text{-}2.1 * 10^4$	Wu *et al*. (2001)
Sediments	Petroleum-contaminated soil	$9.3 * 10^3$	Coates *et al.* (1999)[a]
	Pristine aquatic sediment	$4.6 * 10^3$	Coates *et al.* (1999)[a]
	Mississippi river sediment	$4.3 * 10^3$	Coates *et al.* (1999)[a]
	Gold mine drainage sediment	$4.3 * 10^3$	Coates *et al.* (1999)[a]
Soils	Pristine soil	$2.3 * 10^3$	Coates *et al.* (1999)[a]
	TX soil	22-130	Wu *et al*. (2001)
Natural waters	Florida swamp	$2.3 * 10^4$	Coates *et al.* (1999)[a]
	Pohic Bay	$1.5 * 10^3$	Coates *et al.* (1999)[a]
	Creek Water	0.01-0.17	Wu *et al*. (2001)

The high number of strains isolated sofar by various research groups from different continents, also indicates that (per)chlorate reducers are widely distributed. In Fig. 2, a phylogenetic tree is displayed, based on 16S rDNA sequences of (per)chlorate-reducing bacteria. This figure shows that all (per)chlorate-reducing bacteria belong to the class of *Proteobacteria*. The α, β, γ, and ε-subclasses of the *Proteobacteria* are represented. Fig. 2, shows that the predominant group of (per)chlorate-reducing bacteria belongs to the recently described *Dechloromonas* and *Dechlorosoma* genus (Achenbach *et al*., 2001). Also several strains were isolated which showed similarity to the denitrifying *Pseudomonas stutzeri* (chapter 2 of this thesis).

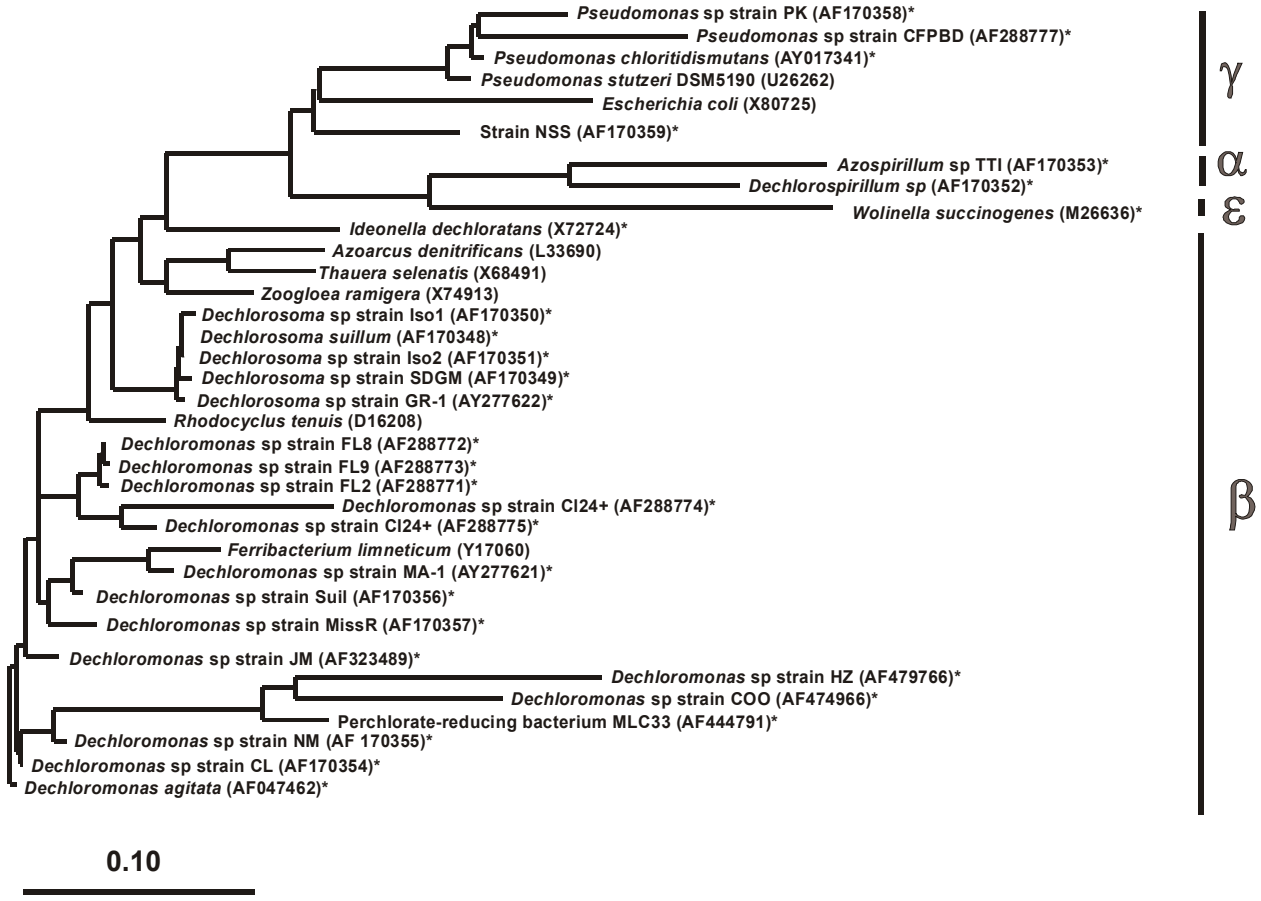

Fig. 2 Phylogenetic tree of (per)chlorate-reducing bacteria and their closest relatives based on total 16S rDNA sequences. (Per)chlorate-reducing bacteria are indicated with (*).

The presence of chlorate reducing bacteria can be expected in wastewater from industries using chlorate, for example for the production of matches. From such polluted wastewater *Acinetobacter thermotoleranticus* was isolated (Stepanyuk *et al*., 1993). However, chlorate reduction was also observed in pristine soils, with no known source of (per)chlorate contamination (Coates *et al*. 1999[a]). Wu *et al*. (2001) did not detect perchlorate reduction when 2 grams of pristine soil was incubated, but perchlorate reduction was observed when a larger soil sample (100 g) was used. Because (per)chlorate has only been produced during the last 100 years, it is doubtful if this anthropogenic (per)chlorate production can explain the widespread occurrence of (per)chlorate-reducing bacteria. Because some (per)chlorate-reducing bacteria are almost phylogenetically similar to organisms (Fig. 2, *Pseudomonas* species) which are not capable to grow by the dissimilation of (per)chlorate, it was suggested that the ability of chlorate reduction is a result of horizontal gene transfer (Achenbach *et al*.,

2001). The function of (per)chlorate-reducing bacteria in the natural chlorine cycle (Öberg, 2002) is not known yet.

1.5 Potential of (per)chlorate-reducing bacteria in bioremediation processes

(Per)chlorate-reducing bacteria can be used for the treatment of perchlorate- and chlorate-containing waste streams and groundwater (Bryan 1966; Bryan and Rohlich, 1954). (Per)chlorate-reducing bacteria were used for the bioremediation of perchlorate-contaminated groundwater using a packed bed biological reactor (Losi *et al*., 2002) and of chlorate and perchlorate contaminated water using permeable barriers containing vegetable oil (Hunter, 2002). The ability of (per)chlorate-reducing bacteria to produce oxygen can also be applied in other bioremediation processes. Benzene, toluene, ethylbenzene and xylene (BTEX) can be degraded under aerobic conditions, but are more persistent under anaerobic conditions (Young and Cerniglia, 1995). Degradation of hydrocarbons is important, to prevent the enlargement of the contamination zone in anaerobic soil layers. Under anaerobic conditions, alternative electron acceptors can be used. Two *Dechloromonas* strains, RCB and JJ, could couple nitrate reduction to anaerobic benzene oxidation (Coates *et al.*, 2001). Also under sulfate and iron-reducing conditions, BTEX compounds could be degraded (Burland and Edwards, 1999; Edward and Grbic-Galic, 1992; Coates *et al*., 1996). However, oxidation rates with these alternative electron acceptors are low. Injection of oxygen into the anaerobic zone to improve degradation is costly and inefficient. Therefore, chlorite dismutation by (per)chlorate-reducing bacteria offers a good alternative strategy to supply extracellular oxygen to the aerobic hydrocarbon-oxidizing population. This was demonstrated by Logan and Wu (2002) who reported increased rates of toluene oxidation which indicated a symbiotic relationship between toluene-degrading bacteria and chlorate-reducing bacteria. Washed cell suspensions of (per)chlorate-reducing cells of strain GR-1 showed formation of oxygen upon the addition of chlorite (Rikken *et al*., 1996). The addition of chlorite in anoxic soil samples, inoculated with starved bacterial cells, showed that [^{14}C]-benzene was rapidly oxidized to $^{14}CO_2$ (Coates *et al*., 1999[b]). Results sofar, indicate that chlorate-reducing bacteria may be used in remediation of BTEX-contaminated soils and groundwater.

1.6 Outline of the thesis

This thesis describes the research on the physiological and biochemical characterization of (per)chlorate-reducing bacteria. At the start of this project, only three (per)chlorate-reducing bacteria had been isolated, described and deposited at culture collections. To gain more insight into the physiological, biochemical and phylogenetic properties of (per)chlorate-reducing bacteria, strictly anaerobic conditions were applied to isolate bacteria which could grow with chlorate as electron acceptor. Together with the already described (per)chlorate-reducing bacteria, the bacteria isolated in this study are of importance to evaluate the diversity of (per)chlorate-reducing bacteria in nature.

Chapter 2 describes the characterization of a novel chlorate-reducing bacterium, *Pseudomonas chloritidismutans* which is phylogenetically similar to the denitrifying *Pseudomonas stutzeri*. Denitrifying bacteria contain a nitrate reductase which shows chlorate reductase activity. Therefore, purification of the chlorate reductase of *P. chloritidismutans* described in chapter 3, gives insight into the properties of the chlorate reductase in comparison to the nitrate reductase. Also a comparison is made to the (per)chlorate reductase of strain GR-1, which is the first (per)chlorate reductase isolated from a chlorate and perchlorate respiring bacterium. Besides this reductase, the chlorate reductase of *Ideonella dechloratans* is used for comparison.

P. chloritidismutans can grow with chlorate as electron acceptor, whereas *P. stutzeri* can not. Due to the absence of the chlorite dismutase, *P. stutzeri* is not able to convert the toxic chlorite into chloride and oxygen. Like other (per)chlorate-reducing bacteria, *P. chloritidismutans* contains a chlorite dismutase. In chapter 4, the purification and characterization of this enzyme is described. A few chlorite dismutases have already been described and were purified from strain GR-1, *Dechloromonas agitata* and *Ideonella dechloratans*. A comparison between these enzymes indicates that chlorite dismutases have many common characteristics.

Chapter 5 describes the characterization of two (per)chlorate-reducing bacteria of which strain MA-1 was isolated from a pristine soil sample. MA-1 is compared to *D. agitata* resulting in the proposition of a new species, *Dechloromonas hortensis*. Another strain, strain ASK-1 which was isolated from a sludge sample treating a chlorate and bromate polluted waste stream, showed similarities to *P. chloritidismutans* on the phylogenetic and physiological level, although, a different morphology was found. Strain GR-1 was previously described, but a phylogenetic analysis was not done. 16S rDNA sequence analysis and DNA-DNA hybridization revealed that strain GR-1 is a *Dechlorosoma suillum* strain. The

physiological and phylogenetic characteristics of *P. chloritidismutans* versus ASK-1 and strain MA-1 versus *Dechloromonas agitata* and *Ferribactericum limneticum* are compared.

In chapter 6, possible (a)biotic processes are discussed in which chlorate may be formed. These processes could be a missing piece in the chlorine cycle and could explain the occurrence of (per)chlorate-reducing bacteria in polluted and pristine sites.

In chapter 7 the main conclusions of this thesis are summarized and discussed in a broader context.

Chapter 2

Pseudomonas chloritidismutans sp. nov., a non-denitrifying chlorate-reducing bacterium

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Abstract

A Gram-negative, facultatively anaerobic, rod-shaped, dissimilatory chlorate-reducing bacterium, strain AW-1^T, was isolated from biomass of an anaerobic chlorate-reducing bioreactor. Phylogenetic analysis of the 16S rDNA sequence showed 100% sequence similarity with *Pseudomonas stutzeri* DSM 50227 and 98·6% sequence similarity with the type strain of *P. stutzeri* (DSM 5190^T). *P. stutzeri* species possess a high degree of genotypic and phenotypic heterogeneity. Therefore, eight genomic groups, termed genomovars, have been proposed based upon delta T_m values, which were used to evaluate the quality of the pairing within heteroduplexes formed by DNA-DNA hybridization. In this study DNA-DNA hybridization between strain AW-1^T and *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T revealed 80·5 % and 56·5 % similarity, respectively. DNA-DNA hybridization between strain *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T revealed 48·4 % similarity. DNA-DNA hybridization indicated that strain AW-1^T is not related at the species level to the type strain of *P. stutzeri*. However, strain AW-1^T and *P. stutzeri* DSM 50227 are related at the species level. Physiological and biochemical properties of strain AW-1^T and both *P. stutzeri* strains were compared. A common characteristic of *P. stutzeri* species is the ability to denitrify. However, in growth experiments, strain AW-1^T could only use chlorate and oxygen as electron acceptor but not nitrate, perchlorate or bromate. Strain AW-1^T is the first chlorate-reducing bacterium described which does not possess another oxyanion reduction pathway. Cell extracts of strain AW-1^T showed chlorate and bromate reductase activity but not nitrate reductase activity. *P. stutzeri* strain DSM 50227 and strain DSM 5190^T could use nitrate and oxygen as electron acceptor, but not chlorate. In cell extracts of both *P. stutzeri* strains, besides nitrate reductase activity also chlorate reductase activity was detected. Chlorite dismutase activity was absent in extracts of both *P. stutzeri* strains but present in extracts of strain AW-1^T. Based on the hybridization experiments and the physiological and biochemical data, we propose that strain AW-1^T should be classified as a new species of *Pseudomonas*. We propose *Pseudomonas chloritidismutans* sp. nov.. The type strain of *Pseudomonas chloritidismutans* sp. nov. is strain AW-1^T (= DSM 13592^T = ATCC BAA-443^T).

Introduction

Contamination of the environment with oxyanions, such as chlorate (ClO_3^-), is caused by human activities. Chlorate is used in a wide range of applications. Chlorate has been used as a herbicide in agriculture (Agaev *et al*., 1986) and it is used for the on-site generation of the bleaching agent chlorine dioxide (ClO_2) in the paper and pulp industry (Germgard *et al*., 1981). The compound may be toxic for certain organisms; chlorate added to drinking water can cause hemolytic anemia in rats (Condie, 1986). In plants and microorganisms, chlorate may compete with nitrate in the nitrate reductase system (van Wijk and Hutchinson, 1995). However, chlorate-reducing bacteria are able to remove this oxyanion by chlorate respiration (Rikken *et al*., 1996). This process seems to be promising compared to conventional chemical and physical treatment technologies (Wallace *et al*., 1998; Malmqvist and Welander, 1992). Strain GR-1 was the first bacterium reported to reduce chlorate completely to chloride (Rikken *et al*., 1996). In the first step chlorate is reduced to chlorite (ClO_2^-) by a chlorate reductase. In the second step chlorite is disproportionated to chloride and oxygen by a chlorite dismutase. These enzymes have been purified and characterized (van Ginkel *et al*., 1996; Kengen *et al.*, 1999). Energy for biosynthesis can be conserved during reduction of chlorate and oxygen. Dissimilatory chlorate reduction has also been studied in *Dechloromonas agitata* strain CKB (Bruce *et al*., 1999; Achenbach *et al*., 2001), *Ideonella dechloratans* (Malmqvist *et al*., 1994) and *Wolinella succinogenes* HAP-1 (Wallace *et al*., 1996). These organisms (except CKB) have been reviewed by Logan (1998). All chlorate reducers were characterized as facultative anaerobes, except *Wolinella succinogenes* HAP-1 which is a microaerophilic bacterium (Wallace *et al*., 1998). The latter strain is the only one which uses H_2 as an electron donor. Besides chlorate, other electron acceptors can be used by chlorate-reducing strains. The use of nitrate is of special interest, because many nitrate reductases are able to reduce chlorate (Hochstein and Tomlinson, 1988). Several denitrifying bacteria reduce chlorate, but in general this reduction is not coupled to growth (De Groot and Stouthamer, 1969; Oltmann *et al*. 1976[a,b]). Moreover, these organisms lack chlorite dismutase, which is required for the removal of the toxic chlorite. Recently, it was described that dissimilatory chlorate-reducing bacteria have a specific chlorate reductase and a specific nitrate reductase (Rikken *et al.,* 1996). It was shown that perchlorate-grown cells of strain GR-1 were unable to reduce nitrate or nitrite indicating that specific (per)chlorate reductases are involved. *Dechloromonas agitata* strain CKB is a dissimilatory chlorate reducer incapable of coupling growth to the reduction of nitrate (Bruce *et al*., 1999). These findings suggest that nitrate and chlorate

reduction pathways are not necessarily related. This paper describes the isolation and characterization of a chlorate-reducing bacterium strain AW-1^T. This strain was enriched from sludge of an anaerobic bioreactor treating a chlorate and bromate polluted waste water (Abma *et al*., 1999). Physiological, biochemical and phylogenetic properties of strain AW-1^T were compared with those of *P. stutzeri* strains DSM 5190^T and DSM 50227.

Materials and methods

Media and growth conditions

Enrichment and cultivation of strain AW-1^T was performed in strictly anaerobic medium containing (l^{-1}): KH_2PO_4, 0.41g; Na_2HPO_4, 0.53 g; $NaClO_3$, 1.06 g; $CaCl_2$, 0.11 g; $MgCl_2$, 0.1 g; NH_4HCO_3, 0.44 g; $NaHCO_3$, 3.73 g; $Na_2S{\cdot}9H_2O$, 0.5 g; 1 ml resazurin solution (0.5 g/l), as well as acid and alkaline trace elements (each 1 ml/liter) and vitamins (1 ml/liter). The medium was boiled and afterwards cooled under N_2 to remove dissolved oxygen. Resazurin was present as an indicator of the redox potential. When oxygen was produced by dismutation of chlorite, the medium turned pink. When all chlorate and oxygen were consumed, the pink color disappeared. The acid trace element solution contained (l^{-1}): $FeCl_2{\cdot}4H_2O$, 1.485 g; H_3BO_3, 0.062 g; $ZnCl_2$, 0.068 g; $CuCl_2$, 0.013 g; $MnCl_2{\cdot}4H_2O$, 0.098 g; $CoCl_2{\cdot}6H_2O$, 0.119 g; $NiCl_2{\cdot}6H_2O$, 0.119 g; and 4.1 ml HCl (37%). The alkaline trace element solution contained the following (l^{-1}): $Na_2SeO_3{\cdot}5H_2O$, 0.026 g: $Na_2WO_4{\cdot}2H_2O$, 0.033 g; $Na_2MoO_4{\cdot}2H_2O$, 0.024 g; and NaOH, 0.40 g. The composition of the vitamin solution was as described by Wolin *et al*. (1963). Bacteria were cultured at 30ºC in 120-ml serum vials with 40 ml medium and a gas phase of 1.5 atm N_2. pH values were calculated using the Henderson-Hasselbach equation. At 30ºC, the α and pK' are respectively 0.665 and 6.348 (Breznak and Costilow, 1994). The percentage of CO_2 in the headspace was changed, while the bicarbonate concentration was kept constant. In this way pH values of 9 (0% CO_2 v/v), 8.5 (1.2% CO_2 v/v), 8 (3.9% CO_2 v/v), 7.3 (20% CO_2 v/v) and 6.6 (100% CO_2 v/v) were obtained. Bottles were sealed with butyl rubber stoppers and aluminum caps. All solutions were sterilized by autoclaving at 121ºC for 30 minutes, except for the vitamin solution which was added filter sterilized. The batch cultures were incubated on an orbital shaker (100 rev/min) in the dark. Substrates were added from 0.8 M stock solutions to give a final concentration of 10 or 20 mM. Occasionally, oxygen was added to the gas phase, and its concentrations was expressed as mmol per liter medium dissolved oxygen. Synthetic medium, nutrient broth and strict anaerobic media were used to test the denitrification and chlorate reduction capacity of the *P. stutzeri* strains and

strain AW-1^T. Synthetic medium was prepared as described by Matsubara *et al.* (1982) with modifications described by Coyle *et al.* (1985). This synthetic medium contained (l^{-1}): L-asparagine·H_2O, 2.0 g; trisodium citrate·$2H_2O$, 7.0 g; KH_2PO_4, 2.0 g; $CaCl_2·2H_2O$, 0.2 g; $MgSO_4·7H_2O$, 4.0; $FeCl_3·6H_2O$, 0.02 g and NaCl; 20 g. The pH was adjusted to 6.6 with 5 N NaOH. Forty ml medium was added to 120 ml serum bottles. N_2 was used as gas phase. Nutrient broth medium contained the following (l^{-1}): peptone, 5.0 g; nutrient broth, 3.0 g. The pH was adjusted to the pH 7.0. For determination of the optimum pH, a pH range of 5-10 was used. The medium was boiled and afterwards cooled under N_2 to remove dissolved oxygen. Forty ml medium was added to 120 ml serum bottles. N_2 was used as gas phase. Nutrient agar plates (pH 7.0) were prepared in the same way, except that 15 g (l^{-1}) agar was added.

Microorganisms

P. stutzeri strain DSM 50227 and *P. stutzeri* strain DSM 5190^T were purchased from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany). Strain AW-1^T was isolated in this study.

Enrichment cultures and isolation of strain AW-1^T

Approximately 2 gram sludge, taken from an anaerobic bioreactor treating chlorate and bromate polluted waste water, was added to strictly anaerobic medium. Chlorate (10 mM) and acetate (10 mM) were used as electron acceptor and electron donor, respectively. Nitrogen was used as gas phase. Enrichment cultures were checked for chlorate reduction and growth by visual and microscopic inspection. After a second subculture, a pure culture of strain AW-1^T was obtained by repeated application of the roll tube dilution method as described by Hungate (1969) using chlorate and acetate as substrates.

Determination of growth parameters

All growth parameters of strain AW-1^T were checked in strictly anaerobic sulfide-reduced medium. The growth rate was determined by measuring the increase in optical density at 430 nm. Doubling times were determined by linear regression of a logarithmically plotted growth curve. Cell counts were obtained by plating dilution series on nutrient agar plates. The following electron donors (10 mM) were tested with ClO_3^- (10 mM) as electron acceptor: acetate, propionate, glucose, maltose, mannitol, malate, lactate, arabinose, hydrogen, glycine, glycerol, formate, gluconate, ethanol, starch, citrate and succinate. The following electron acceptors (10 mM) were tested with acetate (10 mM) as electron donor: perchlorate, chlorate,

chlorite, nitrate, nitrite, bromate, sulfate, selenate, and oxygen. All anions were supplied as sodium salts. The temperature optimum was determined in medium with pH 8.5, in the range of 10-42ºC. The pH optimum was determined in medium at 30ºC using a pH range of 6.6-9. For comparison to the type strain of *P. stutzeri* (DSM 5190^T) the pH optimum was also determined in nutrient broth medium in a pH range of 5-10. The optimum salt concentration was determined at 30ºC and pH 8.5 using a NaCl range of 1-40 g/l. The effect of oxygen on chlorate reduction was investigated. Oxygen was either added together with chlorate at the start of the incubation, or oxygen was added to a culture in which chlorate reduction was already going on.

Cellular characterization

Micrographs were taken using transmission electron microscopy (TEM). The Gram stain type was determined using Gram staining and electron microscopy. Protocols as described previously by Plugge *et al*. (2000), were used.

Phylogenetic identification of strain AW-1^T

The 16S rDNA sequence and GC content of DNA of strain AW-1^T were determined at the German Collection of Microorganisms and Cell cultures. The 16S rDNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA as described by Rainy *et al*. (1996). Approximately 95% of the 16S rDNA gene sequence was analyzed. The GC content of DNA was determined by HPLC as described by Mesbah *et al.* (1989). The 16S rDNA sequence was analyzed with ARB software (Ludwig and Strunk, 1996). The 16S rRNA gene similarity values were calculated by a pairwise comparison of sequences within the alignment. A neighbour-joining tree was constructed based on the evolutionary distance values (Saitou and Nei, 1986). These tools were implemented in the ARB software. Sequences of chlorate-reducing bacteria and the denitrifying strains of *P. stutzeri* were introduced into the ARB software package. These sequences were derived from the NCBI data-base or the ARB software package. In Fig. 4 strain and sequence accession numbers are added to the species names. DNA-DNA hybridization was also done at the DSMZ. DNA from strain AW-1^T (DSM 13592), *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al*. (1977). DNA-DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modification described by Huss *et al.* (1983) and Escara and Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

Preparation of cell extracts

All procedures were done anaerobically. Strain AW-1^T was grown in strictly anaerobic medium with chlorate as electron acceptor. The *P. stutzeri* strains were grown in synthetic media with nitrate as electron acceptor. Whole cells were harvested from 200-ml cultures by centrifugation at 9,000 rpm for 9 min at 10ºC. The cell pellet was washed once with 15 mM potassium phosphate/sodium phosphate buffer (per liter: K_2HPO_4, 1.55 g; $NaH_2PO_4 \cdot H_2O$, 0.85 g), pH 7.2. Cells were suspended in the same buffer (2 ml). To prepare cell extracts, 1 ml cell suspension was sonified for 30 sec and cooled on ice for 30 sec. This cycle was repeated 5 times. The cell debris fraction was removed by centrifugation at 13,000 rpm for 1 min at room temperature. Cell extracts were stored under a N_2 gas phase at 4°C.

Enzyme activity measurements

Chlorate reductase and chlorite dismutase activity were determined in cell extracts of strain AW-1^T and *P. stutzeri* strains DSM 50227 and DSM 5190^T. Chlorate reductase and nitrate reductase activities were determined spectrophotometrically as described previously by Kengen *et al*. (1999) by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm and 30°C. The following electron acceptors (supplied as sodium salts) were tested: perchlorate, chlorate, chlorite, bromate, nitrate, selenate, sulfate, iodite, iodate. In this assay 10 µl of a 0.25 M stock solution and 10 µl cell extract fraction were added to the reaction mixture. Chlorite dismutase activity was determined by measuring the oxygen production with a Clark-type oxygen Electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA). The reaction vessel was kept at 30ºC. The reaction mixture contained 2.8 ml 15 mM potassium phosphate/sodium phosphate buffer (pH 7.2) and 10 µl cell extract or 10 µl whole cells. The reaction was started by injecting a concentrated solution of sodium chlorite into the vessel to give a concentration of 0.2 mM. The reaction was run for 5 min but only the initial linear part was used to calculate the rate. One unit (U) of activity is defined as the amount of enzyme required to convert 1 µmol of chlorite or chlorate per min. The protein content of the cell extract fraction was determined according to the method of Bradford, with bovine serum albumin as standard (Bradford, 1976).

Other analyses

Electron acceptors were analyzed by high-pressure liquid chromatography as described previously by Scholten and Stams (1995). Acetate, hydrogen and oxygen levels were measured by gas chromatography as described by Stams *et al.* (1993).

Chemicals

All chemicals were of analytical grade and obtained from commercial sources.

Results and discussion

Enrichment and isolation of strain AW-1^T

A sludge sample was transferred to a 120-ml serum bottle with strictly anaerobic medium containing chlorate as electron acceptor and acetate as electron donor. In subcultures, within three days growth was observed coupled to the disappearance of chlorate and acetate and the formation of chloride. After a second subculture, a pure culture was finally obtained by repeated application of the roll tube dilution method.

Morphological and physiological characterization of strain AW-1^T

The strain obtained was a Gram-negative, facultatively anaerobic, motile rod-shaped organism (Fig. 1). The size of the bacterium was 0.5-2 μm as measured by transmission electron microscopy.

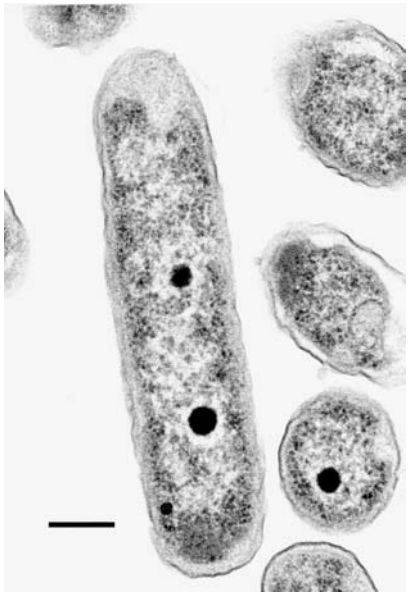

Fig. 1 Electron micrograph showing strain AW-1^T. Electron-dense structures are polyphosphate particles. The bar indicates 0.25 μm.

Colonies in anaerobic agar were circular and pale orange. Colonies on nutrient broth plates were wrinkled, coherent and had a pale brown or sometimes reddish brown color. This is the same morphology as described for *P. stutzeri* by Stolp and Gadkari (1981). Reduction of 14.2 mM chlorate resulted in the oxidation of 10.6 mM acetate and the formation of 15.6 mM chloride (Fig. 2), which resulted in a stoichiometry of 1.0 : 0.75 : 1.1, respectively. This is

close to what can be theoretically expected when chlorate is reduced to chloride with acetate as is described by Rikken *et al.* (1996): ClO_3^- + 0.75 CH_3COO^- → 1.5 HCO_3^- + H^+ + Cl^-. During the exponential growth phase, the intermediate compound in chlorate reduction, chlorite, was not detected.

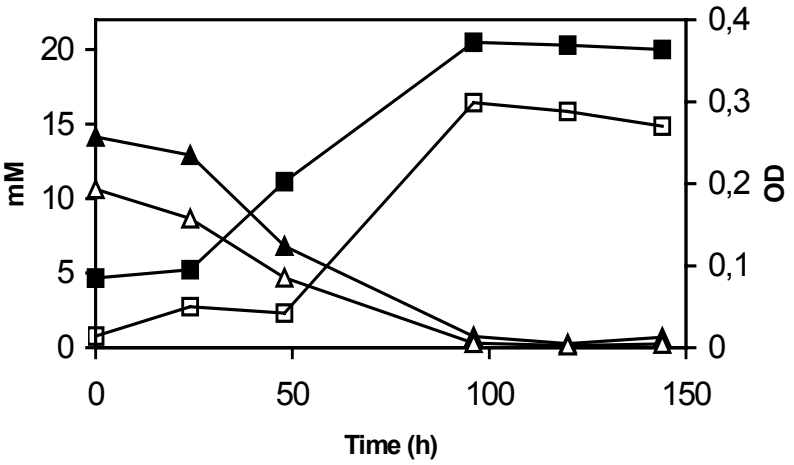

Fig. 2 Growth curve of strain AW-1^T with acetate (Δ) as electron donor and chlorate (▲) as electron acceptor in a batch culture. (■) chloride, (□) optical density (430 nm).

With acetate as electron donor, growth of strain AW-1^T could only be observed when ClO_3^- or O_2 were used as electron acceptors. When ClO_4^-, ClO_2^-, NO_3^-, NO_2^-, BrO_3^-, SO_4^{2-} or SeO_4^{2-} were tested, no growth could be detected. Compared with strain GR-1 (ClO_3^-, ClO_4^-, O_2, NO_3^-, Mn(IV)) (Rikken *et al*., 1996) and *Dechloromonas agitata* strain CKB (ClO_3^-, ClO_4^-, O_2) (Bruce *et al.*, 1999) the electron acceptor range of strain AW-1^T is rather limited. This indicates that a specific chlorate reductase is involved which can not reduce perchlorate or nitrate. Strain AW-1^T differs in this aspect from other chlorate-reducing bacteria. Apparently, strain AW-1^T does not possess another oxyanion reduction pathway. However, a recently isolated bacterium showed also only chlorate reduction (Achenbach *et al.*, 2001). In strictly anaerobic medium strain AW-1^T grew with a doubling time of 1.5 h and 1.2 h, using chlorate and oxygen as electron acceptor, respectively. In a competition experiment the effect of oxygen on chlorate reduction was analyzed. When oxygen and chlorate were both present at the beginning of the experiment, chlorate reduction started only when oxygen had been consumed (Fig. 3A). When oxygen was added to a chlorate–reducing culture, oxygen and chlorate were simultaneously consumed (Fig 3B). Similar results were obtained by Rikken *et al.* (1996) with washed cells of strain GR-1. These results suggest that the expression of

chlorate reductase is inhibited by the presence of oxygen. However, when chlorate reduction proceeds, addition of oxygen does not inhibit the chlorate reduction process itself.

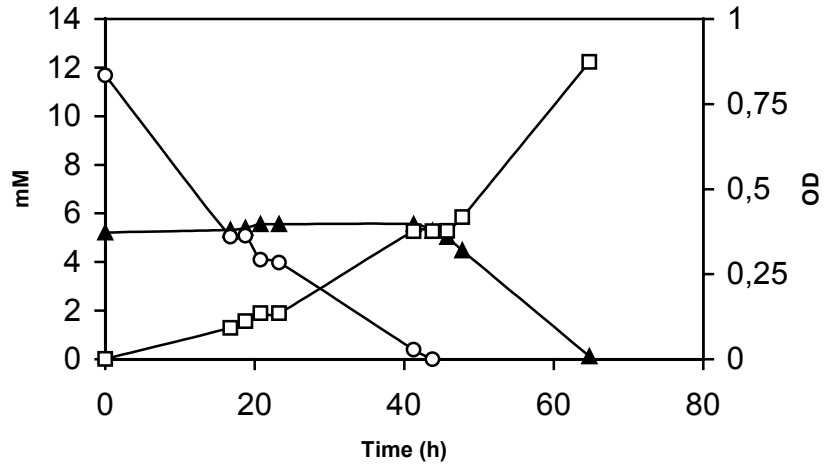

Fig. 3A Competition experiment in strict anaerobic medium with chlorate and oxygen addition at the start of the experiment. (▲) chlorate, (○) oxygen, (□) optical density (430 nm).

With chlorate as electron acceptor, growth of strain AW-1^T was observed with acetate, propionate, glucose, maltose, gluconate, mannitol, glycerol, ethanol, starch or citrate as electron donors and carbon-sources. No growth was observed with malate, succinate, lactate, glycine, arabinose or H_2 (+ CO_2). In the absence of chlorate or oxygen, glucose and maltose were not fermented. The pH range in strictly anaerobic medium was defined by the buffer capacity of bicarbonate/carbon dioxide. A pH range of 6.6 (100% CO_2) till 9.0 (100% N_2) was obtained. In this medium growth occurred within a pH range of 8.0-9.0, with an optimum at pH 8.5. In nutrient broth medium growth occurred within a pH range of 7.0-9.0 with an optimum at pH 7.5. For *P. stutzeri* DSM 5190^T a pH optimum of 7.0 was described, although growth was possible till pH 9 (Van Niel and Allen, 1952). Because strictly anaerobic medium was used for isolation, the temperature and salinity range were determined in this medium. Strain AW-1^T could grow in a NaCl concentration range of 1 to 40 g NaCl per liter. Highest growth rates were obtained between 20 and 40 g/l. However, at these high NaCl concentrations, in the stationary phase cells lysed more rapidly. The optimal temperature was 30ºC with lower and upper limits of 10 and 37ºC.

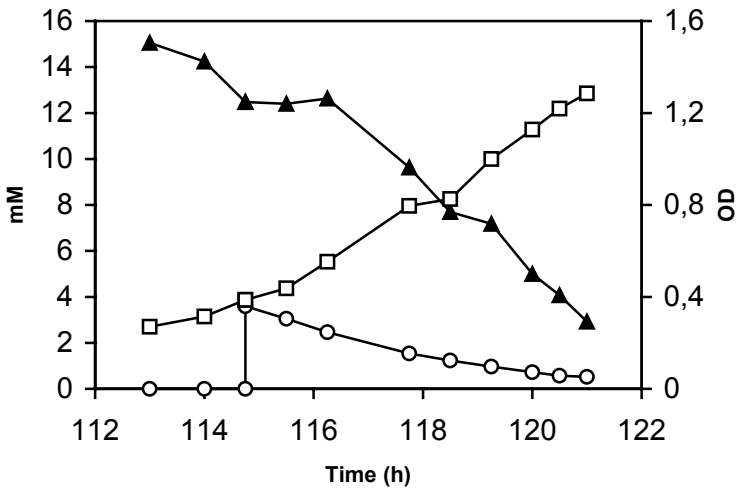

Fig. 3B Competition experiment in strict anaerobic medium with chlorate (▲). At t = 115 hours, oxygen (○) is added to the culture. (□) optical density (430 nm).

Phylogenetic, physiological and biochemical comparison of strain AW-1^T and *P. stutzeri* strains

16S rDNA sequencing showed that strain AW-1^T belongs to the gamma subclass of the *Proteobacteria*. The 16S rDNA sequence of strain AW-1^T showed 100% similarity to the 16S rDNA sequence of *P. stutzeri* DSM 50227 and 98.6% similarity to the 16S rDNA sequence of *P. stutzeri* DSM 5190^T. The 16S rDNA similarity between strain AW-1^T and *P. stutzeri* sp PK was 99.4%. The latter strain was isolated by Coates *et al.* (1999)[a] and it is also a chlorate-reducing bacterium. In Fig. 4 the phylogenetic position of strain AW-1^T is shown. The figure shows that chlorate-reducing bacteria are present in all subclasses of the *Proteobacteria*, making it a phylogenetically diverse group of microorganisms.

The G + C content of strain AW-1^T is 63.9 mol %. The G + C content of *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T are 63.7 ± 0.17 and 65.0 ± 1.00 mol %, respectively (Rosselló *et al*., 1991). DNA-DNA hybridization using strain AW-1^T and *P. stutzeri* DSM 50227 or *P. stutzeri* DSM 5190^T showed 80.5 % and 56.5 % similarity, respectively. DNA-DNA hybridization between *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T showed 48.4 % similarity. It is agreed that when two strains are compared and the similarity of 16S rDNA sequences is greater than 97 % the strains might be members of the same species (Stackebrandt and Goebel, 1994).

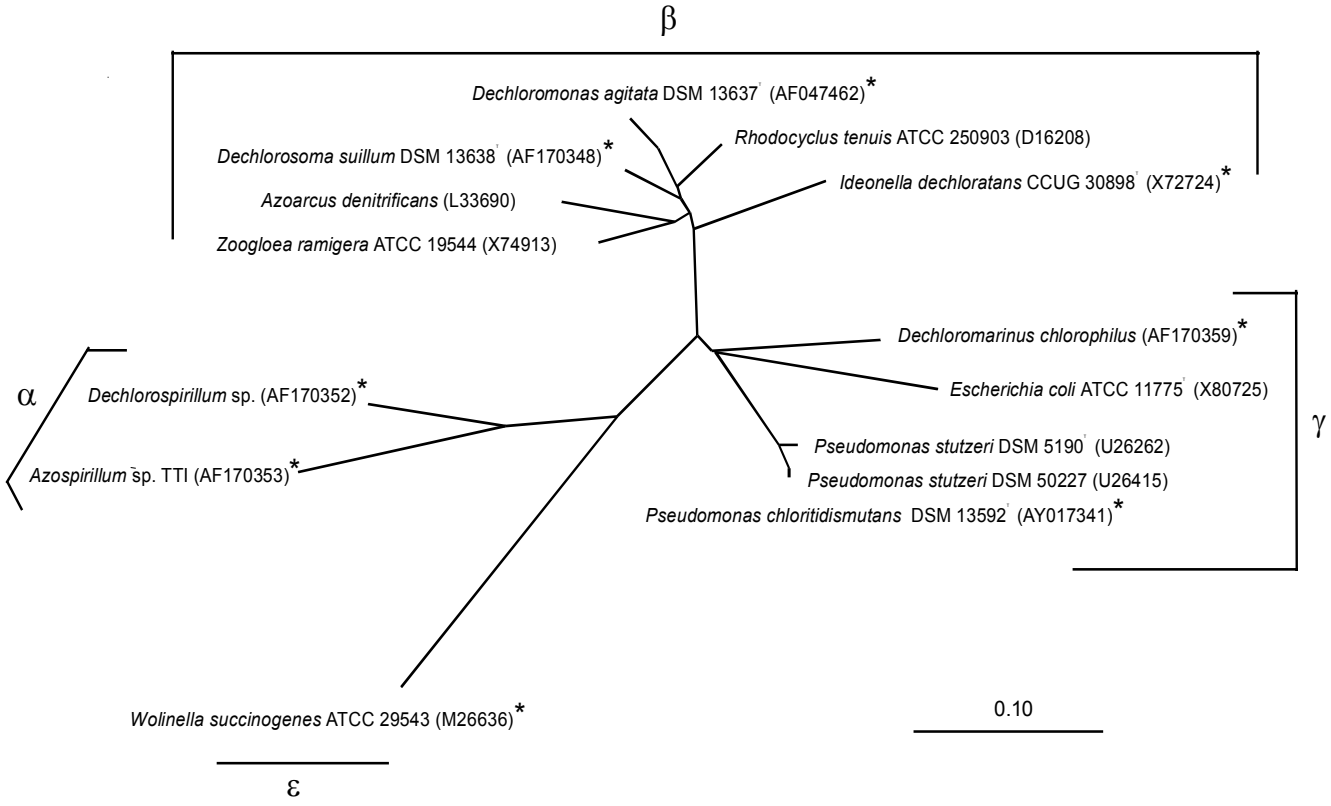

Fig. 4 Neighbor joining tree based on 16S rDNA sequences showing the phylogenetic position of strain AW-1^T among the subclasses of *Proteobacteria*. Chlorate-reducing bacteria are indicated with (*). The bar indicates 0·1 evolutionary distance.

Because the 16S rDNA molecule is small and contains little information, priority is given to DNA-DNA hybridization. To differentiate two species, the DNA similarity should be less than 60-70% (Stackebrandt and Goebel, 1994). According to these criteria, strain AW-1^T, and also strain *P. stutzeri* DSM 50227, are not related to *P. stutzeri* DSM 5190^T at the species level. Instead, strain AW-1^T and *P. stutzeri* DSM 50227 are related at the species level. Many studies have demonstrated that *P. stutzeri* species contain a high degree of phenotypic and genotypic heterogeneity (Stanier *et al*., 1966; Palleroni *et al*., 1970; Rosselló *et al*., 1991). DNA-DNA hybridization (based on calculation of delta T_m values, which were used to evaluate the quality of the pairing within heteroduplexes formed by DNA-DNA hybridization) together with G + C content studies showed the existence of eight genomic

groups, called genomovars (Rosselló *et al*., 1991; Rosselló-Mora *et al*., 1996). This subdivision was confirmed by 16S rDNA sequencing (Bennasar *et al*., 1996), lipid analysis, protein analysis (Rosselló-Mora et al., 1994), macrorestriction fragment analysis of genomic DNA (Ginard *et al*., 1997) and internally-transcribed 16S-23S rRNA gene spacer regions (Guasp *et al*., 2000). Based on differences in 16S rRNA sequences together with differential phenotypic traits, genomovar 6 was renamed to *Pseudomonas balearica* (Bennasar *et al*., 1996). Because DNA-DNA hybridization experiments in this study showed that *P. stutzeri* DSM 5190^T was not related at the species level to strain AW-1^T and *P. stutzeri* DSM 50227, physiological and biochemical characterization between these strains were done for further description of strain AW-1^T. *P. stutzeri* is known to be a vigorous denitrifier that is capable of growth on nitrate, nitrite and nitrous oxide (van Niel and Allen, 1952; Palleroni *et al*., 1970; Bergan, 1981; Zumft, 1997). Growth experiments were done in a strictly anaerobic medium, a synthetic medium and a nutrient broth medium. In all three media, strain AW-1^T was able to couple chlorate reduction to growth, whereas the *P. stutzeri* strains were not. Conversely, nitrate reduction only occurred in synthetic and nutrient broth media inoculated with *P. stutzeri* strains, but not with strain AW-1^T. Apparently, strain AW-1^T is a chlorate reducing bacterium which does not possess another oxyanion reduction pathway. These results were confirmed by the data derived from the enzyme assays. Strain AW-1^T showed only chlorate and bromate reductase activity. The specific activity was 9.0 U/mg and 8.6 U/mg for chlorate and bromate, respectively. Although *P. stutzeri* strains could not couple growth to chlorate reduction, the enzyme assays revealed chlorate reductase and nitrate reductase activity. The specific reductase activity for strain *P. stutzeri* DSM 50227 was 0.9 U/mg and 1.6 U/mg for nitrate and chlorate, respectively. Strain *P. stutzeri* DSM 5190^T showed a specific reductase activity of 4.3 U/mg and 3.4 U/mg for nitrate and chlorate, respectively. These data suggest that *P. stutzeri* contains a nitrate reductase with chlorate-reducing activity. Apparently, the bacterium cannot gain energy from chlorate reduction. This confirms findings from other denitrifying bacteria (De Groot and Stouthamer,1969; Oltmann *et al*. ,1976[b]). *P. stutzeri* cannot grow on chlorate most likely because the chlorite dismutase is lacking; no activity of this enzyme was found in *P. stutzeri* strain DSM 50227 and DSM 5190^T. Chlorate is reduced but the product chlorite is not dismutated into chloride and oxygen. Chlorite may be toxic for these bacteria. Extracts of strain AW-1^T did show chlorite dismutase activity with a specific activity of 134 U/mg of protein. Strain AW-1^T, *P. stutzeri* strain DSM 50227 and DSM 5190^T contain an enzyme to reduce chlorate, but the presence of

chlorite dismutase differs strain AW-1^T from *P. stutzeri* strain DSM 50227 and DSM 5190^T. This enzyme seems to be essential to allow growth with chlorate as electron donor.

Strain AW-1^T showed 100% 16S rDNA similarity and 80·5 % DNA-DNA hybridization similarity with *P. stutzeri* DSM 50227. However, comparison with *P. stutzeri* DSM 5190^T showed that strain AW-1 is not related with the type strain at the species level. Also physiological and biochemical comparison of strain AW-1^T to *P. stutzeri* DSM 50227 and *P. stutzeri* DSM 5190^T showed that these strains were differentiated with respect to the ability of dissimilatory chlorate reduction, chlorite dismutation and denitrification. Therefore, the name *Pseudomonas chloritidismutans* sp. nov. is proposed for strain AW-1^T.

Description of *Pseudomonas chloritidismutans* sp. nov.

Pseudomonas chloritidismutans [chlo.ri.ti.dis'mu.tans. N.L. n. chloris chlorite, L. part. pres. dismutans splitting, N.L. part. pres. chloritidismutans chlorite splitting].

Cells are Gram-negative, facultative anaerobic motile rods. The size of the cells is 0.5-2 μm. Bacteria growing in anaerobic agar are circular and pale orange. Colonies on nutrient broth plates are wrinkled, coherent and have a pale brown or sometimes reddish brown color. In strictly anaerobic sulfide reduced medium the following growth characteristics are: growth range 10-37ºC; optimum 30ºC. Growth in pH range 8.0-9.0, optimum pH 8.5. In nutrient broth medium growth in pH range 7.0-9.0, optimum pH 7.5. Doubling times 1.5 h and 1.2 h for chlorate and oxygen, respectively. Growth occurs in media containing up to 40 g/l NaCl. At high NaCl concentration cells in stationary phase may lyse. Growth on acetate, propionate, glucose, maltose, gluconate, mannitol, ethanol, starch, glycerol or citrate. No growth on malate, succinate, lactate, glycine, arabinose or H_2. Glucose and maltose are not fermented. Chlorate and oxygen are used as electron acceptors. No growth with perchlorate, chlorite, nitrate, nitrite, bromate, sulfate or selenate. Cell extracts contain chlorate- and bromate reductase activity. Chlorite is converted to chloride and oxygen by a chlorite dismutase. The type strain belongs to the gamma subclass of the *Proteobacteria*. 16S rDNA sequence is 98.6 % similar to *P. stutzeri* DSM 5190^T. The sequence has been deposited in the GenBank under accession number AY017341. DNA-DNA hybridization between strain AW-1^T and *P. stutzeri* DSM 5190^T showed 56.5% similarity. The G + C content is 63.9 mol %. The type strain has been deposited in the culture collection of the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany) as DSM 13592^T and the culture collection of the

American Type Culture Collection (ATCC, Manassas, USA) as ATCC BAA-443^T. *Pseudomonas chloritidismutans* was obtained from biomass of an anaerobic bioreactor treating chlorate and bromate polluted waste water.

Acknowledgment

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Chapter 3

Characterization of the Chlorate Reductase from *Pseudomonas chloritidismutans*

Published in modified form as : **Wolterink, A.F.W.M., Schiltz, E., Hagedoorn, P.-L., Hagen, W.R., Kengen, S.W.M. and Stams, A.J.M.** (2003) Characterization of the chlorate reductase from *Pseudomonas chloritidismutans*. J Bacteriol **185**:3210-3213.

Abstract

Pseudomonas chloritidismutans **was recently isolated from biomass of an anaerobic chlorate-reducing bioreactor. This bacterium converts chlorate completely to chloride, with the transient formation of chlorite.** ***P. chloritidismutans*** **is not able to use electron acceptors other than oxygen and chlorate for growth. In this study, the purification and characterization of the chlorate reductase is described. The oxygen-sensitive enzyme showed chlorate and bromate reducing activity. In contrast to the characterized (per)chlorate reductase from strain GR-1, no reductase activity was found with perchlorate or nitrate. The enzyme has a cytoplasmic orientation, which differs from the periplasmic localization of the (per)chlorate reductase of GR-1 and the chlorate reductase of** ***Ideonella dechloratans*** **and the localization of chlorate reductase C of** ***Proteus mirabilis*** **in the membrane. Nevertheless, comparison with membrane bound nitrate reductases (NAR) suggest that the chlorate reductase is likely to be membrane bound in order to take part in the respiratory chain. The molecular mass of the enzyme is approximately 167 kDa, with subunits of 97, 38 and 34 kDa likely arranged in an heterotrimer $\alpha_1 \beta_1 \gamma_1$ composition. N-terminal sequence analysis showed partial sequence similarity of the β-subunit to β-subunits of nitrate reductases of denitrifying bacteria, selenate reductase of** ***Thauera selenatis*** **and the chlorate reductase of** ***I. dechloratans*****. No comparable N-terminal sequence was found for the α and γ-subunits. The K_m and V_{max} value for chlorate is 159 µM and 51 U/mg, respectively. Chlorate reductase showed maximum activity at pH of 7.5 and at a temperature of 75ºC. Metal analysis demonstrated the presence of iron and molybdenum. Quantitative electron paramagnetic resonance spectroscopy showed unusual signals with all g-values greater than the free-electron value, g_e=2.002, which are likely to be associated to Mo(V). Mo(V) is also found in other oxyanion reductases. The results of this chapter suggest that the chlorate reductase of** ***P. chloritidismutans*** **is essentially different from the (per)chlorate reductase of strain GR-1, the chlorate reductase of** ***I. dechloratans*** **and nitrate reductases.**

Introduction

Pseudomonas chloritidismutans (strain AW-1^T) is a recently isolated facultative anaerobic chlorate-reducing bacterium (Wolterink *et al*., 2002). In chlorate-reducing bacteria, chlorate is first reduced to chlorite by a chlorate reductase and in a second reaction chlorite is disproportionated to chloride and oxygen by a chlorite dismutase. These enzyme activities have been demonstrated in cell-free extracts of *P. chloritidismutans* (Wolterink *et al*., 2002). Two closely related strains of *P. chloritidismutans, Pseudomonas stutzeri* DSM 50227 and DSM 5190^T (with 16S rDNA similarities of 100% and 98.6% , respectively) were not able to grow by dissimilatory chlorate reduction. Accordingly, both *P. stutzeri* strains lack chlorite dismutase activity. However, in addition to nitrate reductase activity, these strains showed chlorate reductase activity, which has been observed before for other denitrifying bacteria (De Groot and Stouthamer, 1969; Oltmann *et al*., 1976[a,b]). From *Salmonella typhimurium*, a trimethylamine-N-oxide reductase was purified which showed chlorate reductase activity (Kwan and Barrett, 1983). However, also this bacterium could not grow with chlorate.

P. chloritidismutans could be differentiated from other chlorate-reducing bacteria. Cell extracts of *P. chloritidismutans* showed besides chlorate reductase activity only bromate reductase activity. However, *P. chloritidismutans* could not grow with bromate as electron acceptor. Apparently, *P. chloritidismutans* did not possess an oxyanion reduction pathway other than the chlorate reduction pathway. Other chlorate-reducing bacteria can also use perchlorate or nitrate for growth (Logan, 1998; Achenbach *et al*., 2001).

Sofar, three enzymes have been purified and characterized as (per)chlorate reductases. From the denitrifying strain *Proteus mirabilis*, a chlorate reductase C and two nitrate reductases have been purified (Oltmann *et al*., 1976[a,b]). The only known substrate for chlorate reductase C is chlorate, which is reduced to chlorite. It was not demonstrated that *Proteus mirabilis* can to couple the reduction of chlorate to growth. A second (per)chlorate reductase has been purified from strain GR-1 (Kengen *et al*., 1999). One enzyme is responsible for both chlorate and perchlorate reduction. Besides (per)chlorate, nitrate, iodate and bromate are also reduced by the (per)chlorate reductase of strain GR-1. Perchlorate-grown cells of strain GR-1 are unable to convert nitrate or nitrite, indicating that another nitrate reductase may be involved in nitrate-grown cells (Rikken *et al*., 1996). A third chlorate reductase has been isolated from *Ideonella dechloratans* (Danielsson Thorell *et al*., 2003). The enzyme is composed of three subunits, and catalyzed besides chlorate reduction, nitrate, iodate, bromate and selenate reduction.

In the present paper, the purification and characterization of the chlorate reductase of *P. chloritidismutans* is reported.

Materials and methods

Organisms and Culture Conditions

P. chloritidismutans (DSM13592) was grown under strictly anaerobic medium at 30°C, as described before (Wolterink *et al*., 2002). Strain GR-1 (DSM 11199) was grown in a mineral medium supplemented with 0.02 g of yeast extract/liter, as described previously (Kengen *et al*., 1999; Rikken *et al*., 1996). Chlorate (10 mM) was used as electron acceptor, while acetate (10 mM) was the electron donor and carbon source. For cultivation, 3 liter bottles were filled with 2 liter medium. The bottles were closed with butyl rubber stoppers and the gas phase was exchanged for 100% N_2.

Preparation of cell extract

Chlorate reductase of *P. chloritidismutans* showed to be sensitive to oxygen. Therefore, the preparation of cell extracts and all purification steps were done under anaerobic conditions in an anaerobic glove box. Cells were collected by centrifugation at 9,000 rpm for 10 min at 4°C. The cell pellet was suspended (1:2 [wt/vol]) in 15 mM potassium/sodium phosphate buffer, pH 7.2 (= buffer A). The cells were disrupted by ultrasonic disintegration (Sonics and Materials inc., Danbury, CT) at 40 K_c/sec for 30 sec followed by cooling for 30 sec on ice. This cycle was repeated 5 times. Cell debris and whole cells were removed by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant fraction was subjected to an ultracentrifuge step, 110,000 × g for 1 h at 4ºC. This resulted in a red supernatant, containing the cytoplasmic and periplasmic fraction. The pellet was suspended in buffer A (= membrane fraction).

Enzyme purification

All purification columns were placed in an anaerobic glove box containing an atmosphere of 96% N_2 and 4% H_2. Oxygen was removed from the buffer solutions by flushing with N_2 gas. Enzyme purification was started by loading the red supernatant fraction onto a Q-Sepharose column (2 by 30 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The chlorate reductase eluted just after the chlorite dismutase, at the start of a linear gradient of 0 to 1 M potassium chloride in 50 mM Tris-HCl buffer, pH 7.5. Active fractions were pooled

and desalted by ultrafiltration (150 ml stirred cell, filter pore size 10 kDa, Filtron Technology). The concentrated enzyme fraction was diluted with 10 mM Tris-HCl, pH 7.2. This fraction was loaded on a hydroxy apatite column equilibrated with 10 mM Tris-HCl, pH 7.2. Again, the chlorate reductase eluted from the column at the start of a linear gradient of 10 mM Tris-HCl, pH 7.2 to 450 mM potassium phosphate, pH 7.2. Active fractions were combined and loaded onto a Mono-Q column which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Chlorate reductase eluted from the column at the start of a linear gradient of 0 to 1 M potassium chloride in 50 mM Tris-HCl buffer, pH 7.5. A 400 µl aliquot of the active fraction was subsequently loaded onto a Superdex 200 column (1.6 by 70.5 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl. The chlorate reductase was eluted in the same buffer, and fractions were kept at 4ºC for further analysis.

Enzyme assays

Chlorate reductase activity was measured anaerobically in stoppered glass cuvettes, by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm and 30ºC in a Hitachi spectrophotometer (U-2010) (Kengen *et al*., 1999). The assay mixture consisted of 1 ml of 50 mM Tris-HCl buffer (pH 7.5), containing 0.5 mM MV. The assay mixture was reduced by adding a small amount of a sodium dithionite solution (0.2 M) until an absorbance of 1.5 was reached. The reaction was started by adding 10 µl of a chlorate solution (100 mM) and 10 µl of cell or enzyme fraction. In samples with high activities appropriate dilutions were made. The following electron acceptors (all sodium salts) were tested in the same way: ClO_4^-, ClO_2^-, NO_3^-, NO_2^-, BrO_3^-, SO_4^{2-}, SeO_4^{2-}, IO_3^- or IO_4^-. Kinetic parameters were obtained by a computer-aided direct fit of the Michaelis-Menten curve. The chlorate concentration was varied between 10 µM and 4 mM (final concentration). The enzyme activity was measured at different pH values (5.5-10) and temperatures (30-90ºC). Chlorite dismutase and catalase activity were determined by measuring the oxygen production with a Clark-type Electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA). The reaction vessel was kept at 30°C. The reaction mixture contained 2.8 ml buffer A (pH 7.2). The reaction was started by injecting a concentrated solution of sodium chlorite into the vessel to give a final concentration of 0.2 mM. 10 µl of enzyme fraction was added and for samples with high activities, appropriate dilutions were made. The reaction was run for 5 min but only the initial linear part was used to calculate the rate. One unit (U) is defined as the amount of enzyme required to convert 1 µmol of substrate per minute. The protein content of the enzyme

fractions was determined according to the method of Bradford, with bovine serum albumin (BSA) as standard (Bradford, 1976).

Localization of chlorate reductase

For localization of the chlorate reductase, a fresh culture of *P. chloritidismutans* or strain GR-1 was centrifuged for 10 min at 9,000 rpm at 4ºC. The cell pellet was suspended (1:2 [wt/vol]) in EDTA-buffer (50 mM Tris, 50 mM EDTA, 170 mM Na_2CO_3, pH 9) (Sebban *et al*., 1995). This cell suspension was incubated for 30 min at room temperature followed by a centrifugation step (13,000 rpm, 10 min at 4ºC). The red supernatant (periplasmic fraction) was separated from the pellet. The pellet was suspended in buffer A (1:2 [wt/vol]). The cells (checked with light microscopy) were disrupted by ultrasonic disintegration as described above. Cell debris were removed by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant fraction was subjected to ultracentrifugation at 110,000 × g for 1 h at 4ºC. This step yielded a red supernatant, which is the cytoplasmic fraction. The pellet (membrane fraction) was suspended in 2 ml buffer A. A more gentle method to disrupt intact cells is a freeze-thaw procedure (Yoshimatsu *et al*., 2000). A cell pellet was suspended in buffer A. After the suspension was frozen in liquid N_2, it was thawed by immersing it in running water (40º). This procedure was repeated four times. A small amount of DNAse I (Sigma) was added to reduce the viscosity due to the released DNA. The solution was centrifuged at 13,000 rpm, 10 min at 4ºC. The supernatant (cell-free extract) was centrifuged at 45,000 rpm (110,000 × g) for 1 h at 4ºC. The supernatant (cytoplasmic and periplasmic fraction) was separated from the precipitate. The precipitate (membrane fraction) was suspended in buffer A.

Gel electrophoresis and determination of the molecular mass

Sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) with a 12% acrylamide gel. The gels were stained with Coomassie Brilliant blue R250. To calculate the molecular mass of the subunits of the chlorate reductase, the gel was calibrated using the following standard proteins (size in kDa): myosin (200), ß-galactosidase (116), phosphorylase b (97), serum albumin (66), ovalbumin (45), and carbonic anhydrase (31). The elution volume on a calibrated Superdex 200 column of the chlorate reductase was used to estimate the molecular mass of the native enzyme. The Superdex 200 column was calibrated with high molecular weight markers (size in kDa) : blue

dextran (2000), thyroglobulin (669), ferritin (440), catalase (232), aldolase (158), bovine serum albumin (67), ovalbumin (43), chymotrypsinogen (25) and ribonuclease (14).

N-terminal Sequencing of the subunits

For determination of the N-terminus of the subunits, the subunits were separated using SDS-PAGE as described above. However, gels were not stained with Coomassie brilliant blue R250. The subunits were transferred from the SDS polyacrylamide gel onto a Sequi-Blot^{TM} PVDF Membrane (Biorad) by blotting with a Biorad mini-protean blot module. Blotting was carried out in a buffer containing 200 ml CAPS (3-cyclohexylamino-1-propanesulfonic acid)-buffer (22.13 g CAPS l^{-1}, pH 11), 200 ml methanol and 1.6 l milli-Q. After staining of the transferred protein with Ponceau S staining solution (0.2% Ponceau S, 3% TCA (trichloroacetic acid) and 3% sulfosalicylic acid), the bands were cut from the membrane. N-terminal sequencing of the electroblotted proteins was performed in an automated protein sequencer with on-line analysis of the phenylthiohydantoin amino acids (Models 477A and 120A, Applied Biosystems, Foster City, USA) following the manufacturers instructions. N-terminal sequences were compared to other sequences using the protein-protein Blast program available at the website of the National Center for Biotechnology Information (NCBI).

EPR of chlorate reductase and metal analysis

Electron paramagnetic resonance spectra (EPR) were recorded on a Bruker ER-200D spectrometer with peripheral equipment and data handling as described previously (Pierik and Hagen, 1991). The modulation frequency was 100 kHz. The presence of metals was measured by inductively coupled plasma mass spectrometry (ICP-MS) (Elan 6000, Perkin-Elmer).

Materials

Q-sepharose, Superdex 200 and Mono-Q HR 5/5 were purchased from Pharmacia (Woerden, The Netherlands). Hydroxyapatite (CHT5-I, Bio-Scale), protein standards for gel filtration and SDS-PAGE standards were purchased from Bio-Rad (Veenendaal, The Netherlands). Methyl viologen and sodium dithionite were from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). All other chemicals were of analytic grade.

Results

Purification of the chlorate reductase

The first purification step was anion-exchange chromatography with Q-sepharose. This was different from the purification of the chlorate reductase from strain GR-1 (Kengen *et al*., 1999), which was a cation-exchange column (S-sepharose). The chlorate reductase was separated from a red colored fraction, which eluted just before the chlorate reductase, and contained the chlorite dismutase. On the other columns used, the chlorate reductase eluted at the start of the linear gradient. These fractions had a brown-yellow color. In Table 1 the results of the purification are shown.

Table 1 Summary of the purification of chlorate reductase form *P. chloritidismutans*

Step	Volume (ml)	Protein concentration (mg/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Soluble fraction	13	27.5	726	2	100	1
Q-Sepharose	20	2.8	335	6	46.1	3
Hydroxy apatite	5	3.5	155	8.7	21.4	4.4
Mono-Q	2	3.3	95	14.4	13.1	7.2
Superdex 200	10	0.18	75	42	10.3	21

Analysis by SDS-PAGE of the different column fractions is shown in Fig. 1. The molecular weight for the native enzyme was about 167 kDa as determined by gel filtration using the calibrated Superdex 200 column. Three bands were obtained, with molecular masses of 97, 38 and 34 kDa, when the Superdex 200 fraction was run on SDS-PAGE (Fig. 1, lane 5).

Catalytic activity

For chlorate, K_m and V_{max} values of 159 μM and 51 U/mg were found, respectively. Besides chlorate, only bromate served as electron acceptor. Under standard conditions, a specific activity of 50 and 26 U/mg, was found for chlorate and bromate, respectively. Cell extract lost chlorate reductase activity when stored under air. When purified chlorate reductase was stored under 100% oxygen atmosphere, the activity decreased by 50% in 45 min. The temperature optimum of the enzyme was 75ºC. The optimum pH for the chlorate

reductase was pH 7.5. At pH 5.5 and 10, the specific activity was approximately 50% of the optimal value at pH 7.5.

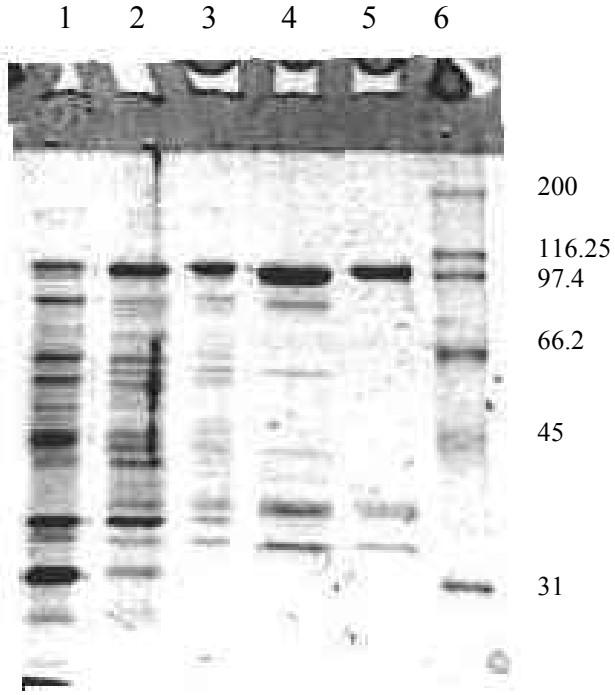

Fig. 1 SDS-PAGE of the chlorate reductase from *P. chloritidismutans*. *Lane 1*, soluble fraction; *lane 2*, Q-Sepharose pool; *lane 3*, hydroxyapatite pool; *lane 4*, MonoQ pool; *lane 5*, Superdex 200 pool; *lane 6*, set of marker proteins with indicated molecular masses. Coomassie Brilliant Blue R250 was used to stain the proteins.

Analysis of the N-terminal sequences

Table 2 shows the N-terminal sequence of each subunit. The α-subunit and γ-subunit gave no similarity to known N-terminal sequences. However, the β-subunit showed similarity to several N-terminal sequences of the β-subunits of nitrate reductases. The β-subunit of the dimethylsulfide dehydrogenase of *Rhodovulum sulfidophilum* (McDevitt *et al*., 2002), the β-subunit of the selenate reductase of *Thauera selenatis* (Schröder *et al*., 1997), the (per)chlorate reductase of strain GR-1 (Kengen *et al*., 1999) and the chlorate reductase of *Ideonella dechloratans* (Danielsson Thorell *et al.,* 2003) revealed several identical amino acids.

Table 2 N-terminal sequences of the three chlorate reductase subunits of *P. chloritidismutans*. Similarity of β-subunit was compared to N-terminal sequences of other reductases

α subunit	L	N	M	L	E	P	V	G	E	T	L	A	S	E	Y	P	Y	X	K							
β subunit			T	**V**	**K**	X	**Q**	**L**	**S**	**M**	**V**	**L**	**D**	**L**	**N**	**K**	E	**I**	**G**	G	Q	**T**	**X**	**T**	A	**A**
(a)							**Q**	V	A	**M**	**V**	**L**	N	**L**	D	**K**	C	**I**	**G**	C	H	**T**	C	S	V	T	C	K	N	T	W	T
(b)			G	**V**	D	H	**Q**	V	A	**M**	**V**	M	**D**	**L**	**N**	**K**	C	**I**	**G**	C	**Q**	**T**	C	**T**	I	**A**	C	K	N	L	W	T
(c)		M	K	I	**K**	A	**Q**	V	A	**M**	**V**	**L**	N	**L**	D	**K**	C	**I**	**G**	C	H	**T**	C	S	V	T	C	K	N	T	W	
(d)			K	A	**K**	R	**Q**	**L**	V	T	**V**	I	**D**	**L**	**N**	**K**	C	L	**G**	C	**Q**	**T**	C	**T**	V	**A**						
(e)				S	Q	R	**Q**	**L**	A	Y	**V**	F	**D**	**L**	**N**	L	X	**I**	**G**	X	H	**T**	**X**	**T**	M	**A**	X	K	Q	L	W	T
(f)	A	N	V	M	K	A	P	R	R	**Q**	**L**	T	Y	**V**	T	**D**	X	**N**														
(g)				M	S	Q	R	**Q**	V	A	Y	**V**	F	**D**	**L**	**N**	**K**	C	**I**	**G**	C	H	**T**	C	**T**	M	**A**	C	K	Q	L	W	T
(h)					**V**	**K**	R	**Q**	I	**S**	**M**	**V**	**L**	**D**	**L**	**N**	**K**	C	**I**	**G**	C	**Q**	**T**	C	**T**	S	**A**						
γ subunit	E	X	S	E	Q	N	P	N	I	L	E	I	K	P	G	D	T	V	K	V	X	T										

(a)	NarH *S. carnosus*	acc nr: AAC82543
(b)	β subunit nitrate reductase *H. marismortui* subsp *marismortui*	acc nr: CAB89112
(c)	β subunit nitrate reductase *S. aureus* subsp *aureus* MU50	acc nr: NP372920
(d)	Anaerobic ethylbenzene dehydrogenase subunit B, *Azoarcus* sp EB1	acc nr: AF337952
(e)	β subunit selenate reductase *T. selenatis*	Schröder *et al*. 1997
(f)	β subunit chlorate reductase strain GR-1	Kengen *et al*., 1999
(g)	β subunit chlorate reductase *I. dechloratans*	Danielsson Thorell *et al*., 2003
(h)	β subunit dimethylsulfide dehydrogenase *Rhodovulum sulfidophilum*	McDevitt *et al*., 2002

acc nr: α subunit, Swiss-Prot P83448; β subunit, Swiss-Prot P83449; γ subunit, Swiss-Prot P83450

Localization

Activity of chlorate reductase, chlorite dismutase and catalase were determined in different cell fractions (Table 3). Chlorate reductase activity was mainly found in the cytoplasmic fraction. In contrast to the chlorate reductase, chlorite dismutase activity was predominantly found in the periplasmic fraction. Catalase activity was only found in the cytoplasmic fraction (Table 3). This indicates that the method used in this study is effective to discriminate between a periplasmic and cytoplasmic localization of enzymes. Localization activity measurements previously done with cells of strain GR-1, showed chlorate reductase activity in the periplasmic fraction (Kengen *et al*., 1999). However, in that study, a different protocol was used, which included the preparation of spheroplasts. Therefore, in the present study, cells of strain GR-1 were also subjected to the EDTA treatment. Following this procedure,

chlorate reductase activity was again found in the periplasmic fraction (3.4 U/mg). No activity was found in the cytoplasmic fraction, confirming the earlier results.

Table 3 Localization of chlorate reductase, chlorite dismutase and catalase activity in cell fractions
Periplasmic fraction (P), Cytoplasmic fraction (C), Membrane fraction (M). n.a., no activity

	ClO_3^- red (U/mg)	ClO_3^- red (U total)	ClO_2^- dism (U/mg)	ClO_2^- dism (U total)	Catalase (U/mg)	Catalase (U total)
AW-1, fraction P	6.2	7.5	236	284	n.a.	n.a.
AW-1, fraction C	29.6	47.2	20.6	32.7	15.7	24.9
AW-1, fraction M	3.8	2	9.3	4.8	3.3	1.7

By disrupting cells with a ultrasonic oscillating device, it was shown earlier that nitrate reducing activity was easily released into the soluble fraction (Yoshimatsu *et al*., 2000). In that study a more gentle method, a freeze-thaw procedure, was used to purify nitrate reductase from the membrane fraction. This procedure was also used to see if chlorate reductase could be purified from the membrane fraction. Using the freeze-thaw method, again chlorate reductase activity was mainly found in the soluble fraction and resulted in the highest chlorate reductase activity in cytoplasmic- periplasmic fraction (95 U), compared to the membrane fraction (8 U).

EPR and metal analysis

Anaerobically purified enzyme exhibited an unusual signal with all g-values greater than the free-electron value, g_e=2.002, namely g_z=2.076 and g_{xy}=2.024 (Fig. 2, trace A). Double integration versus an external copper standard gives a spin count of 0.2 S=1/2 per 167 kDa $\alpha_1 \beta_1 \gamma_1$ heterotrimer. The signal is very similar in shape and integrated intensity to that of the $\alpha_3\beta_3$ heterohexameric (per)chlorate reductase from strain GR-1 (Kengen *et al*., 1999). We have previously suggested that this signal could come from Mo(V) with Se coordination (Kengen *et al*., 1999). In line with this suggestion we find the intensity of the present signal to reduce to 0.13 spins upon incubation with sodium dithionite; it further diminishes to 0.05 spins upon light reduction with the deazaflavin/EDTA system. The present enzyme did not contain selenium as was measured with ICP-MS.

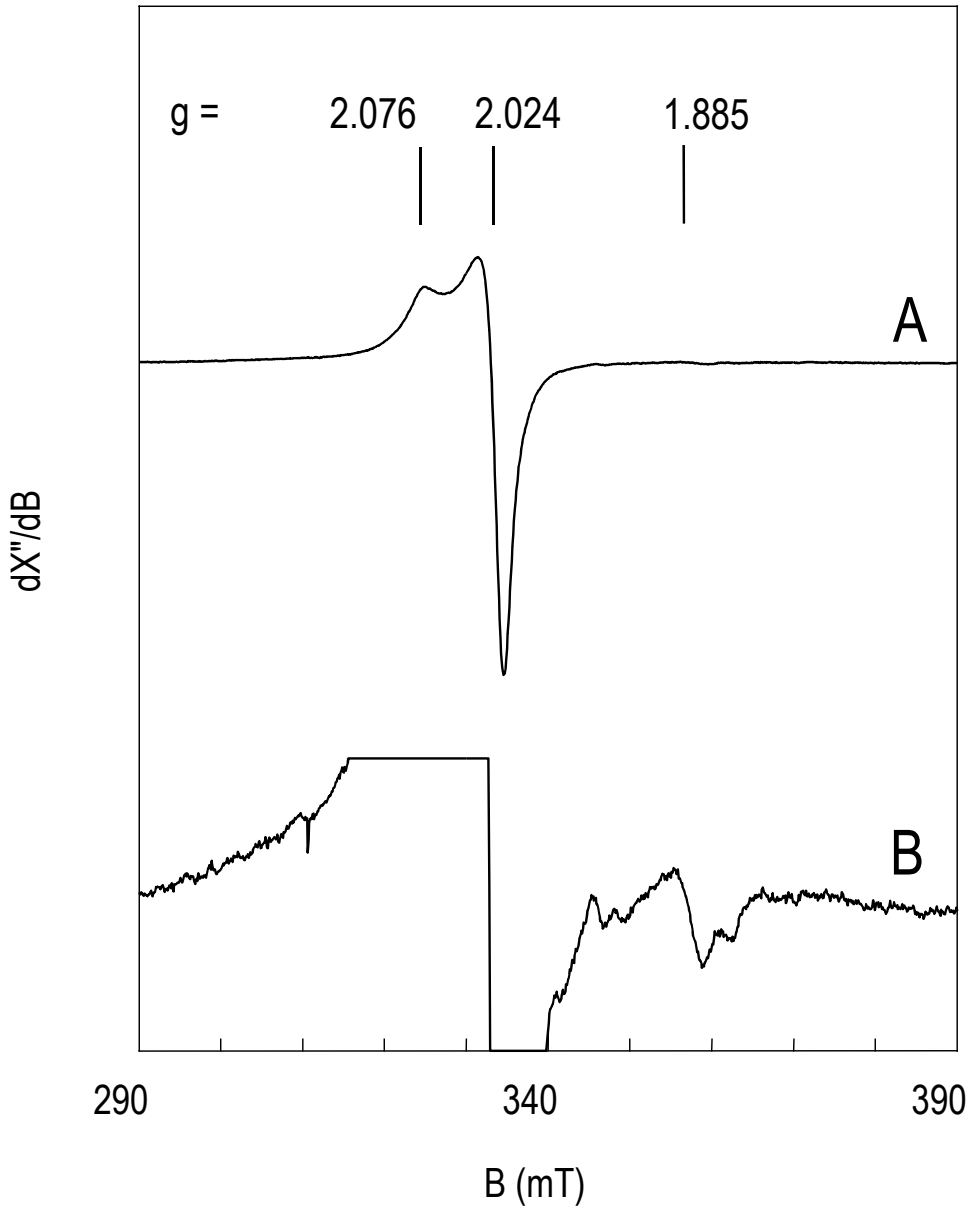

Fig. 2 EPR spectra of the chlorate reductase of *P. chloritidismutans*. The enzyme concentration was 2.3 mg/ml or 14 μM. Trace A is from the enzyme as anaerobically isolated; trace B is from dithionite reduced enzyme. The amplification for trace B is 25x that for trace A. EPR conditions: microwave frequency, 9.43 GHz; microwave power, 126 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 16 K.

The mol ratio of [Fe]/[Mo]≈16 was determined with ICP-MS, for strain GR-1 a mol ratio of [Fe]/[Mo]≈11 was found (Kengen *et al*., 1999). Moreover, in the GR-1 enzyme a complex 2[4Fe-4S]$^{1+}$ spectrum developed upon dithionite reduction with an intensity of approximately 2 spins; oxidation with potassium ferricyanide afforded a [3Fe-4S]$^{1+}$ signal equivalent to 1

spin (Kengen *et al*., 1999). In contrast to this previous observation we found for the *P. chloritidismutans* enzyme only very weak Fe/S signals. The Fe/S spectrum integrates to approximately 1% of the protein concentration. Addition of dithionite causes an increase to some 0.02 spins (Fig. 2, trace B). The amplitude does not increase further upon light reduction.

Discussion

The purified chlorate reductase of *P. chloritidismutans* showed to have many characteristics not described earlier for other known (per)chlorate reductases (Table 4). In addition to the reduction of chlorate, the enzyme showed only bromate reductase activity. Because bromate could not be used as an electron acceptor for growth (Wolterink *et al*., 2002), the chlorate reductase of *P. chloritidismutans* is clearly not involved in another oxyanion dissimilatory reduction pathway. Strain GR-1 contains a single enzyme responsible for both the perchlorate and chlorate reduction activity. Reductase activity was also found for bromate, iodate and nitrate (Kengen *et al*., 1999). For the chlorate reductase C of *Proteus mirabilis* only chlorate reduction was reported; other electron acceptors than nitrate were not tested (Oltmann *et al*., 1976[a,b]). The recently described chlorate reductase of *I. dechloratans*, did not show reductase activity with perchlorate. Besides chlorate reduction, reductase activity was also found for bromate, nitrate, iodate or selenate (Danielsson Thorell *et al*., 2003).

The location of the chlorate reductase of *P. chloritidismutans* in the cytoplasmic fraction was different from the (per)chlorate reductase from strain GR-1, the chlorate reductase of *I. dechloratans* and the chlorate reductase C from *Proteus mirabilis* (Table 4). In contrast to the chlorate reductase, chlorite dismutase activity was predominantly found in the periplasmic fraction. The absence of catalase activity in the periplasmic fraction indicated the effectiveness of the localization method used. It is known that nitrate reductases (NAR) are almost exclusively located in the cytoplasmic membrane (Danneberg *et al*., 1989). By disrupting cells of *H. marismortui*, with a ultrasonic oscillating device, nitrate reducing activity was easily released into the soluble fraction (Yoshimatsu *et al*., 2000). Using a more gentle method (freeze-thaw procedure) cells of *H. marismortui* were disrupted and the nitrate reductase was extracted from the resulting membrane fraction. Therefore, the enzyme was considered to be an extrinsic membrane protein that binds to the surface of the cytoplasmic membrane by hydrophobic interaction. This freeze-thaw procedure was also used for cells of *P. chloritidismutans* which resulted in highest chlorate reductase activity in the

cytoplasmic/periplasmic fraction (95.7 U) compared to the membrane fraction (8.2 U). Therefore, we suggest that the chlorate reductase of *P. chloritidismutans* is cytoplasmic.

Table 4 Characteristics of (per)chlorate reductases of *Proteus mirabilis*, strain GR-1, *P. chloritidismutans* and *Ideonella dechloratans*

	ClO_3^- Reductase C *P. mirabilis**	ClO_4^-/ClO_3^- reductase strain GR-1**	ClO_3^- reductase *P. chloriti-dismutans*	ClO_3^- reductase *I. dechloratans****
Localization	membrane	periplasm	cytoplasm	periplasm
Electron acceptor	chlorate[a]	perchlorate, chlorate, nitrate, iodate and bromate	chlorate, bromate[b]	chlorate, bromate iodate, nitrate, and selenate[c]
Sizes of subunits (in kDa)	75, 63, 56	95, 40	97, 38, 34	94, 35.5, 27
Composition native enzyme	heterotrimer ($\alpha_1 \beta_1 \gamma_1$)	trimer of heterodimers (α_3-β_3)	heterotrimer ($\alpha_1 \beta_1 \gamma_1$)	heterotrimer ($\alpha_1 \beta_1 \gamma_1$)
EPR parameters of Mo(V) (g values)	--	1.976 2.016 2.091	2.024, 2.076	--
V_{max} (U/mg) for ClO_3^-	--	13.2	51	--
K_m (μM) for ClO_3^-	--	less than 5	159	850

([a]) electron acceptors other than ClO_3^- and NO_3^- were not tested

([b]) ClO_4^-, ClO_2^-, NO_3^-, NO_2^-, IO_4^-, IO_3^-, SO_4^{2-} or SeO_4^{2-} were tested but did not show reductase activity

([c]) ClO_4^-, dimethyl sulfoxide (DMSO) and tri-methylamine-N-oxide (TMAO) did not show activity

* Oltmann *et al*. (1976)[a]

** Kengen *et al*. (1999)

*** Danielsson Thorell *et al*. (2003)

For the chlorate reductase of *P. chloritidismutans* three bands of 97, 38 and 34 kDa were found on SDS-PAGE (Fig. 1). The molecular weight of the whole enzyme was about 167 kDa as determined by gel filtration. These results suggest that all subunits are present in a 1:1:1 stoichiometry and that the chlorate reductase is a heterotrimer (α_1 β_1 γ_1). The same stoichiometry was found for the chlorate reductase C from *Proteus mirabilis* and the chlorate reductase of *I. dechloratans*. However the molecular weight of the native enzyme was 183 and 160 kDa for *P. mirabilis* and *I. dechloratans*, respectively. Chlorate reductase of strain GR-1 showed a different stoichiometry resulting in a different molecular weight of the native

enzyme (420 kDa).This enzyme was found to consist of an α and β subunit, but a third γ subunit was suggested.

The Michaelis-Menten constant (K_m = 159 μM) describing the affinity of the enzyme for chlorate, was higher than the K_m value found for strain GR-1 (< 5 μM) but lower than the K_m value found for the chlorate reductase of *I. dechloratans* (850 μM). The K_m value of the chlorate reductase of *P. chloritidismutans* and *I. dechloratans* is comparable to the values of nitrate reductases for nitrate (0.1 to 1.3 mM) (Hochstein and Tomlinson, 1988). Comparing the V_{max} values, *P. chloritidismutans* showed a higher value (51 U/mg) than strain GR-1 (13 U/mg).

Optimal growth conditions for *P. chloritidismutans* were determined at pH 8.5 and at a temperature of 30ºC in strictly anaerobic medium (Wolterink *et al*., 2002). The purified enzyme has a pH optimum of 7.5 and a temperature optimum of 75ºC. This is remarkable because *P. chloritidismutans* is a mesophilic bacterium, which does not grow above 42ºC. The trimethylamine N-oxide (TMAO) reductase of *Salmonella typhimurium* also has a higher temperature optimum (75ºC) than the optimal growth temperature, which was 37ºC (Kwan and Barrett, 1983). Remarkably, besides the reduction of TMAO, this enzyme also showed chlorate and bromate but not nitrate reduction activity. *P. chloritidismutans* could not grow with TMAO as electron acceptor and cell extracts of *P. chloritidismutans* did not show TMAO reductase activity. The optimal temperature of the chlorate reductase of *P. chloritidismutans* is higher than the optimum found for the chlorate reductase of strain GR-1, which was 45ºC.

A Blast search in databases did not result in any similar N-terminal sequence for the α- and γ-subunit of the chlorate reductase. However, a significant similarity was found with β-subunits of several oxyanion reductases (Table 2). Especially high similarity was found for the N-terminal sequence of the β subunit of the nitrate reductase of *H. marismortui* subsp *marismortui* (61%) (Yoshimatsu *et al*., 2000), the anaerobic ethylbenzene dehydrogenase subunit B of *Azoarcus* sp EB1 (60%) (Johnson *et al*., 2001), the chlorate reductase of *I. dechloratans* (Danielsson *et al*., 2003) and the dimethylsulfide dehydrogenase of *Rhodovulum sulfidophilum* (McDevitt *et al*., 2002). In addition to a similarity in N-terminal sequence of the β-subunit of the (per)chlorate reductase of *P. chloritidismutans*, chlorate reductase of *I. dechloratans* and selenate reductase of *Thauera selenatis*, all enzymes had a similar subunit composition (a trimeric α_1 β_1 γ_1 complex). However nitrate, nitrite and chlorate were not

reduced at detectable rates by the selenate reductase (Schröder *et al*., 1997). The chlorate reductase of *I. dechloratans* showed selenate reductase activity (Table 4).

The unusual all-g > 2, signal (Fig. 2) is apparently a characteristic of (per)chlorate reductases as it has now been found for two different sources. The nature of the signal is not understood, but it appears to represent an intermediate redox state, likely associated with Mo(V). The lack of Fe/S EPR signals is puzzling, however, a few examples are known of [4Fe-4S] cluster containing enzymes in which (part of) the clusters are EPR silent (Hagen *et al*., 2000). Nevertheless, based on these results, we suggest that the Fe in the protein is nonheme iron. Metal analysis revealed the absence of selenium, which was previously found in the chlorate reductase of strain GR-1. However, molybdenum and iron were detected. The molar ratio of [Fe] to [Mo] of ≈ 16 was different from the molar ratio of [Fe] to [Mo] of ≈ 11 found for strain GR-1.

It is not yet clear, how the respiratory pathway is arranged in chlorate-reducing bacteria. The (per)chlorate reductase of strain GR-1 and the chlorate reductase of *I. dechloratans* are located in the periplasm, whereas the chlorate reductase described here is orientated in the cytoplasm. This means that the way in which energy is conserved in the bacterium must also be different. Comparison with membrane bound nitrate reductases (NAR) suggest that the chlorate reductase has to be membrane bound in order to take part in the respiratory electron chain. Because the γ-subunit in *Paracoccus denitrificans* was proposed to be a transmembrane anchor that immobilizes the complex of the α and β-subunits at the cytoplasmic side of the membrane (Berks *et al*., 1995), the γ-subunit in *P. chloritidismutans* could have the same function.

In denitrifiers, besides membrane-bound nitrate reductases, also periplasmic reductases can be found. The periplasmic nitrate reductase of *Alcaligenes eutrophus* (Warnecke-Eberz and Friedrich, 1993; Siddiqui *et al*., 1993) consists of two subunits which show resemblance with the composition of the chlorate reductase of strain GR-1. However, the subunit sizes differed (87 and 17 kDa versus 95 and 40 kDa for *A. eutrophus* and strain GR-1, respectively). Localization experiments with *P. chloritidismutans*, indicated that the chlorite dismutase has a periplasmic orientation (Table 3). This opposite location of the enzymes involved in chlorate conversion, implies that transport systems are required to transport chlorate from the periplasm to the cytoplasm and to transport chlorite back to the periplasm. Because chlorite is highly oxidative, it might be favorable to translocate chlorite directly. We do not have any insight of the transport of chlorate and chlorite. However, a chlorate/chlorite antiport system

might be most appropriate to avoid an accumulation of toxic chlorite in the cell. The opposite position is also favorable for the oxygen sensitive chlorate reductase because oxygen is produced in the periplasm. For *Azospirillum brasilense* Sp7, a denitrifying bacterium with a cytoplasmic nitrate reductase and a periplasmic nitrite reductase (Danneberg *et al*., 1989), it was proposed that nitrate uptake might proceed via an energy-independent nitrate/nitrite antiport system as in *Paracoccus denitrificans* (Boogerd *et al*., 1983; 1984). This process prevents intracellular accumulation of toxic levels of nitrite and allows further detoxification in the periplasm through the action of nitrite reductase. Regulation studies in denitrifiers have indicated that oxygen inhibition of nitrate uptake, is a common regulatory mechanism in nitrate respiration (Hernandez and Rowe, 1988). Physiological studies with *P. chloritidismutans* showed that oxygen inhibited chlorate reduction when both compounds were present at the start of a growth experiment (Wolterink *et al*., 2002). When chlorate reduction is ongoing, addition of oxygen did not inhibit chlorate reduction. The translocation of chlorite during chlorate reduction by means of an antiport system might avoid oxygen inhibition.

The (per)chlorate reductase of strain GR-1, the chlorate reductase of *I. dechloratans* and the chlorate reductase of *P. chloritidismutans* were shown to be molybdenum enzymes. In an overview by Hille (1996), about mononuclear molybdenum enzymes, the dimethyl sulfoxide (DMSO) reductase family is described. It is remarkable that in this DMSO reductase family two other enzymes are present, the TMAO reductase (Kwan and Barrett, 1983) and the dissimilatory nitrate reductases, which both have chlorate reductase activity. Also the N-terminal sequence similarity of the β-subunit of the chlorate reductase of *I. dechloratans*, the chlorate reductase of *P. chloritidismutans* and the dimethylsulfide dehydrogenase of *R. sulfidophilum* show that not only the presence of molybdenum is a characteristic of enzymes belonging to the dimethyl sulfoxide reductase family.

The results presented in this paper suggest that the chlorate reductase of *P. chloritidismutans* is different from the nitrate reductases, with respect to localization, substrate specificity and the corresponding physiological role. The chlorate reductase was also different from the (per)chlorate reductase of strain GR-1 and the chlorate reductase of *I. dechloratans* (substrate specificity, localization, N-terminal sequence of subunits). A gene cluster for chlorate metabolism in *I. dechloratans* was described (Danielsson Thorell *et al*., 2003). The genes (*clr*) encoding chlorate reductase are arranged as *clr*ABDC, where *clr*A, *clr*B and *clr*C genes encode the subunits and *clr*D gene encodes a specific chaperone. The arrangement of the genes is similar to the disposition of the genes encoding some of the

enzymes identified as homologues of chlorate reductase in database searches, like *T. selenatis ser*ABDC, encoding selenate reductase (Krafft *et al*., 2000). Further upstream, a gene for chlorite dismutase was found in the opposite direction. The arrangement of the chlorate reductase of *P. chloritidismutans* is a subject for future research.

Chapter 4

Purification and characterization of a chlorite dismutase from *Pseudomonas chloritidismutans*

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Abstract

The chlorite dismutase of *Pseudomonas chloritidismutans* was purified from the periplasmic fraction in one step by hydroxy apatite chromatography. The enzyme followed Michaelis Menten kinetics with V_{max} and K_m values of 443 U/mg and 84 µM, respectively at pH 6 and 25ºC. The activity was enhanced by kosmotropic salts like phosphate and sulfate. Chaotropic salts like nitrate and chlorate decreased the activity, which was in accordance with the Hofmeister series. Chlorite dismutase was inhibited by cyanide, azide, but not by hydroxyl amine or 3-amino-1,2,3-triazole. The enzyme had a molecular mass of 110 kDa and consisted of four 31-kDa subunits. A pyridine-NaOH-dithionite-reduced chlorite dismutase revealed a Soret peak indicative for protoheme IX. One molecule of tetrameric enzyme contained 1.5 molecules of protoheme IX. High concentrations of chlorite resulted in disappearance of the Soret peak which coincided with an activity loss. EPR-measurement showed one axial high-spin signal which is characteristic for a high-spin heme with a symmetric environment. The chlorite dismutase reaction carried out in the presence of ^{18}O-labeled water, did not result in the formation of $^{34}O_2$ or $^{36}O_2$, indicating that water is not a substrate in the reaction. N-terminal sequence analysis showed no similarity to other sequences of chlorite dismutases. However, a similar internal sequence was found for a hypothetical protein of *I. dechloratans* which was located downstream of the chlorite dismutase gene. PCR with primers designed for the 3'-end of the chlorite dismutase gene of *I. dechloratans* and the hypothetical protein indicated that the organization of the chlorite dismutase gene in *I. dechloratans* and *P. chloritidismutans* is different.

Introduction

One of the key reactions in the dissimilatory reduction of perchlorate and chlorate, is the conversion of chlorite into chloride and oxygen, catalyzed by a so-called chlorite dismutase. Sofar, chlorite dismutases have been purified and characterized from strain GR-1 (van Ginkel *et al*., 1996), *Dechloromonas agitata* (Coates *et al*., 1999[a]), and *Ideonella dechloratans* (Stenklo *et al*., 2001). These enzymes showed to have a homotetrameric composition and absorption spectra characteristic for protoheme IX. The chlorite dismutases were all located in the periplasm. Because of the apparent splitting of ClO_2^- into Cl^- and O_2, the enzyme involved has been designated as chlorite dismutase. But in the strict sense this is not correct, because the reaction is not a dismutation but an intramolecular redox reaction. The valence of Cl is reduced from +3 in ClO_2^- to -1 in Cl^-, while the valence of oxygen is oxidized from -2 in ClO_2^- to 0 in O_2. Therefore, the systematic name for this enzyme should be chloride: oxygen oxido-reductase or chlorite O_2-lyase (EC 1.13.11.49). Nevertheless, chlorite dismutase was used in all articles describing this type of enzyme.

Recently, antibodies were raised against the purified chlorite dismutase from *D. agitata* to develop an immuno probe (O'Connor and Coates, 2002). The antiserum was active against both cell lysates and whole cells of *D. agitata*, when this strain was grown under anaerobic conditions. No response was found when aerobically grown cells were used. The developed immunoprobe was specific for chlorate-reducing bacteria and cross-reactivity with non-chlorate-reducing bacteria was not observed. The dot blot response obtained for several cell lysates of chlorate-reducing bacteria was different, suggesting that there may be some differences in the antigenic sites of these chlorite dismutases. Besides an immuno probe against the chlorite dismutase, a metabolic probe was constructed based on the chlorite dismutase gene sequence of *D. agitata* (Bender *et al*., 2002). The constructed probe hybridized to all (per)chlorate-reducing bacteria tested and failed to hybridize to non-(per)chlorate-reducing bacteria. Results indicated that the 3' end of the chlorite dismutase gene is more conserved than the 5' end and likely encodes the protoheme IX binding region observed in mature proteins. An alignment of the chlorite dismutase sequence of *D. agitata* and *I. dechloratans* showed 71% similarity at the amino acid level. No other proteins were more than 24% similar to the product encoded by the *D. agitata* chlorite dismutase gene. These results suggested that the chlorite dismutase enzyme is unique and highly

conserved among (per)chlorate-reducing bacteria (Bender *et al*., 2002; O'Connor and Coates, 2002; Coates *et al*., 1999[a]).

In the present paper, the purification of chlorite dismutase of *Pseudomonas chloritidismutans* is described. This chlorate-reducing bacterium differs from most other (per)chlorate-reducing bacteria in that the chlorate reductase is specific for chlorate and bromate and is not able to use perchlorate and nitrate (Wolterink *et al*, 2003). Also the chlorate reductase of *P. chloritidismutans* and the (per)chlorate reductase of strain GR-1 (Kengen *et al*., 1999) were quite different. Localization experiments had already shown that in *P. chloritidismutans* the dismutase had a periplasmic orientation. The present paper describes a simple protocol for the purification of the chlorite dismutase from the periplasmic fraction. The chlorite dismutase of *P. chloritidismutans* was characterized and compared to chlorite dismutases which have been described.

Materials and methods

Bacterial strains

P. chloritidismutans was isolated from sludge of an anaerobic bioreactor treating a chlorate and bromate polluted waste stream (Abma *et al*., 1999). This bacterium was grown in strictly anaerobic medium as described before (Wolterink *et al*., 2002). Acetate and chlorate, both 10 mM, were used as electron donor and acceptor, respectively. *D. agitata* (DSM 13637), *D. suillum* (DSM 13638), *P. stutzeri* (DSM 50227) were purchased from the DSMZ. *Ideonella dechloratans* was purchased from the Culture Collection of the University of Göteborg (CCUG). Strain GR-1 (DSM 11199), strain ASK-1 (DSM 15671) and strain MA-1 (DSM 15637) were available in our laboratory. Strain GR-1, *D. agitata* and *D. suillum* were grown in medium described for strain GR-1 (Rikken *et al*., 1996) with modification described by Kengen *et al*. (1999). Strain ASK-1 and strain MA-1 were grown in medium described for *P. chloritidismutans* with modifications in the gas phase (N_2/CO_2 (80%/20%) instead of N_2) and the sulfur source (0.2 g/l Na_2SO_4 instead of 0.5 g/l Na_2S). *P. stutzeri* DSM 50227 was grown as described previously (Wolterink *et al*., 2002). *I. dechloratans* was grown as described by Stenklo *et al*. (2001).

Enzyme purification

For purification of the chlorite dismutase from the periplasmic fraction, the cell pellet (2 to 3 g wet weight) was suspended in 10 ml 0.05 M Tris-HCl buffer (pH 9.5) and 0.05 M EDTA, and incubated for 30 min at room temperature (Sebban *et al*., 1995). The suspension was centrifuged for 10 min at 13,000 rpm at 4°C. The cell pellet was checked for the presence of whole cells by light microscopy. The supernatant (the periplasmic fraction) was subjected to ultracentrifugation at 45,000 rpm (110,000 x g) for 1 h at 4ºC and the red supernatant was removed from the pellet (membrane debris). The supernatant was diluted with an equal volume of 10 mM Tris-HCl buffer (pH 7.2). Purification of the enzyme was carried out using an ÄKTA*fplc* (fast performance liquid chromatography; Amersham Pharmacia Biotech). Four ml of the diluted supernatant was loaded on a hydroxy apatite column (CHT5-I; BIORAD; column dimensions 10 x 64 mm) equilibrated with 10 mM Tris-HCl, pH 7.2. The chlorite dismutase eluted from the column at the start of a linear gradient of 10 mM Tris-HCl, pH 7.2 to 450 mM potassium phosphate, pH 7.2. Fractions of chlorite dismutase were kept at 4ºC for further analysis or stored at -20ºC.

Determination of the molecular mass and N-terminal sequence

Protein fractions were checked by sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The 12% acrylamide gels were stained with Coomassie Brilliant blue R250. For calculation of the molecular mass of the subunit(s) a marker was applied with the following standard proteins (size in kDa): myosin (200), β-galactosidase (116), phosphorylase b (97), serum albumin (66), ovalbumin (45), and carbonic anhydrase (31). Estimation of the size of the native enzyme was done by loading a 400 µl aliquot of chlorite dismutase on a Superdex 200 column (1.6 by 70.5 cm) equilibrated in 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl. The Superdex 200 column was calibrated with the following molecular weight markers (size in kDa): ferritin (440), catalase (232), bovine serum albumin (67), ovalbumin (43), chymotrypsinogen (25), and ribonuclease (14).

N-terminal sequencing of a chlorite dismutase solution was performed in an automated protein sequencer with on-line analysis of the phenylthiohydantoin amino acids (Models 477A and 120A, Applied Biosystems, Foster City, USA) following the manufacturers instructions (Schiltz *et al.*, 1991). N-terminal sequences were

compared to other sequences using the protein-protein Blast program available at the website of the National Center for Biotechnology Information (NCBI).

Activity assay

Chlorite dismutase activity was measured with a Clark-type Electrode (Yellow Spring instruments, Yellow Springs, Ohio, USA). The biological oxygen monitor was calibrated by relating the concentration of chlorite added to the measurement cell (100, 200 or 300 µM) to the amount of oxygen produced. An example of the calibration curve is shown in Fig. 1 (measurements were done in triplicate).

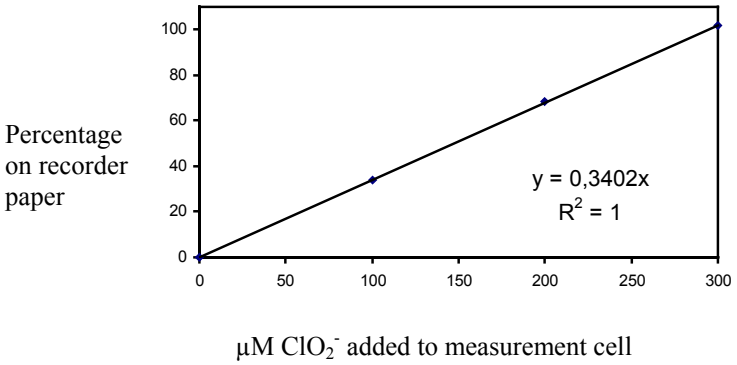


Fig. 1 Calibration curve of biological oxygen monitor. Calibration revealed that 1% is 2.94 µM O_2.

Under standard condition, 2.8 ml of buffer (100 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 6) was added to the sample chamber together with 10 µl of a stock solution of sodium chlorite solution to give a final concentration of 0.2 mM. The reaction was run for 5 min and the initial linear part was used to calculate the rate. One unit (U) is defined as the amount of enzyme required to convert 1 µmol of chlorite per minute. The enzyme activity was measured at different temperatures between 15 and 40ºC (in 15 mM phosphate (Na_2HPO_4/NaH_2PO_4) buffer, pH 7.2). For technical reasons, analysis of the activity above 40ºC was not possible. At the optimal temperature of 25ºC, the activity was measured at different pH values, ranging from 5 to 8.5 using Na_2HPO_4/ NaH_2PO_4 buffers. Kinetic parameters (K_m and V_{max}) were determined at different sodium phosphate concentrations (100 mM and 1 M) at pH 6 and 25ºC. The chlorite concentration was varied between 1 and 500 µM. Data were processed by a computer-aided direct fit to the Michaelis-Menten curve. The k_{cat} values at the different phosphate concentrations were calculated for determination of k_{cat}/K_m, which

is used to express the kinetic perfection. The protein content was determined according to Bradford with bovine serum albumin as standard (Bradford, 1976).

Spectroscopy

Visible and ultraviolet spectra were recorded on a Hitachi U-2010 (Hitachi Science Systems, Hitachinaka, Japan) at 30ºC. The pyridine hemochrome absorbance spectrum was measured in 20% (w/v) pyridine, and 0.1 M NaOH in 50 mM potassium phosphate (pH 7.0). Sodium dithionite (2 mM final concentration) was added prior to the measurement in a stoppered cuvette, flushed with N_2 gas. The heme content was calculated using an absorbance coefficient of ε_{418} = 191,500 $M^{-1}cm^{-1}$ (Falk, 1964). X-band EPR spectra were recorded on a Bruker ER-200D spectrometer with peripheral equipment and data handling as described before (Pierik and Hagen, 1991). EPR spectra were recorded in phosphate buffers (pH 6) of 10 mM and 500 mM. The presence of iron (Fe^{3+}) was measured by inductively coupled plasma mass spectrometry (ICP-MS) (Elan 6000, Perkin-Elmer).

Inhibition and stabilization of chlorite dismutase

The effect of inhibitors was tested by adding the following compounds in a final concentration of 71 µM or 685 µM: cyanide, azide, hydroxylamine, EDTA, H_2O_2 or 3-amino-1,2,4-triazole. The inactivation of the chlorite dismutase by the substrate chlorite was analyzed by adding 56 mM chlorite (final concentration) to an enzyme solution. After 1 min, the activity was measured and a UV-VIS spectrum of the protoheme was made. After 10 min, again 56 mM chlorite (final concentration) was added and activity and UV-VIS spectrum were determined. The amount of enzyme present in the assay mixture was found to influence the specific activity. Therefore, undiluted and a 10 times diluted solution of chlorite dismutase was added to the assay. The specific activity (U/mg) and the total amount of oxygen produced (µM) was determined at final chlorite concentrations of 100, 200 and 300 µM.

The effect of Hofmeister salts on the activity of chlorite dismutase was determined. The following salts were added to the assay mixture at concentrations between 0 to 2 M: Na_2HPO_4/NaH_2PO_4; KH_2PO_4/K_2HPO_4, solutions were set to pH 6. All solutions of the following salts were made in 10 mM Tris-HCl: $NaClO_3$; $NaNO_3$;

NaCl; NH_4Cl; $(NH_4)_2SO_4$; Na_2SO_4; $NaHCO_3$. The pH was adjusted to 6 with 1 M HCl or 1 M NaOH.

Incubation with $H_2{}^{18}O$

To determine whether oxygen originates from chlorite or water, an experiment was set up using isotopically labeled water. Labeled water (water-^{18}O, 98 atom % ^{18}O) was purchased from ISOTEC (Sigma-Aldrich). Serum flasks (10 ml) were closed with butyl rubber stoppers, and the gas phase was exchanged for 100% helium. To these flasks, the following compounds were added; 100 µl water-^{18}O or 100 µl water-^{16}O (44.5% v/v), 10 µl ClO_2^- (final concentration 45 mM), and 100 µl enzyme solution (final concentration = 0.09 mg/ml); 100 µl water-^{18}O (81.7% v/v), 10 µl ClO_2^- (final concentration 125 mM), and 10 µl enzyme solution (final concentration 0.02 mg/ml). Oxygen production was clearly visible as small bubbles, which appeared when the three compounds were combined. The produced oxygen was analyzed for $^{32}O_2$, $^{34}O_2$ and $^{36}O_2$ using a gas chromatography / mass spectrometer (GC/MS) equipped with a capillary column (Packard, innowax 30 m * 0.25 mm, split ratio 25:1). Helium gas was used as carrier gas, the inlet pressure was 1 kPa and the column temperature was 40ºC.

PCR amplification of chlorite dismutase genes using degenerated primers

Degenerated primers D_1 and D_2 were designed based on the amino acid sequences of the chlorite dismutase of *I. dechloratans* and *D. agitata* (Danielsson Thorell *et al*., 2002; Bender *et al*., 2002). Degenerated primer D_3 and D_4 were designed based on the amino acid sequence of the N-terminal sequence of the chlorite dismutase of *P. chloritidismutans*. Primers had the following sequences (for all positions, except primer D_4, reverse (r) and forward (f) primers were designed: D_1f 5'-AAGAACG CSGAYTGGTGGAA-3' ; D_2r 5'-ACGTGGTAYTTRTTYTCNGG-3' ; D_3f 5'-ATGTCSCAYGA YTCNATGTC-3' ; D_4r 5'-TTCATSCCGTCYTTYTTCAT-3'.

PCR amplification was done in a 50 µl PCR reaction mixture containing ; 0.2 µM of each primer; 200 µM of dATP, dCTP, dGTP and dTTP; 3 mM $MgCl_2$, 5 µl 10X PCR buffer and 1 U *Taq* DNA Polymerase (Invitrogen, Life Technologies). To each PCR-mixture, 1 µl of DNA template was added. DNA template was obtained by centrifuging 2 ml of cell culture. The pellet was suspended in 50 µl TE buffer (10 mM

Tris, 1 mM EDTA, pH 8). This cell suspension was incubated at 95ºC for 10 min, and subsequently used as DNA template solution. PCR products were obtained with a T1 thermocycler (Biometra, Göttingen, Germany). The PCR-mixture was preheated to 95ºC for 6 min. 35 amplification cycles were performed, including denaturation for 40 sec at 94ºC, primer annealing for 1 min at different temperatures (depending on primer between 40-48ºC), and elongation for 1 min at 72ºC. Finally an extension for 4 min at 72ºC was done. PCR products were checked on an 1% agarose gel. PCR products were made visible using UV-light detection of ethidium bromide stained gels.

Partial gene sequencing of chlorite dismutase of *P. chloritidismutans*

The PCR products from *P. chloritidismutans*, produced with primer pairs D_1f and D_2r or D_3f and D_4r, were purified with QIAquick PCR Purification Kit (QIAGEN). PCR products were ligated into a pGEMt Easy vector and transferred into competent *E. coli* cells (Promega). White colonies were checked for the insert with the selected primers. Positive colonies were then used for a PCR reaction with primers T7 and SP6 (primers based on the ligation sites of the vector). PCR products were purified as described above. These PCR products were used for sequencing by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) with a Li-Cor automatic sequencing system (model 4000L, Westburg, Leusden, The Netherlands) using fluorescently labeled SP6 or T7 primers.

Results and discussion

Enzyme assay

The activity of the chlorite dismutase was affected by the amount of enzyme present in the assay. In Table I, the results are shown of enzyme assays using undiluted and 10 times diluted chlorite dismutase. In the presence of the same concentration of chlorite, not only the specific activity dropped, but also the amount of oxygen which could be produced was less. These results suggest that the enzyme is inactivated by chlorite at low enzyme concentrations. Therefore, prior to the measurement of chlorite dismutase activity, the enzyme solution was checked for complete chlorite conversion.

Table I Inactivation of chlorite dismutase

µM ClO_2^- added	Undiluted chlorite dismutase		10 times diluted chlorite dismutase	
	spec. activity (U/mg)	O_2 produced (µM)	spec. activity (U/mg)	O_2 produced (µM)
100	399	98	25	34
200	628	205	59	69
300	870	300	120	96

O_2 production measured in 1 M phosphate buffer at 25ºC

Enzyme purification

Localization experiments performed previously, had shown that the chlorite dismutase has a periplasmic orientation (Wolterink *et al*., 2003). A periplasmic location was also suggested for the chlorite dismutase of strain GR-1 and *Ideonella dechloratans*. For this reason the protocol of Sebban *et al.* (1995) was used to wash the periplasmic fraction from the cells. A centrifugation step resulted in a red supernatant, the periplasmic fraction (lane 2, Fig. 1). A suspension of the cell pellet was checked by light microscopy and intact cells were seen. No spheroplasts were formed. After the ultracentrifuge step, the red periplasmic fraction was diluted (1:1) with 10 mM Tris-HCl buffer (pH 7.2) and subsequently loaded on a hydroxyapatite column. The chlorite dismutase, which eluted from the column at the start of the gradient, was separated from another red-colored fraction which did not bind to the column and did not show dismutase activity. In Table II, the purification scheme is shown.

Table II Purification of chlorite dismutase from *Pseudomonas chloritidismutans*

	Volume (ml)	protein Concentration (mg/ml)	specific activity (U/mg)	total activity (U)	yield (%)	purification (-fold)
Periplasmic extract	4	2.98	21	252	100	1
Hydroxy apatite column	4	0.21	208	175	69.4	9.9

O_2 production measured in 15 mM PO_4 buffer at 30ºC

The purification factor of 9.9 indicates that the chlorite dismutase is an abundant protein in the periplasm. A similar result was found for *I. dechloratans* (Stenklo *et al*. 2001). A 8 fold purification was obtained starting with a periplasmic fraction.

Gel electrophoresis and determination of the molecular mass

The purified chlorite dismutase was loaded on a SDS-PAGE. This resulted in a single band at 31 kDa (Fig. 2, lane 3). On a calibrated Superdex 200 column the molecular weight of the native enzyme was determined as 110 kDa. This suggests a homotetrameric structure. Similar results were found for the chlorite dismutases from strain GR-1 and *I. dechloratans* (Table IV).

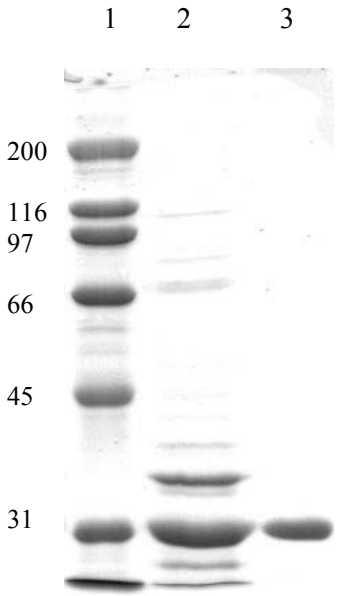


Fig. 2 SDS-PAGE of chlorite dismutase from *P. chloritidismutans*. Lane 1, marker proteins with indicated molecular molecular masses; lane 2, periplasmic fraction; lane 3, hydroxy apatite fraction.

Spectral characteristics

The pyridine-NaOH-dithionite-reduced chlorite dismutase showed peaks indicative of protoheme IX (Fig. 3; Soret, 418 nm; β max, 526; α, max 557) (Falk, 1964). The chlorite dismutase showed a Soret peak at 411 nm. A shift of the Soret band to 433 nm was seen when the enzyme was reduced with dithionite. The addition of cyanide shifted the Soret peak to 420 nm. This formation of a cyanide complex was also seen for the chlorite dismutase of strain GR-1 and indicates the presence of ferric heme in the enzyme.

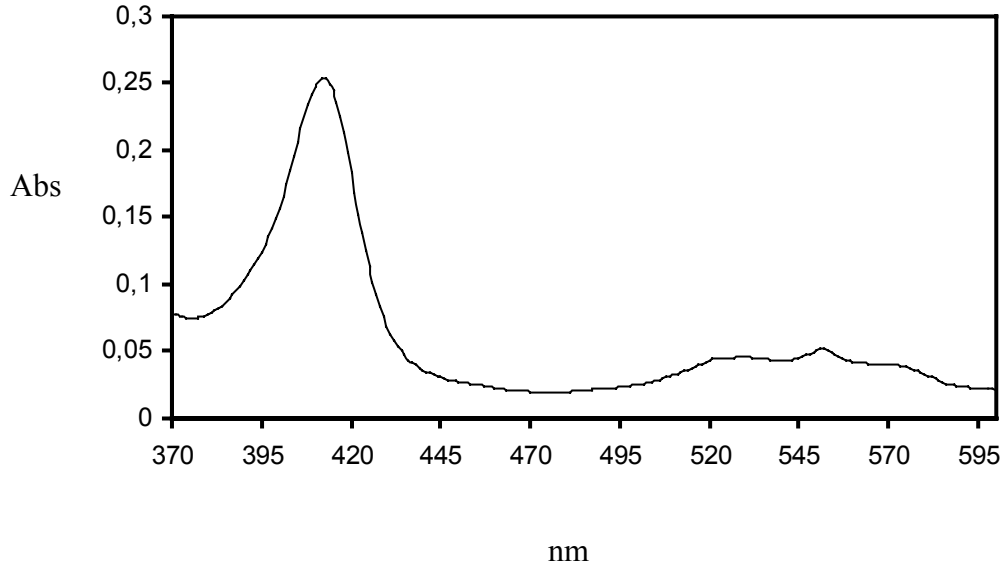


Fig. 3 Visible light spectrum of purified chlorite dismutase

The heme content was determined to be 1.5 molecules of heme per molecule of tetrameric chlorite dismutase, based on a molar extinction coefficient of 191,500 M^{-1} cm^{-1}. One molecule of tetrameric enzyme contained 1.7 and 2.4 molecules of protoheme IX for strain GR-1 and *I. dechloratans*, respectively. For GR-1 *and P. chloritidismutans* an extinction coefficient of 191,000 M^{-1} cm^{-1} at 418 nm was used, while 24,000 M^{-1} cm^{-1} at 556-540 nm was used for *I. dechloratans*. The heme content of the enzyme is lower than that expected for a homotetrameric enzyme. Stenklo *et al*. (2001) suggested that the exposure of the enzyme to chlorite caused bleaching of the heme absorption which resulted in a loss of activity. This observation was also done by van Ginkel *et al*. (1996), where the decrease of chlorite dismutase activity at high chlorite concentrations was attributed to the oxidation of the heme. By adding two times 56 mM (final concentration) chlorite to the chlorite dismutase of *P. chloritidismutans*, the disappearance of the Soret peak coincided with an activity loss of 80% after the first and 90% (of the starting activity) after the second addition of chlorite. In this respect, the chlorite dismutase is bleached by high concentrations of substrate. This is in agreement with the results found for the 10 times diluted enzyme solution. However, another reason for the incorrect number of heme could be caused by the way the enzyme is purified. It was already mentioned that the fraction which did not bind to the hydroxy apatite column, did not show any chlorite dismutase activity. However, this red-colored fraction showed also a Soret peak identical to that of the chlorite dismutase. This could be an indication, that during purification, heme is

removed from the enzyme. The chlorite dismutase of strain GR-1 and *P. chloritidismutans* contained 0.7 and 0.55 atoms of iron per monomer, respectively.

EPR measurements

For the chlorite dismutase of *P. chloritidismutans* only one axial high-spin EPR-signal was detected (Fig. 4, line A).

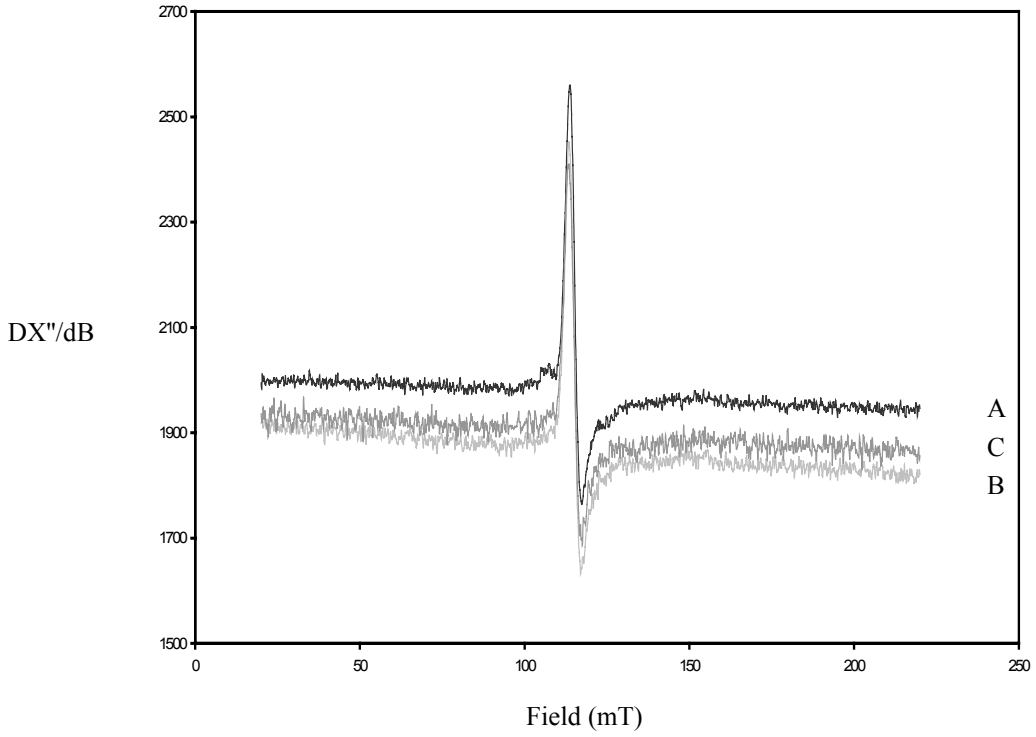


Fig. 4 Electron paramagnetic resonance spectra of *P. chloritidismutans* chlorite dismutase. Trace A is from the enzyme as isolated (in 10 mM sodium phosphate). Trace B is chlorite dismutase in 0.5 M sodium phosphate. Trace C is chlorite dismutase incubated in 0.5 M sodium phosphate for 30 min. EPR conditions : microwave frequency, 9.432 GHz; Microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 10.2 K.

For the ferric chlorite dismutase of strain GR-1 (Hagedoorn *et al*., 2002) and *I. dechloratans* (Stenklo *et al*., 2001), a mixture of two high-spin signals and one low-spin EPR signal were observed, which are characteristics of a high-spin heme with an asymmetric environment. The one axial high-spin EPR-signal for ferric chlorite dismutase of *P. chloritidismutans* indicates a high spin heme with a symmetric environment.

Kinetic characterization

The enzyme was active between pH 5 and pH 8.5, with an optimum at pH 6. The enzyme was active between 15 and 45ºC, with an optimum at 25 ºC. The V_{max} and K_m values at 100 mM sodium phosphate, pH 6 and at 25ºC were 443 U/mg and 84 µM, respectively. The k_{cat}/K_m (M^{-1} sec^{-1}) value at 100 mM sodium phosphate was $2.7*10^6$. This k_{cat}/K_m value is in the same range as found for the chlorite dismutases of strain GR-1 and *I. dechloratans* (Table III).

Table III Characteristics of chlorite dismutases of several (per)chlorate-reducing bacteria

	Strain GR-1*	*I. dechloratans***	*P. chloritidismutans*
subunit size (kDa)	32	25	31
relative molecular mass (kDa)	140	115	110
BOM-T (ºC)	30	25	30
BOM-pH	7.2	7	6
BOM-buffer (mM PO_4^{2-})	14.2	100	100
V_{max} (U/mg)	2200	4300	443
K_m (µM)	170	260	84.2
k_{cat} (s^{-1})	1200	1800	229
k_{cat}/K_m (M^{-1} sec^{-1})	$7.1*10^6$	$6.9*10^6$	$2.7*10^6$

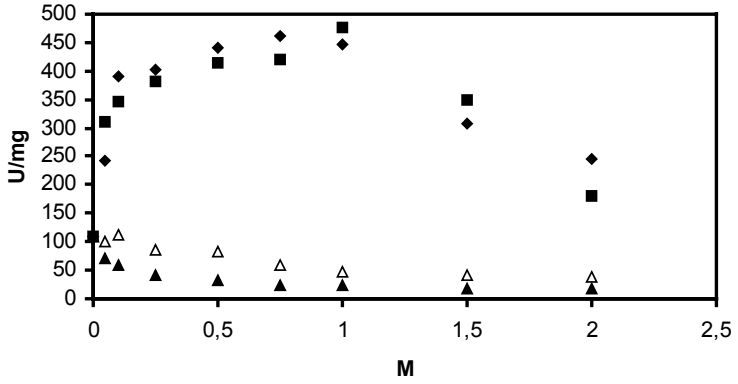
* ; van Ginkel *et al*., 1996

**; Stenklo *et al*., 2001

The activity of chlorite dismutase was found to be strongly influenced by the type and concentration of salts added in the assay. The results are shown in Fig. 5A and B. When phosphate or sulfate was used, activity increased at higher salt concentrations. When chlorate or nitrate was used, the opposite was observed. These results are in line with the Hofmeister series which describes the effects of salts on protein structure (Jensen *et al*., 1995). The stabilizing effects of kosmotropic salts (SO_4^{2-} and PO_4^{3-}) on enzymes are caused by the promotion of intermolecular and intramolecular hydrophobic interactions of the protein which are due to the water structuring effects of the ions. This water structuring effectiveness is ranked for anions according to the Hofmeister series following the order $PO_4^{3-} > F^- > SO_4^{2-} > Cl^- > Br^- > I^- > BrO_3^- > ClO_4^-$. Increase of chaotropic salts like nitrate and chlorate reduce the activity. The increase of the concentration of kosmotropic salts results in higher V_{max} and K_m

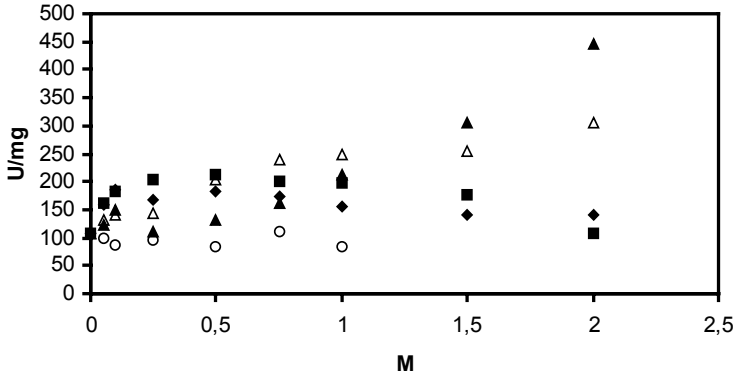
values (for 1 M phosphate the V_{max} and K_m values were 4650 U/mg and 833 µM, respectively).

To rule out the possibility that the activity increase by a kosmotropic salt (PO_4^{3-}) is due to interaction of this salt with the active center (protoheme), EPR measurements were done with chlorite dismutase in 10 mM and 0.5 M phosphate solution (both at pH 6). The high-spin EPR-signal did not change (Fig. 4, line B and C). Experiments done with NO (Hagedoorn *et al*., 2002) showed that this compound bound to the heme which resulted in disappearance of the two high spin EPR-signals and the appearance of a low-spin EPR-signal. Activity increase by phosphate therefore seems to be due to water structuring effects.



A

Filled diamond:	Na_2HPO_4 / NaH_2PO_4
Filled square:	K_2HPO_4 / KH_2PO_4
Filled triangle:	$NaClO_3$
Open triangle:	$NaNO_3$



B

Filled diamond:	NaCl
Filled square:	NH_4Cl
Filled triangle:	$(NH_4)_2SO_4$
Open triangle:	Na_2SO_4
Open circle:	$NaHCO_3$

Fig. 5 Stimulation or inhibition of chlorite dismutase activity from *P. chloritidismutans*. Experiments were done at 25ºC and pH 6. The conversion of chlorite into chloride and oxygen did not alter the pH of the buffer solution.

Inhibition studies

Inhibition studies showed that cyanide and azide were effective inhibitors (Table IV). The chlorite dismutase of strain GR-1 was not inhibited by azide (20 mM) (van Ginkel *et al*., 1996). Chlorite dismutase of *P. chloritidismutans* is in this respect quite distinct from the chlorite dismutase of strain GR-1. The chlorite dismutases of strain GR-1 and *P. chloritidismutans* were not inhibited by 3-amino-1,2,4-triazole, which is an effective inhibitor of catalases (Diaz and Wayne, 1974; Jouve *et al*., 1983). The chlorite dismutase of *P. chloritidismutans* was not inhibited by hydroxyl amine, while the chlorite dismutase of strain GR-1 was.

Table IV Percentage inhibition of chlorite dismutase by various compounds

Inhibition concentrations	71 μM	685 μM
Cyanide	83	95
Hydroxyl amine	0	0
Azide	44	89
3-amino-1,2,4-triazole	0	7.5
EDTA	13	30
H_2O_2	6	37

Assay conditions were; ClO_2^- concentration 200 μM, 15 mM PO_4^{2-}, pH 6

The inhibition of the chlorite dismutase of *P. chloritidismutans* is distinct from inhibition of catalases with respect to 3-amino-1,2,4-triazole and of chlorite dismutase of strain GR-1 with respect to hydroxyl amine and azide.

Involvement of water

To investigate if water is involved in the dismutation reaction, ^{18}O labeled water was used and the evolution of $^{32}O_2$, $^{34}O_2$ or $^{36}O_2$ was monitored using GC-MS. During all conditions tested, only $^{32}O_2$ was measured, indicating that despite the presence of different concentrations of ^{18}O labeled water, water did not take part in the reaction. So, oxygen is only formed out of chlorite.

N-terminal sequence analysis

Analysis of the N-terminus of the chlorite dismutase of *P. chloritidismutans* revealed the following amino acid sequence:

NH_3-A D T M S H D S M S K D S M S K D A M K K D G M K

A BLAST search in the database (NCBI) did not show similarity to the N-terminal sequences of the chlorite dismutases of *I. dechloratans* (Stenklo *et al*., 2001) or *D. agitata* (Bender *et al*., 2002). However, similarity was found with internal fragments of two hypothetical proteins which showed the same repetitive sequence (M S H/K D S/A):

P. chloritidismutans	2	D T M S H D S M S K D S M S K D A M K----------K D G M K	25
		D M + H D S M + K D S M S K D M K K D G M K	
Rhodopseudomonas	35	D G M A H D S M A K D S M S K D G M K K D T M A K D G M K	63
palustris		(length 79 aa; ZP_00012081; gi:22964475)	

P. chloritidismutans	4	M S H D S M S K D S M S K D A M K K D G M	24
		M S H D S M S K D + M S K D + M K D M	
Burkholderia fungorum	28	M S H D S M S K D A M S K D S M A K D S M	48
		(length 83 aa; ZP_00033827; gi:22988766)	

Remarkably, also similarity was found to an internal fragment of a hypothetical protein (ORF 2) of *Ideonella dechloratans*:

P. chloritidismutans	2	D T M S H D S M S K D S M S K D A M K K D G M K	25
		D M D + M + K + S M + K + A M K D M K	
I. dechloratans	24	D G M K K D T M A K E S M A K E A M T K D D M K	47
		(length 74 aa; AJ296077.1; gi:11121511)	

The gene for this hypothetical protein is located downstream of the chlorite dismutase (*cld*) gene and has been designated as ORF2. A schematic representation of the *cld* gene and ORF 2 is depicted in Fig. 6. The function of this hypothetical protein of *I. dechloratans* is not known. Future research has to give insight in the possible relation with the nearby located chlorite dismutase encoding gene

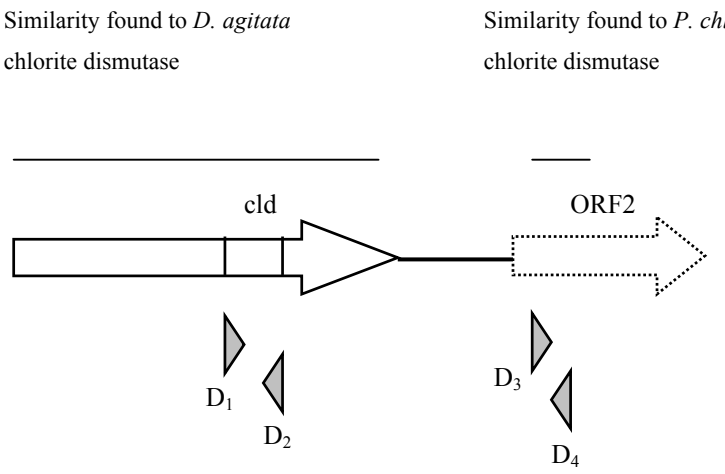


Fig. 6 The architecture of the *Ideonella dechloratans cld* gene and ORF2. The position of the degenerated primers used in this study are shown as arrowheads (for all positions, forward and reverse primers were designed, except for position D_4).

PCR amplification of chlorite dismutase genes

Amplification of the *cld* gene and ORF2 of *I. dechloratans* using primer pairs D_1f-D_2r, D_1f-D_4r and D_2f-D_4r should yield PCR products of 201, 556 and 355 base pairs, respectively. When the organization of the *cld* and ORF2 is the same in all (per)chlorate-reducing bacteria, identical PCR products are expected. The predicted amino acid sequence of the chlorite dismutase of *D. agitata* was found to be 71% similar to that of *I. dechloratans* (Fig. 6) (Danielsson Thorell *et al*., 2002). The degenerated primers D_1f-D_2r were based on the conserved amino acid sequence shared between both proteins. As expected, use of this primer pair generated a PCR product of approximately 0.2 kb for both *I. dechloratans* and *D. agitata* (lane 2, 5; Fig. 7). Using the same primer pair on *P. chloritidismutans* DNA, a substantially larger PCR product of approximately 0.6 kb was observed (lane 3, Fig. 7). Thus, *P. chloritidismutans* may contain a different chlorite dismutase gene. Sequence analysis of this 0.6 kb PCR product of *P. chloritidismutans* showed no similarity to other gene sequences. Using the other primer pairs (D_1f-D_4r and D_2f-D_4r; lane 6 and 10 in Fig. 7, respectively), the expected bands were only found for *I. dechloratans*. Thus, the *I. dechloratans* organization of *cld* and ORF2 (Fig. 6) is unique. Moreover, *P. chloritidismutans* does not contain a *cld*-like gene as found in *I. dechloratans* and *D. agitata*. As expected, genomic DNA of the denitrifying strain *Pseudomonas stutzeri* DSM 50227, did not show any PCR product with the primer pairs used. Previous

research had shown that cell extracts of *P. stutzeri* DSM 50227 did not show chlorite dismutase activity (Wolterink *et al*., 2002).

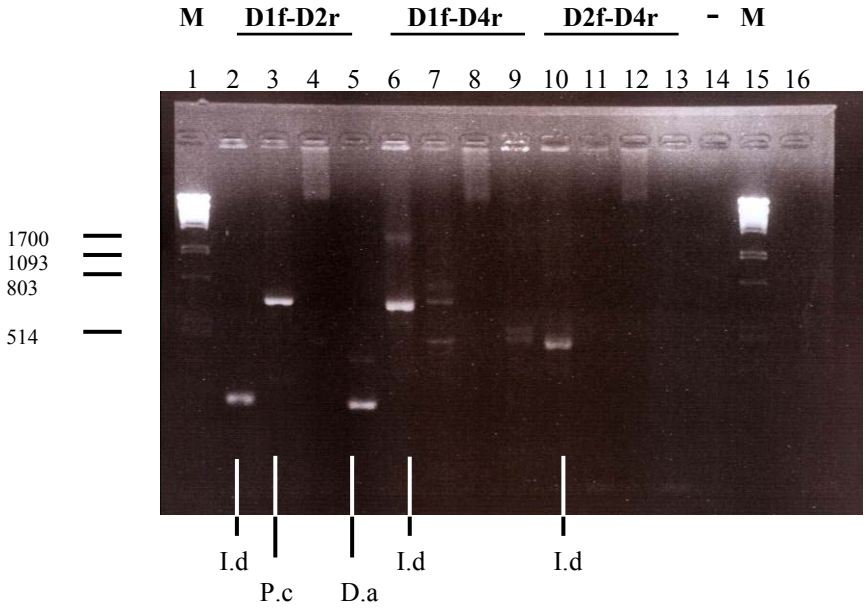


Fig. 7 Agarose gel with PCR products using degenerated primers. Lane 1 and 15, HindIII marker. Lane 2-5, primers D1-D2; Lane 6-9, primer D1-D4; Lane 10-13, primer D2-D4; f, forward; r, reverse; Lane 14, negative control (water); Lane 16 empty lane. DNA of the following species was used in PCR; I.d., *I.dechloratans* (Lane 2 , 6, 10); P.c., *P. chloritidismutans* (lane 3, 7, 11); *P. stutzeri* DSM 50227 (lane 4, 8, 12); D.a., *D. agitata* (lane 5, 9, 13).

Primer pair D_1f-D_2r can be used to amplify the 3' end of the *cld* gene for *I. dechloratans* and *D. agitata*. It was already demonstrated that the 3' end of the *cld* gene is more conserved than the 5' end, and likely encodes the protoheme IX binding region observed in mature proteins (Bender *et al*., 2002). However, as mentioned above, using the primer pair D_1f-D_2r, another PCR product (0.6 kb) was detected for *P. chloritidismutans*. This primer set was also tested for other (per)chlorate-reducing bacteria to see whether this conserved 3' region of the *cld* gene of *I. dechloratans* and *D. agitata* could be detected. However, none of the bacteria tested, reacted in the same way (Fig. 8). These results indicate that the *cld* gene sequence is apparently different from the *cld* genes of *I. dechloratans* and *D. agitata*. Moreover, these results also question the possibility to use similar sequences for the development of a general metabolic probe as reported by Bender *et al*. (2002).

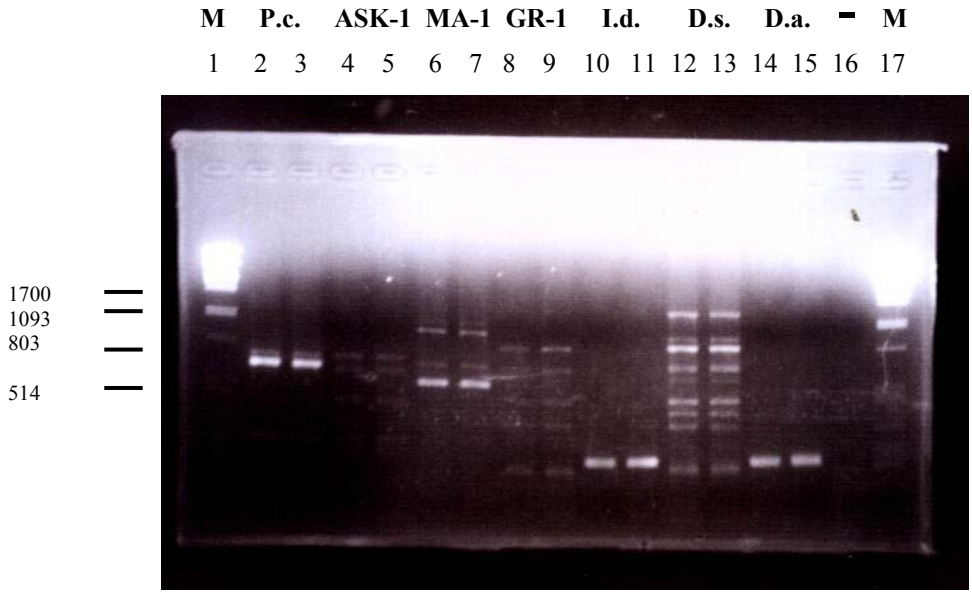


Fig. 8 PCR products using degenerated primers D1f-D2r (twice). The following strains were used; P.c; *P. chloritidismutans* (lane 2,3); ASK-1, strain ASK-1 (lane 4, 5); MA-1, strain MA-1 (lane 6,7); GR-1, strain GR-1 (lane 8,9); I.d., *I. dechloratans* (lane 10, 11); D.s., *D. suillum* (lane 12, 13); D.a., *D. agitata* (lane 14, 15); lane 16, negative control; lane 1, 17, Hind III marker.

DNA isolated from *P. chloritidismutans* was amplified in a PCR reaction using the degenerated primers D_3f and D_4r. The PCR product was purified and ligated into pGEMt Easy vectors and transferred into competent *E. coli* cells. White colonies were screened for insert by doing a PCR with SP6 and T7 primers. PCR products were purified and their sequence was analyzed. The obtained DNA sequence coding for the N-terminale region of the chlorite dismutase (AY450915) was translated and compared to the N-terminal amino acid sequence:

MSHDSMSKDHMSKDSMSKDGM	Derived from DNA sequence
MSHDSMSKDSMSKDAMKKDGM	N-terminal sequence

The result confirmed the presence of the gene sequence coding for the N-terminus of the chlorite dismutase in *P. chloritidismutans*.

This sequence and the obtained DNA fragment can be used in future investigations to obtain the complete gene of the chlorite dismutase of *P. chloritidismutans*. All chlorite dismutases isolated so far showed similar biochemical characteristics including localization, subunit composition and presence of a Soret peak. However, the genes coding for these enzymes differ for *P. chloritidismutans* and *D. agitata* or *I. dechloratans* and further insight in their predicted primary structure may be instrumental in determining their functional characterization.

Chapter 5

Characterization of (per)chlorate-reducing bacteria isolated from chlorate contaminated and pristine environments and taxonomic description of strain GR-1

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Abstract

Recent studies on the occurrence of (per)chlorate-reducing bacteria, resulted in the characterization of various strains capable of dissimilatory (per)chlorate reduction. Phylogenetic analysis showed that these bacteria were members of the *Proteobacteria*. Strains were isolated from polluted samples but also from pristine sites. However, only strains isolated from polluted sites were characterized in detail and deposited in culture collections. Here, the characterization of a perchlorate-reducing bacterium strain MA-1 and a chlorate-reducing bacterium strain ASK-1 is described. Strain ASK-1 and strain MA-1 were isolated from a chlorate polluted and a pristine sample, respectively. Both isolates are members of the *Proteobacteria*. For strain MA-1, the 16S rDNA similarity to *Dechloromonas agitata* (97.6%) was not in accordance with similarity in DNA-DNA reassociation studies (36.5%). Therefore, strain MA-1 is classified as a novel species, *Dechloromonas hortensis* sp. nov. For strain ASK-1 and a previously described strain GR-1, DNA-DNA hybridization studies indicated that these bacteria were related at the species level to *Pseudomonas chloritidismutans* and *Dechlorosoma suillum*, respectively. The latter strain showed to be a later subjective synonym of *Azospira oryzae*. Although strain ASK-1 is related at the species level to *P. chloritidismutans*, the morphology and growth requirements were different.

Introduction

The occurrence of perchlorate and chlorate in natural environments is as far as we know only due to human activities. Chlorate is used as herbicide or as catalyst in matches and used for the onsite production of chlorine dioxide (ClO_2) which is used as bleaching agent in paper and pulp industries. Perchlorate is used as rocket propellant in defense and aerospace industries. In the United States, discharge of perchlorate-containing waste streams has been identified as the major source of perchlorate pollution in drinking water supplies (Renner, 1998, U.S. Environmental Protection Agency, 2000). Because of the chemical stability and high solubility of perchlorate and chlorate, microbial processes have been primarily used to remediate perchlorate pollution (Urbansky, 2002). (Per)chlorate-reducing bacteria can remove (per)chlorate from the environment by reducing (per)chlorate to chlorite by a (per)chlorate reductase (Kengen *et al*., 1999) and subsequently chlorite is converted to chloride and oxygen by a chlorite dismutase (van Ginkel *et al*., 1996). Research of van Ginkel *et al*. (1995), Coates *et al*. (1999[a]) and Wu *et al*. (2001) showed that (per)chlorate-reducing bacteria are widespread

in nature. Various (per)chlorate-reducing bacteria have been isolated from polluted sites. For example, *Acinobacter thermotoleranticus* was isolated from a waste stream of a match factory (Stepanyuk *et al*., 1993). Previous studies on the ubiquity and diversity of (per)chlorate-reducing bacteria, resulted in the description of *Dechloromonas* and *Dechlorosoma* species which represent the most predominant (per)chlorate-reducing bacteria in the environment (Coates *et al*., 1999[a]). This was confirmed by a study of Logan *et al.* (2001) in which ten chlorate-respiring bacteria were isolated and 16S rDNA sequencing suggested that most isolates could be classified within the genera *Dechloromonas* and *Dechlorosoma*. (Per)chlorate reducing activity was not only observed in chlorate or perchlorate polluted samples but also in pristine aquatic sediment and pristine soil (Coates *et al*., 1999[a]). Wu *et al*. (2001) observed perchlorate reduction when instead of 2 g, 100 g pristine soil was inoculated, and van Ginkel *et al*. (1995) observed chlorate reduction in public garden soil. Although, bacterial strains have been isolated from pristine soil samples (Coates *et al*.,1999[a]), none of these isolates have been characterized or made available by deposition in culture collections.

In this study the isolation of two novel strains, strain MA-1 and strain ASK-1 is described. Strain MA-1 is a (per)chlorate-reducing bacterium isolated from garden soil. The chlorate-reducing bacterium strain ASK-1 was isolated from sludge of a bioreactor, treating a bromate and chlorate polluted waste-stream. From this sample also *P. chloritidismutans* was isolated (Wolterink *et al*., 2002). For a third strain, strain GR-1, which has been described before (Rikken *et al*. 1996), a phylogenetic analysis was performed, which is included here.

Materials and methods

Enrichment cultures and isolation of strain MA-1 and strain ASK-1

Enrichment and cultivation of strain MA-1 and strain ASK-1 were performed in anoxic medium as was described previously for *P. chloritidismutans* with the following modifications: the gas phase used was N_2/CO_2 (80%/20%) and instead of 0.5 g of Na_2S, 0.2 g of Na_2SO_4 was used as sulfur source. For the isolation of both strains, chlorate (10 mM) and acetate (10 mM) were used as electron acceptor and electron donor, respectively. The pH of the medium was 7.2 and the batch cultures were incubated at 30ºC on an orbital shaker (100 rpm) in the dark. For enrichment and isolation of strain ASK-1, sludge was taken from an anaerobic bioreactor treating chlorate and bromate polluted waste water (Abma *et al*., 1999). This sludge sample has also been used for the isolation of *P. chloritidismutans* (Wolterink *et*

al., 2002). Strain ASK-1 was obtained by repeated application of the roll tube dilution method as described by Hungate (1969). For the isolation of strain MA-1, approximately 2 gram garden soil was added to 40 ml medium as described above. Enrichment cultures were checked for chlorate reduction. Up till now, (per)chlorate-reducing bacteria are facultative anaerobic organisms. Therefore, aerobic plating techniques using nutrient broth plates were used to obtain a pure culture of strain MA-1.

Determination of growth parameters

Substrates were added from 0.8 M stock solutions to give a final concentration of 10 mM. The following electron donors (10 mM) were checked with chlorate (10 mM) as electron acceptor: acetate; propionate; glucose; maltose; mannitol; malate; lactate; arabinose; hydrogen; glycine; glycerol; formate; gluconate; ethanol; starch; citrate or succinate. The following electron acceptors (10 mM) were tested with acetate (10 mM) as electron donor: perchlorate; chlorate; chlorite; nitrate; bromate; sulfate or oxygen. All anions were supplied as sodium salts. The reduction of Fe^{3+} (applied as Fe^{3+}-citrate) was only tested for strain MA-1. Fe^{3+} reduction was analyzed with a protocol described by Lovley and Phillips (1987), in which the reduction product (Fe^{2+}) was monitored spectrophotometrically by the reaction with ferrozine at 562 nm. Different pH values of the medium were obtained by changing the concentration of CO_2 in the gas phase, and they were calculated using the Henderson-Hasselbach equation as was described earlier (Wolterink *et al*., 2002). Growth was checked at the following temperatures: 10; 20; 30; 37; 50ºC.

Other micro-organisms

Strain GR-1 (DSM 11199) was grown in a medium as described before (Rikken *et al*., 1996) with modification as described by Kengen *et al*. (1999). *P. chloritidismutans* (DSM 13592) was grown in the medium described by Wolterink *et al*. (2002) and *P. stutzeri* DSM 50227 was grown in the medium described by Matsubara *et al*. (1982) with modifications described by Coyle *et al*. (1985).

Other analyses

Organic and inorganic compounds were analyzed using protocols described by Scholten and Stams (1995) and Stams *et al.* (1993). Gram-type was determined using Gram staining with protocols as described previously by Plugge *et al*. (2000).

Phylogenetic identification of the strains ASK-1, MA-1 and GR-1

The 16S rDNA sequence of strain ASK-1, MA-1 and GR-1 were determined at the German Collection of Microorganisms and Cell cultures (DSMZ). Approximately 95% of the 16S rDNA gene sequence was analyzed. The 16S rDNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA as described by Rainey *et al*. (1996). The 16S rDNA sequence was analyzed with ARB software (Ludwig and Strunk, 1996). The accession number of the 16S rDNA sequence of the strains are as follows: strain ASK-1, AY277620; strain MA-1, AY277621; strain GR-1, AY277622. For comparison of 16S rDNA molecules the following strains were used; *Pseudomonas chloritidismutans*, AY017341; *Pseudomonas stutzeri* DSM 50227, U26415; *Dechloromonas agitata*, AF047462; *Dechlorosoma suillum*, AF170348 and *Azospira oryzae* (AF011347). DNA-DNA hybridization was also done at the DSMZ. DNA was isolated by chromatography on hydroxyapatite by using the procedure of Cashion *et al*. (1977). DNA-DNA hybridization between DNA isolated from strain GR-1 and *D. suillum*; strain MA-1 and *D. agitata*; strain ASK-1 and *P. chloritidismutans*, was carried out as described by de Ley *et al*. (1970), with the modification described by Huss *et al*. (1983) and Escara and Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Enzyme activity measurements

For preparation of cell extracts, all procedures were done anaerobically using an anaerobic glovebox containing H_2 / N_2 (4% / 96%) gas (Wolterink *et al*., 2002). Oxyanion reductase activities were determined spectrophotometrically as described previously by Kengen *et al*. (1999) by monitoring the oxidation of reduced methyl viologen at 578 nm and 30ºC. Chlorite dismutase activity was determined as described by Wolterink *et al*. (2002) by measuring the oxygen production with a Clark-type oxygen Electrode (Yellow Springs Instruments, Yellow Springs, Ohio, USA). One unit (U) of activity is defined as the amount of enzyme required to convert 1 µmol of chlorite (or chlorate, bromate, nitrate) per min. The protein content of the cell extract fraction was determined according to the method of Bradford, with bovine serum albumin as standard (Bradford, 1976).

Electron microscopy

Cells of strain GR-1 were fixed in 4% formaldehyde in 0.01M phosphate buffered saline (PBS, pH 7.6), washed, dispersed in PBS and adsorbed to glow-discharged carbon-stabilized formvar-coated nickel grids, negatively stained using 2.0% ammonium molybdate (pH 5.1) or

2.0% potassium phosphotungstate (pH 6.0) and analyzed in a Philips TECNAI 12 electron microscope at a operating voltage of 80 kV. Images were digitally stored and analyzed using analySIS (Soft-imaging software, Germany). Images were printed after gray value modification.

Results and discussion

Characterization of strain ASK-1 and strain GR-1

The 16S rDNA sequence of strain ASK-1 was 100% similar to that of *P. chloritidismutans* and *Pseudomonas stutzeri* DSM 50227 (Wolterink *et al*., 2002). DNA-DNA hybridization between strain ASK-1 and *P. chloritidismutans* showed 79% similarity. To differentiate two species, DNA-DNA similarity should be less than 60-70% (Stackebrandt and Goebels, 1994). Therefore, strain ASK-1 is related at the species level to *P. chloritidismutans*. Both bacteria are chlorate-reducing bacteria, which can only grow with oxygen and chlorate as electron acceptors. Utilization of electron donors and acceptors for growth is presented in Table 1. The observed doubling time was 1.5 h and 10.6 h for *P. chloritidismutans* and strain ASK-1, respectively.

Despite the phylogenetic and physiological similarities, a morphological difference was seen when chlorate-grown cells were plated under aerobic conditions on nutrient broth plates. *P. chloritidismutans* formed yellow-brown circular, dry colonies (Fig. 1A) and strain ASK-1 formed white circular, dry colonies (Fig. 1B). Cell extract of chlorate-grown cells of *P. chloritidismutans* (grown in strictly anaerobic medium, Fig. 1C) had a red-brownish color, whereas cell extract of chlorate-grown cells of strain ASK-1 (grown in anoxic medium, Fig. 1D) showed to be white.

Table 1 Electron donor and acceptor usage of the chlorate-reducing bacterium strain ASK-1 and *P. chloritidismutans* and the perchlorate-reducing bacterium strain GR-1

	electron donor utilized	electron donor not utilized	electron acceptor utilized	electron acceptor not utilized
Strain ASK-1	acetate, glycerol, glycine, gluconate, glucose, mannitol, propionate	arabinose, citrate, ethanol, lactate, malate, maltose, starch, succinate	ClO_3^-, O_2	ClO_4^-, NO_3^-, BrO_3^-, SO_4^{2-}
P. *chloritidismutans**	acetate, propionate glucose, maltose, gluconate, mannitol, ethanol, starch, glycerol, citrate	malate, succinate lactate, glycine arabinose, H_2	ClO_3^-, O_2	ClO_4^-, NO_3^-, BrO_3^-, SO_4^{2-}
Strain GR-1**	acetate, propionate, caprionate, malate, succinate	glycine, formate glycolate, citrate glucose, arabinose mannose, mannitol maltose, gluconate N-acetylglucosamine adipate, phenyl acetate	ClO_3^-, O_2 ClO_4^-, NO_3^-, Mn(IV)	BrO_3^-, SO_4^{2-} IO_4^-, SeO_4^{2-} Fe(III)

* Wolterink *et al*., 2002
** Rikken *et al*., 1996

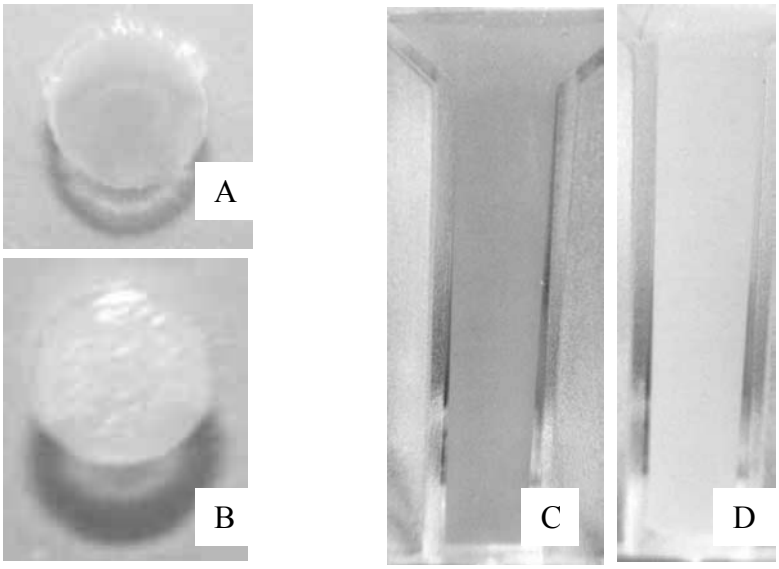

Fig. 1 Colony of *P. chloritidismutans* (A) and strain ASK-1 (B) grown on nutrient broth medium with oxygen as electron donor. Cell extract of *P. chloritidismutans* (C) and strain ASK-1 (D) from batch cultures, both grown with chlorate and acetate as electron acceptor and donor, respectively. This Figure has been reproduced in color on the back cover.

The color difference between strain ASK-1 and *P. chloritidismutans* might be caused by the amount of chlorite dismutase present in the cell. Purification of the chlorite dismutase of *P. chloritidismutans* showed that this enzyme had a reddish color, caused by the protoheme IX (see this thesis, chapter 4). The observed activity in cell extracts of *P. chloritidismutans* and strain ASK-1 were 134 U/mg and 6.3 U/mg, respectively, indicating that in strain ASK-1 less chlorite dismutase is present. Colonies of strain GR-1 growing anaerobically with chlorate on agar plates were circular and red. White colonies were obtained when the bacterium was growing with oxygen as electron acceptor (Rikken *et al*., 1996). Because perchlorate and chlorate reduction only occurred in the absence of oxygen, the color difference observed with strain GR-1 could also be caused by the presence of chlorite dismutase. The chlorite dismutase in this strain also contained a protoheme IX (van Ginkel *et al*., 1996)

Strain GR-1 was one of the first dissimilatory perchlorate-reducing bacteria that was described (Rikken *et al*., 1996). The (per)chlorate reductase and chlorite dismutase were purified and characterized for the first time from using strain. However, a taxonomic characterization of this perchlorate-reducing bacterium was still lacking. The only phylogenetic characteristic known sofar, is that this bacterium belongs to the β-*Proteobacteria*. The sequence analysis reported here showed that the 16S rDNA sequence of strain GR-1 was 99 % similar to that of *Dechlorosoma suillum*. DNA-DNA hybridization between these two strains revealed 85.1 % similarity. Based on the DNA-DNA hybridization threshold value, strain GR-1 is related at the species level to *Dechlorosoma suillum*. However, quite recently, it was shown that 16S rDNA sequence of *D. suillum* (AF170348) showed highest similarity to that of *Azospira oryzae* (AF011347) (99.9%). This sequence was not included in earlier phylogenetic analysis of *D. suillum* (Achenbach *et al*., 2001). The 16S rDNA sequence of *A. oryzae* was already available in 1995 (Hurek and Reinhold-Hurek, 1995) and the name *Azospira oryzae* was validated in 2000 (Reinhold-Hurek and Hurek, 2000). *A. oryzae* and *D. suillum* shared most of the phenotypic characteristics like carbon source utilization. Both bacteria are able to fix nitrogen, which is a main feature of *A. oryzae*. In both bacteria a *nifH*-homologous gene was detected and the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) showed that the *nifH* gene is functional and nitrogenase is present. The only difference was the inability of *A. oryzae* of dissimilatory perchlorate reduction. The DNA-DNA hybridization level of 90-92 % is above the suggested limit for species identity and together with the evidence described above, *D. suillum* is therefore a later subjective synonym of *Azospira oryzae* (Tan and Reinhold-Hurek, 2003). Hence strain GR-1 should be classified as *Azospira oryzae*.

In Fig. 2 an electron microscope picture is depicted of strain GR-1. The size of the rod-shaped bacteria are 1.8 (± 0.2) by 0.60 (± 0.05) μm and contain a single polar flagella.
The picture shows electron transparent globules, varying in size and number per cell. These globules possibly contain polybetahydroxybutyrate or other kinds of polybetahydroxyalkanoates (Thalen *et al*., 1999). The storage compounds are made when there is an excess of energy in comparison to other nutrients. Strain GR-1 contains fimbriae, by which it can be attached to surfaces (picture not shown).

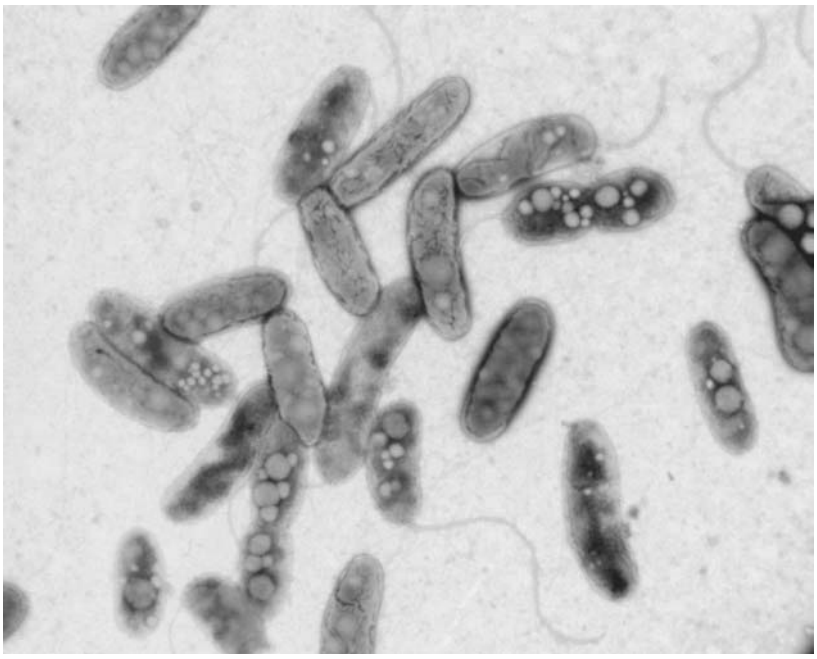

Fig. 2 Electron-microscope picture of cells of strain GR-1

Characterization of strain MA-1

Strain MA-1 was isolated from garden soil with no known history of (per)chlorate pollution. Gram-staining showed that strain MA-1 is a Gram-negative bacterium. The 16S rDNA sequence of strain MA-1 showed 99.9% and 97.6% similarity to those of *Dechloromonas* sp. strain SIUL (which is not a validated species) and *Dechloromonas agitata*, respectively. The 16S rDNA sequence similarity between strain MA-1 and *Ferribacterium limneticum* was 97.5%. This strain is not available in culture collections preventing further comparison. DNA-DNA hybridization between *D. agitata* and strain MA-1 showed 36.5% similarity, indicating that strain MA-1 is not related at the species level to *D. agitata*. Therefore, the name *Dechloromonas hortensis* sp. nov. is proposed for strain MA-1. Strain MA-1 did not grow in anoxic medium reduced with sulfide, which also indicated that

sulfide cannot serve as alternative electron donor, as was described for *D. agitata*. In growth experiments, Fe(III)-citrate reduction by strain MA-1 was tested, because this is a characteristic of the related *Ferribacterium limneticum*. Like other chlorate-reducing bacteria (Coates *et al*, 1999[a]), strain MA-1 could not grow by Fe(III) reduction. It was already concluded that *Ferribacterium limneticum* is in fact a member of a separate genus (Achenbach *et al*, 2001). A comparison of these three strains is given in Table 2.

Table 2 Characteristics of strain MA-1, *Ferribacterium limneticum* (Cummings *et al*., 1999) and *Dechloromonas agitata* (Achenbach *et al*., 2001)

	e-donors	e-acceptors	sample	morphology
Strain MA-1	acetate propionate	ClO_4^-, ClO_3^- O_2, NO_3^-	garden soil	rod shaped, single or in flocs
F. limneticum	acetate, formate lactate, benzoate	Fe^{3+}, NO_3^-, fumarate	mining impact lake sediment	straight or slightly curved rod shaped single or chains of 2-4 cells
D.agitata	acetate, propionate butyrate, lactate succinate, fumarate, malate, Fe^{2+}	ClO_4^-, ClO_3^-, O_2	waste pulp sludge from pulp and paper plant	straight or slightly curved rod shaped

Optimal growth of strain MA-1 was obtained at pH 7.2 and at a temperature of 30ºC. In cell extracts, the specific activity of chlorate reductase was 3.12 U/mg while the chlorite dismutase had a specific activity of 155 U/mg.

Description of *Dechloromona*s Achenbach *et al*. (2001)

Rod-shaped, Gram-negative cells, 0.5×2 µm, non-spore-forming, non-fermenting, facultative anaerobe. Cells are motile by a single polar flagellum and occur singly or in chains of two or three cells. A strictly respiring, complete oxidizer that oxidizes acetate with O_2, ClO_3^-, ClO_4^- or NO_3^- as alternative electron acceptors. Perchlorate and chlorate are completely reduced to chloride. Cells contain *c*-type cytochrome(s). Type strain is *Dechloromonas agitata*.

Description of *Dechloromonas hortensis* sp. nov.

Dechloromonas hortensis (hor.ten'sis. L. masc./fem. Adj. hortensis belonging to the garden).

Strain MA-1 is characterized as a Gram-negative, facultatively anaerobic, motile bacterium. Colonies on (aerobic) nutrient broth plates are circular and have a yellow color. In anoxic medium, which was not reduced with sulfide, optimal growth is obtained at pH 7.2 and at a temperature of 30ºC. Growth occurs with acetate and propionate as electron donors. No growth is found with citrate, gluconate, glucose, mannitol, maltose, starch, ethanol, methanol or sulfide. Perchlorate, chlorate, nitrate and oxygen are used as electron acceptors. Cell extract contains perchlorate-, chlorate-, nitrate- and bromate reductase activity. Chlorite is converted to chloride and oxygen. The specific activity of the chlorite dismutase in cell extracts is 155 U/mg. The strain belongs to the β-*Proteobacteria*. The 16S rDNA sequence is 99.9% similar to *Dechloromonas* spec. strain SIUL (99.9%). The highest similarity to a validated species is *Dechloromonas agitata* (97.6%). DNA-DNA hybridization between strain MA-1 and *Dechloromonas agitata* shows 36.5% similarity. Based on the DNA-DNA hybridization threshold value, strain MA-1 is not related at the species level to *D. agitata*. Therefore, *Dechloromonas hortensis* is proposed for strain MA-1. The strain has been deposited in the Deutsche Sammlung von Mikro-organismen und Zellculturen GmbH under DSM 15637 and at the American Type Culture Collection under ATCC BAA-776. The strain was obtained from a garden soil.

Emended description of Azos*pira oryzae* Reinhold-Hurek and Hurek 2000

Azospira oryzae (o.ry'zae. N.L. fem. N. *Oryza* genus name of rice; N.L. gen. n. *oryzae* from rice, referring to its frequent occurrence in association with rice roots).

The following description is based on the data of Reinhold-Hurek *et al*. (1993), Reinhold-Hurek and Hurek (2000), Achenbach *et al*. (2001) and Tan and Reinhold-Hurek (2003). On VM ethanol medium, colonies are translucent, pinkish to salmon-colored, smooth, convex with an entire margin. Growth optimum at 37ºC. Sources of isolates are surface-sterilized roots of Gramineae such as Kallar grass [*Leptochloa fusca* (L.) Kunth] or rice (*Oryza*) species, grown in the Punjab of Pakistan or Nepal, Philippines and Italy, respectively, resting stages (sclerotia) of basidiomycetes (Pakistan) and (per)chlorate-reducing enrichments from

samples collected from a primary treatment lagoon of swine waste in the USA. The type strain is strain 6a3^T (=LMG 9096^T), which has a G+C content of 65.2 mol% and was isolated from Kallar grass.

Description of *Azospira oryzae* strain GR-1

Strain GR-1 is a Gram-negative, oxidase-positive, motile rod-shaped organism. Cells can grow with O_2, ClO_4^-, ClO_3^-, NO_3^-, or Mn(IV) but not with BrO_3^- or SO_4^-, ClO_2^-, IO_4^-, SeO_4^{2-} or Fe(III) as electron acceptor. With ClO_4^- as electron acceptor, the following fatty acids and dicarboxylic acid can be used as sole carbon and energy source: acetate, propionate, caprionate, malate, succinate. The strain does not catabolize glycine, formate, glycolate or citrate and glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, adipate or phenyl acetate are not assimilated. The bacterium does not have β-glucosidase, β-galactosidase or protease activity and does not form indole from tryptophan. The bacterium grows anaerobically with chlorate at pH 7.2 and at a temperature of 30°C. Colonies of strain GR-1 growing on agar plates are circular and red. White colonies are obtained when the bacterium is growing with oxygen as electron acceptor. 16S rDNA sequence analysis reveals 99 % similarity between strain GR-1 and *Dechlorosoma suillum*. DNA-DNA hybridization between these two strains shows 85.1 % similarity. *D. suillum* shows to be a later subjective synonym of *Azospira oryzae*. 16S rDNA sequence analysis reveals 99 % similarity between strain GR-1 and *A. oryzae*. The strain is therefore referred to *Azospira oryzae* strain GR-1. The strain was deposited in the Deutsche Sammlung von Mikro-organismen und Zellculturen GmbH under DSM 11199 and in the American type Culture Collection under ATCC BAA-777. The strain was obtained from activated sludge from a plant treating predominantly domestic waste water

Description of *Pseudomonas chloritidismutans* strain ASK-1

The bacterium obtained is Gram-negative, motile and facultative anaerobic organism. ClO_3^- and O_2 are used as electron acceptor during growth, but not ClO_4^-, NO_3^-, BrO_3^- or SO_4^-. Electron donors which can be used are; acetate, glycerol, glycine, gluconate, glucose, mannitol or propionate. The following compounds cannot be used; arabinose, citrate, ethanol, lactate, malate, maltose, starch or succinate. Colonies growing aerobically on nutrient broth

agar plates are circular and white. The doubling time is 10.6 h using anoxic media with chlorate and acetate as electron acceptor and donor, respectively. Cell extracts, shows chlorate and bromate reductase activity (specific activity (U/mg) of 6.1 and 4.5, respectively). No reductase activity is found for the following compounds; ClO_4^-, NO_3^- or SO_4^-. ClO_2^- dismutase activity is present (specific activity 6.3 U/mg). Comparison of 16S rDNA sequences shows 100% similarity to *P. chloritidismutans*. DNA-DNA hybridization between strain ASK-1 and *P. chloritidismutans* showed 79% similarity. Therefore, strain ASK-1 is named, *Pseudomonas chloritidismutans* strain ASK-1. The strain was deposited at the DSMZ under DSM 15671 and at the ATCC under ATCC BAA-775. The strain was obtained from sludge taken from a bioreactor, treating a bromate and chlorate polluted wastewater.

Acknowledgements

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Chapter 6

Origin of chlorate

A.F.W.M. Wolterink, S.W.M. Kengen and A.J.M. Stams

Abstract

The presence of chlorate (ClO_3^-) in natural environments is due to human activity because chlorate is used as herbicide or for the production of chlorine dioxide. Because there are no natural sources of chlorate known, it is remarkably that chlorate-reducing bacteria are quite abundant in the environment. The fact that these bacteria have been isolated from chlorate-polluted as well from pristine sites suggests that there maybe other sources of chlorate. The possible formation of chlorate by biotic and abiotic processes is discussed. Chlorate could be a by-product of biological formation of antibiotics. Chlorine is activated as hypochlorous acid by the enzymes, perhydrolases or haloperoxidase. These enzymes show neither substrate specificity nor regioselectivity and produce free hypochlorous acid as the chlorinating agent. By chemical decomposition of hypochlorous acid, chloric and hydrochloric acid maybe formed.

The observation of nitrate in rainwater was due to the fixation of atmospheric nitrogen and oxygen by lightning discharges. The presence of chlorine compounds in coastal areas could indicate that the same process may lead to chlorate formation.

Introduction

During the last decade, several (per)chlorate-reducing bacteria were isolated and characterized. These bacteria are capable of the reduction of (per)chlorate into chlorite by a (per)chlorate reductase. Chlorite is subsequently converted into chloride and oxygen by a chlorite dismutase. This route of dissimilatory (per)chlorate reduction (Fig. 1) was first demonstrated by Rikken *et al*. (1996) for strain GR-1.

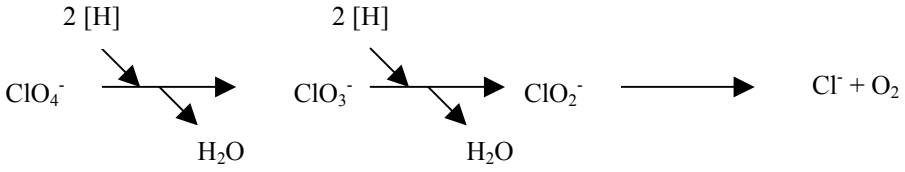

Fig. 1 Proposed reduction pathway of perchlorate catalyzed by strain GR-1

The interest in the metabolic features of these bacteria has increased because this type of organisms could be applied in solving pollution problems. One example is the removal of (per)chlorate from polluted sites (Hunter, 2002; Losli *et al*., 2002). The occurrence of (per)chlorate in the environment is due to the production of perchlorate in the space and army industry, where it is used as propellant in rocket fuel. This oxyanion is released in the

environment by disarmament programs. Chlorate is used as herbicide or for the on-site production of chlorine dioxide in paper- or textile industry. In addition, oxygen which is produced by the conversion of chlorite, maybe used in bioremediation strategies. In anaerobic zones in soils which are polluted with BTEX compounds (benzene, toluene, ethylbenzene and xylene), (per)chlorate-reducing bacteria can supply oxygen to the aerobic hydrocarbon-oxidizing populations (Coates *et al*., 1999[b]; Logan and Wu, 2002).

For chlorate and perchlorate the only known natural source is found in a nitrate deposit in Chile (Achenbach *et al*., 2001). In 1884, the first commercial electrolytic chlorate production process was described by F. Hurter, H. Gall and A. de Montlaur (Mellor, 1946). The overall cell reaction for the electrolysis of a salt solution to form sodium chlorate can be expressed by the following equation; $NaCl + 3H_2O$ + electricity (6 Faradays) $\rightarrow NaClO_3 + 3\ H_2$ (Mellor, 1946; http://people.delphi.com). It can be assumed that from 1884 onward, the environment became polluted with chlorates produced by man.

Taken this into account, some aspects are remarkable. (Per)chlorate-reducing bacteria can be isolated from various environments, including polluted as well as pristine locations (Wu *et al*., 2001; Coates *et al*., 1999[a]). It is relatively easy to isolate (per)chlorate-reducing bacteria, and this has led to the description of several species in the class of *Proteobacteria* (Fig. 2, Chapter 1 of this thesis). Enzymes were purified from (per)chlorate-reducing bacteria which were specific for perchlorate and chlorate (Kengen *et al*., 1999), and for chlorate only (Wolterink *et al*., 2003; Danielsson Thorell *et al*., 2003). Species isolated in the Netherlands are similar to species which were isolated in the United States of America when the 16S rDNA sequences were compared. In Table 1, a comparison is made between species isolated on the different continents.

Table 1

Isolated in The Netherlands	Isolated in the U.S.A.	16S rDNA similarity	
Strain GR-1	*Dechlorosoma suillum*	99 %	(this thesis, chapter 5)
P. chloritidismutans	*P. stutzeri* strain PK	99 %	(Wolterink *et al*., 2002)
Strain MA-1	*Dechloromonas agitata*	97.6 %	(this thesis, chapter 5)

When there are no natural sources of chlorate and perchlorate known, the occurrence of (per)chlorate-reducing bacteria must be explained by the contamination of the environment with these oxyanions by man over the last one hundred years. From an evolutionary

perspective, 100 year is short, for bacteria to develop two different enzymes which can reduce perchlorate and chlorate (strain GR-1; Kengen *et al*., 1999) or chlorate only (*P. chloritidismutans*; Wolterink *et al*., 2003). Achenbach *et al*. (2001), found it surprising that a phylogenetic diverse group of bacteria could have evolved the ability to grow by dissimilatory (per)chlorate reduction in such a short time frame. In the past, it was thought that the ability of denitrifying bacteria to reduce chlorate was a result of the activity of nitrate reductases (De Groot and Stouthamer, 1969; Oltmann *et al*., 1976[a,b]). It has been suggested that dissimilatory (per)chlorate reduction is the result of horizontal gene transfer event (Achenbach *et al*. 2001). However, purified (per)chlorate reductases showed to be different in substrate specificity, localization, subunit composition. More data about (per)chlorate reductases and enzyme characteristics are needed to compare nitrate reductases and (per)chlorate reductases on a biochemical and genetical level in order to find out if (per)chlorate reductases have evolved from nitrate reductases.

We discuss here the option that chlorate could have been formed from other sources. In that case, bacteria were already adapted to this kind of electron acceptor, before man started to release chlorate into the environment. In this communication, a light is shed on possible abiotic and biotic processes, leading to chlorate.

Biotic formation of chlorate

Sofar, no natural sources of chlorate have been found and therefore the presence of this compound is believed to be due to anthropogenic activities. Similar thoughts have existed for xenobiotic compounds. It was believed that chlorinated organic compounds were xenobiotic, meaning that these compounds were made by man only. Öberg (2002) proposed that chlorinated organic compounds are not xenobiotic because: (1) more than 1,000 naturally produced chlorinated compounds have been identified; (2) it is more rule than the exception that organisms are able to convert chloride to organic chlorine; (3) the vast majority of naturally produced organic chlorine is neither persistent nor toxic; (4) organic chlorine is as abundant or even more abundant than chloride in soil.

The production of chlorinated organic compounds was shown for several microorganisms. Chlorinated anisyl metabolites (CAM) are biosynthesized by various fungi like, *Lepista*, *Stropharia* and *Bjerkandera* spp. (de Jong *et al*., 1994[a]). These compounds are produced by wood- and forest litter-degrading fungi in the laboratory as well as in the natural environment. Besides antibiotic properties, the CAM alcohols act as substrate for extracellular aryl alcohol

oxidase, generating H_2O_2 for the lignin degrading peroxidases (de Jong et al., 1994[b]). The natural production of trichloroethylene and perchloroethylene was demonstrated for various marine macroalga (*Asparagopsis taxiformis*) and microalga (*Porphyridium purpureum*) (Abrahamsson *et al*., 1995).

The chlorine-containing antibiotic caldariomycin is produced in the fungus *Caldariomyces fumago*. The reaction is carried out by a heme-containing haloperoxidase which requires hydrogen peroxide and chloride for catalysis (Fig. 2) (van Pée, 2001).

A

$$\text{CPO-Fe} + H_2O_2 \longrightarrow \text{CPO-Fe=O} + H_2O$$
$$\text{CPO-Fe=O} + Cl^- \longrightarrow \text{CPO-Fe-O-Cl}$$
$$\text{CPO-Fe-O-Cl} + H^+ \longrightarrow \text{CPO-Fe} + \text{HOCl}$$
$$\text{HOCl} + \text{A-H} \longrightarrow \text{A-Cl} + H_2O$$

B

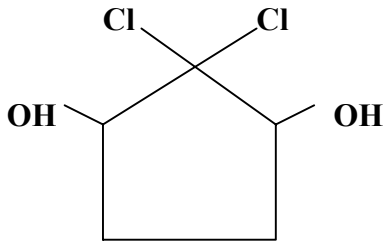

Fig. 2 A : Reaction mechanism of chloroperoxidase (CPO). Fe, heme iron; CPO-Fe, native chloroperoxidase; CPO-Fe=O, compound I; CPO-Fe-O-Cl, enzyme-bound hypochlorous acid; A-H, organic substrate.
B : Molecular structure of caldariomycin.

Pseudomonas and *Streptomyces* strains showed to contain another type of enzyme with halogenating activity, the perhydrolases. These enzymes do not contain heme. The reaction of catalysis is shown in Fig. 3 (van Pée, 2001).

$$\text{E-Ser-OH} + \text{AcOH} \longrightarrow \text{E-Ser-O-Ac} + H_2O$$
$$\text{E-Ser-O-Ac} + H_2O_2 \longrightarrow \text{E-Ser-OH} + \text{Ac-O-O-H}$$
$$\text{Ac-O-O-H} + Cl^- + H^+ \longrightarrow \text{AcOH} + \text{HOCl}$$
$$\text{HOCl} + \text{A-H} \longrightarrow \text{A-Cl} + H_2O$$

Fig. 3 Reaction mechanism of perhydrolases. E-Ser-OH, serine residue at the active site; AcOH, acetic acid; E-Ser-O-AC, acetyl-enzyme intermediate; Ac-O-O-H, peracetic acid; A-H, organic substrate.

Both enzymes show neither substrate specificity nor regioselectivity with regard to the halogenating reaction (Sundaramoorthy *et al*., 1998). Biochemical investigation showed that the enzymes produced free hypochlorous acid as the chlorinating agent. The production of free hypochlorous acid leads to a variety of chemical reactions. This means that it can react

with compound A-H as is depicted in Fig. 2 and 3, but it can react with any other molecule. Free hypochlorous acid, being unstable, decomposes into chloric and hydrochloric acid: 3 HOCl = $HClO_3$ + 2HCl (Mellor, 1946). This chemical process can occur whenever hypochlorous acid molecules are present. In this way, biotic chlorate formation is a by-product of antibiotic and xenobiotic (chlorinated organic compound) formation. The by-product chlorate is probably not produced in sufficient amounts for a bacterium to gain energy for all cellular processes. The production of chlorate may have initiated the evolution of chlorate reductases. Alternatively, chlorate may have been converted by nitrate reductases. In either cases chlorite is formed. Thus the conversion of chlorate must have coincided with the evolution of a chlorite dismutase. This detoxification can be compared to the degradation of the highly reactive radical, superoxide ion (O_2^-). This ion is formed by a large number of oxidases (aldehyde oxidase, NADP oxidase) in aerobes. To prevent damaging effects in the cell, two enzymes, the superoxide dismutase and catalase are needed to convert the superoxide radicals into harmless oxygen (Schlegel, 1993). Recent investigation of chlorite dismutases suggested that these enzymes maybe abundantly present among microorganisms. The gene sequences of the chlorite dismutases of *Ideonella dechloratans* (Stenklo *et al*., 2002) and *Dechloromonas agitata* (Bender *et al*., 2002) were analyzed and a BLAST 2.2 analysis indicated that the chlorite dismutase sequences were 71% similar at the amino acid level. Both bacteria were detected using a *D. agitata* probe to the 3'-end of the chlorite dismutase gene (Bender *et al*., 2002). Also a weak hybridization signal was obtained from *Magnetospirillum magnetotacticum*. A genome search for this organism revealed the presence of a putative chlorite dismutase. However, physiological experiments with *M. magnetotacticum* showed that this bacterium could not grow by (per)chlorate respiration. The functional role of the putative chlorite dismutase gene in this organism remains unknown.

Besides the putative chlorite dismutase in *M. magnetotacticum*, an internal sequence of a hypothetical protein of *Burkholderia fungorum* showed high similarity to the N-terminal sequence of the chlorite dismutase of *P. chloritidismutans* (Fig 4).

Pseudomonas chloritidismutans	4	M S H D S M S K D S M S K D A M K K D G M	24
		M S H D S M S K D + M S K D + M K D M	
Burkholderia fungorum	28	M S H D S M S K D A M S K D S M A K D S M	48

Fig. 4 Alignment of the N-terminal sequence of chlorite dismutase of *P. chloritidismutans* and an internal sequence of a hypothetical protein of *B. fungorum*

Several strains of *B. fungorum* (Coenye *et al*., 2001) have been isolated from the white-rot fungus *Phanerochaete chrysosporium* (Seigle-Murandi *et al*., 1996). It has been shown that *B. fungorum* is very closely associated with the fungus and attempts to eliminate the bacteria from the fungus were unsuccessful. The fungal secondary metabolite 2-chloro-1,4-dimethoxybenzene was isolated from *Ph. chrysosporium* (Valli and Gold, 1991). This is a co-factor which prevents the formation of an inactive form of the lignin peroxidase. Since *B. fungorum* was very successful in degradation of the aromatic compounds derived from degradation of lignin by white-rot fungi, the suggestion was made that there is a symbiotic relation between *B. fungorum* and *Ph. chrysosporium*. Because only the N-terminale sequence of the chlorite dismutases of *P. chloritidismutans* is known, comparison of the characteristics of the genes (sizes, initiation sites) can not be made. Nevertheless, the resemblance of an internal fragment of the sequence of a hypothetical protein of *B. fungorum* to the N-terminal sequence of the chlorite dismutase of *P. chloritidismutans* could be an indication that a chlorite dismutase maybe present in *B. fungorum*, that is involved in the reduction of chlorate which is formed as a by-product of the formation of 2-chloro-1,4-dimethoxybenzene.

Abiotic chlorate formation in liquids

Chlorate is made by man using an electrolytic cell. During the electrolysis of a solution of sodium chloride (NaCl), a number of simultaneous reactions take place which results in the following overall cell reaction; $NaCl + 3H_2O$ + electricity (6 Faradays) $\rightarrow NaClO_3 + 3\ H_2$ (Mellor, 1946; http://people.delphi.com). In nature lightning might be the driving force of these reactions. When lightning channels hit the surface of the sea, chlorate can theoretically be formed. We performed an experiment to test this. Unfortunately, we were not able to demonstrate lightning-mediated chlorate formation yet (Fig. 5).

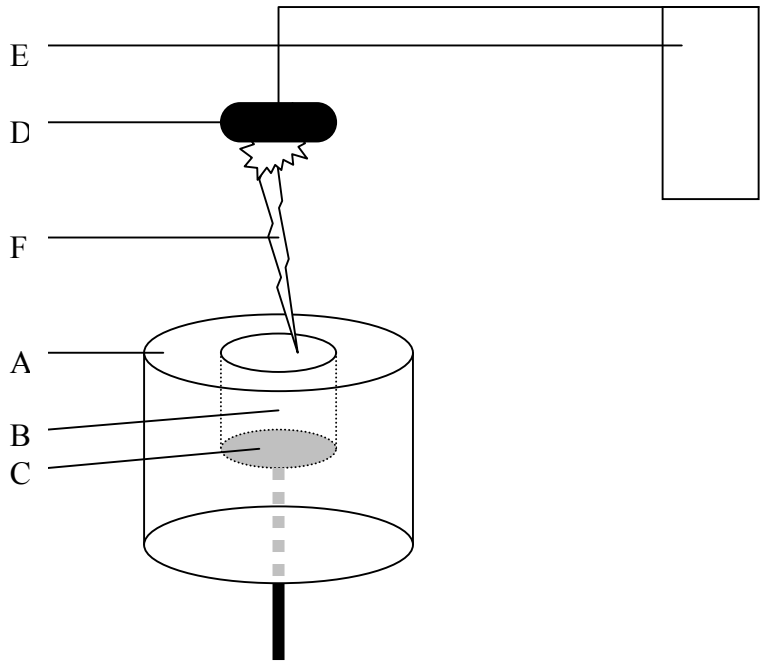

Fig. 5 Setup of the lightning experiment. A massif lexan cylinder (A), with a 100 ml reservoir (B), is connected via a copper plate to a copper pin (grounded electrode)(C). (Lightning) discharges were generated with a lightning surge, a large spark generator (2 MV, 1-50 us se generator) (E) which was connected to a high-voltage electrode (D). The following solutions were tested: seawater; 20 g/L NaCl solution; 20 mM sodium hypochlorite (NaOCl) aqueous solution (Aldrich). Each solution was hit ten times by a discharge channel. The solutions were kept at 4°C and analyzed with a Dionex DX-100 HPLC system (Dionex, Breda, The Netherlands) equipped with a IONPAC AS9-SC analytic column and ED40 electrochemical detector. For detection of low concentrations of anions, the detector was set to SRS 50 mA, range 10 µS (standard condition, SRS 50 mA, range 100 µS). Potassium fluoride (KF; 50 µM) was used as internal standard in a 20 mM mannitol solution (standard condition, 2 mM KF). For sample preparation, 500 µl mannitol/KF solution was added to 500 µl sample (standard conditions, 500 µl mannitol/KF solution, 450 µl milliQ water, 50 µl sample). A carbonate/bicarbonate solution was used as mobile phase (0.515 g/L Na_2CO_3*$10H_2O$; 0.144 g/L $NaHCO_3$). When seawater (Cl^-, 19.353 g/kg; http://earthref.sdsc.edu) or a NaCl solution (20 g/L) were analyzed after these solutions were hit by lightning, no detectable formation of chlorate was observed. Apparently, the conditions are not comparable as those in an electrolytic cell. During the lightning experiment with NaOCl, gas bubbles appeared on the surface of the solution. The gas formation was not monitored but probably Cl_2 was formed. When the solution was analyzed afterwards, no NaOCl was detected. However, an increase of 14.3 µM chlorate was detected. These experiments show that exposure of Cl^--containing solutions to lightning does not result in the detectable formation of chlorate. The significance of the formation of ClO_3^- from NaOCl is probably low.

Abiotic chlorate formation in gas phase

In 1827, von Liebig (1827) proposed that the NO_3^- which was observed in rainwater, was produced via the fixation of atmospheric N_2 and O_2 by lightning discharges. The temperature in a discharge tube reaches 30,000 K and the gas is completely ionized plasma. Oxygen and nitrogen are partly dissociated to O and N. NO formation occurs in the reaction between O and N_2 or N and O_2 (Goldenbaum and Dickerson, 1993). NO is further oxidized to NO_2 with ozone (O_3) . HNO_3 is produced in the reaction between NO_2 and OH radicals, which is the main oxidizing agent in the troposphere (Stockwell *et al*., 1999). Production of NO_x (NO + NO_2) in terms of mass of nitrogen, was 22.5 ± 2.9 mg per spark, yielding an annual global NO_x production of 9.7 Tg(N) yr^{-1} (Cook *et al*., 2000). By Lee *et al*. (1997) the NO_x emission in Tg(N) yr^{-1} was estimated to be 5, 22 and 7 for lightning, fossil fuel combustion and soil microbial production, respectively.

The emissions of nitrogen oxides by natural lightning processes, could indicate the formation of other compounds as well. For chlorine gas (Cl_2), no research has been done to see what will happen in a thunder storm. The presence of this compound in coastal sites near Long Island, New York, was shown by Spicer *et al*. (1998). Model predictions showed that also other chlorine species like ClO, HOCl and HCl, were present.

For the abiotic and biotic formation of chlorate, experimental evidence is lacking. In future, research has to be done to see if chlorate forming processes other than the electrolytic process, occur in nature.

Acknowledgment

The authors would like to thank Lex van Deursen and Rob Kerkenaar at the Technical University of Eindhoven (Power Engineering Division) for providing the possibility to use and assistance in using the large spark generator.

Chapter 7

Summary and concluding remarks

In 1999, at the start of this project, little information was available about (per)chlorate-reducing bacteria. At that time a few bacterial strains had been isolated from reactors in which (per)chlorate reduction was observed. The process of (per)chlorate reduction was investigated in more detail for strain GR-1. It was shown that the reduction of perchlorate and chlorate by a (per)chlorate reductase, leads to the formation of chlorite and that chlorite is subsequently converted into chloride and oxygen. This intermediate formation of chlorite was never detected in batch or continuous culture, but came apparent through the purification and characterization of chlorite dismutase of strain GR-1. However, it remained unknown whether this (per)chlorate reduction pathway is common for bacteria, which can grow by dissimilatory (per)chlorate reduction. Before the results of strain GR-1 became available, it was thought that chlorate-reducing bacteria reduced chlorate via a nitrate reductase. Denitrifying bacteria contain a nitrate reductase which also exhibits chlorate reductase activity. However, none of these denitrifying bacteria were able to grow using chlorate or perchlorate

To get more insight in the (per)chlorate reduction pathway and to find out how ubiquitous (per)chlorate-reducing bacteria are distributed in the environment, the current research was initiated. In this study, several (per)chlorate-reducing bacteria were isolated and characterized. The isolation and characterization of the chlorate-reducing strain AW-1, from a bioreactor treating a chlorate and bromate polluted waste stream, enabled the comparison of denitrifying bacteria with (per)chlorate-reducing bacteria (Chapter 2). Despite a very close phylogenetic relationship between strain AW-1 and two *Pseudomonas stutzeri* strains, the former was not able to use nitrate as electron acceptor, and the latter two were not able to use chlorate. These results confirmed that separate pathways exist for chlorate and nitrate reduction. In accordance, chlorite dismutase activity was only present in strain AW-1 and it was therefore proposed to name strain AW-1, *Pseudomonas chloritidismutans* sp. nov.. *P. chloritidismutans* was differentiated from other described (per)chlorate-reducing bacteria, by its inability to use perchlorate.

Chapter 5 describes a bacterium, strain MA-1, capable to grow with perchlorate, chlorate, nitrate and oxygen. Strain MA-1 was isolated from a soil sample, that was not polluted with

chlorate or perchlorate. 16S rDNA sequence analysis showed similarity to the (per)chlorate-reducing bacterium *Dechloromonas agitata* and *Ferribacterium limneticum*. This latter strain was only capable of dissimilatory reduction of nitrate and Fe^{3+}. Both strain MA-1 and *D. agitata* were not able to grow by Fe^{3+} reduction. Moreover, *D. agitata* was not able to grow with nitrate. DNA-DNA hybridization experiments showed that strain MA-1 was not related at the species level to *D. agitata*. Therefore, the name *Dechloromonas hortensis* sp. nov. was proposed for strain MA-1.

Another chlorate-reducing bacterium, strain ASK-1, was isolated from the same sludge sample *P. chloritidismutans* was isolated from (Chapter 5). 16S rDNA sequence analysis and DNA-DNA hybridization experiments between strain ASK-1 and *P. chloritidismutans* showed that these strains belonged to the same species. Strain ASK-1 could only grow with chlorate and oxygen as electron acceptor as was found for *P. chloritidismutans*. However, the morphology and growth requirements were different. The previously isolated strain GR-1 had not yet been taxonomically described. Therefore, 16S rDNA sequence analysis and DNA-DNA hybridization experiments were included in this chapter. It was found that strain GR-1 was related at the species level to another (per)chlorate-reducing strain, *Dechlorosoma suillum*, which was recently renamed as *Azospira oryzae*.

Chlorate and perchlorate are man made. The (per)chlorate-reducing bacteria which were characterized and deposited in culture collections, have all been isolated from (per)chlorate polluted sites. The isolation of (per)chlorate-reducing *D. hortensis* from a pristine soil sample, indicated that there might be a natural source of chlorate. Such (a)biotic processes by which chlorate can be formed are discussed in Chapter 6. Firstly, chlorate formation might be related to the production of antibiotics. During the biosynthesis of certain antibiotics, hypochlorous acid is formed, which may lead to various chlorinated compounds, including chlorate. Secondly, another process by which chlorate could be formed is lightning. Lightning experiments have already shown that nitrate is formed from atmospheric nitrogen and oxygen. The presence of chlorine gas and other chlorine species (ClO, HOCl and HCl) in coastal areas, could be indicative that similar processes may occur for chlorine species. For either of both alternatives, experimental evidence is lacking. However, these (a)biotic chlorate forming processes could explain the widespread presence of chlorate-reducing bacteria in the environment.

To get more insight into the pathway of chlorate reduction and how reduction of chlorate is coupled to ATP synthesis, the chlorate reductase of *P. chloritidismutans* was purified and characterized (Chapter 3). The oxygen sensitive chlorate reductase showed only reductase

activity with chlorate and bromate which was different from the purified (per)chlorate reductase of strain GR-1, which in addition showed activity with perchlorate, nitrate and Mn(V). The chlorate reductase of *Ideonella dechloratans* also showed no reductase activity with perchlorate. The cytoplasmic localization of the chlorate reductase of *P. chloritidismutans* was different from the periplasmic orientation of the (per)chlorate reductase of strain GR-1 and of the chlorate reductase of *I. dechloratans*. The molecular weight of the native enzyme was 167 kDa and consisted of 3 subunits of 97, 38 and 34 kDa. The same stoichiometry ($\alpha_1\beta_1\gamma_1$) was found for the chlorate reductase of *I. dechloratans* but this was different from the stoichiometry found for the (per)chlorate reductase of strain GR-1 ($\alpha_3\beta_3$). The β-subunit of the chlorate reductase of *P. chloritidismutans* revealed sequence similarity to β-subunits of various other reductases or dehydrogenases. The chlorate reductase from *P. chloritidismutans* was found to contain Mo and Fe, as was the case for the reductase of strain GR-1. EPR analysis confirmed this. The presence of molybdenum is not unusual because sofar, all enzymes that show chlorate reductase activity contain molybdenum (chlorate reductase of *P. chloritidismutans* and *I. dechloratans*, (per)chlorate reductase of strain GR-1, nitrate reductase, trimethylamine N-oxide (TMAO) reductase). These enzymes are positioned in the dimethyl sulfoxide (DMSO) reductase family.

The chlorite dismutase was purified from *P. chloritidismutans* (Chapter 4) and this enzyme showed similar characteristics when compared to chlorite dismutases of strain GR-1, *Dechloromonas agitata* and *I. dechloratans*. The localization (periplasm), subunit composition (homotetramer), subunit size and the spectral characteristics (heme group, Fe-protoporphyrin IX) were the same.

Experiments with ^{18}O-labelled water showed that no $^{36}O_2$ or $^{34}O_2$ labeled oxygen was produced, indicating that water is not a substrate in the conversion of chlorite and that oxygen is entirely produced out of chlorite.

The N-terminal sequence of the chlorite dismutase of *P. chloritidismutans* had no similarity with the N-terminal sequences of other chlorite dismutases. Instead, similarity was found with an internal sequence of a hypothetical protein of *I. dechloratans*. The gene coding for this hypothetical protein (partial ORF2) was located downstream of the gene of the chlorite dismutase. PCR experiments showed that the chlorite dismutase gene arrangement of *P. chloritidismutans* was not similar to the organization of the chlorite dismutase gene and partial ORF2 which was found for *I. dechloratans*.

It is not yet clear, how the respiratory pathway is arranged in chlorate-reducing bacteria. However, based on the information which was obtained in this research and in analogy with

nitrate respiration pathway, a schematic model is proposed as is shown in Fig. 1. The chlorate reductase and chlorite dismutase are located in different cell fractions. In this way the oxygen generating process is separated from the oxygen sensitive chlorate reductase. However, the oxygen sensitive (per)chlorate reductase of strain GR-1 showed to have the same periplasmic location as the chlorite dismutase. A chlorate/chlorite antiport system is suggested to translocate chlorate into the cytoplasm and the highly oxidative chlorite into the periplasm.

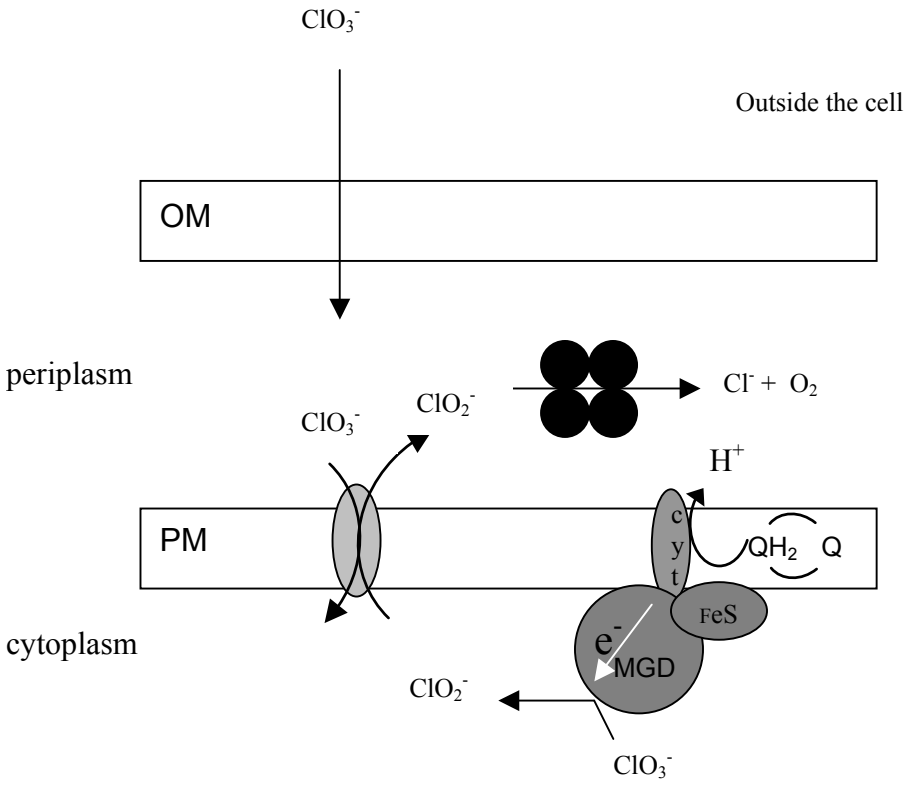

Fig. 1 Schematic model presentation of chlorate reduction pathway in *P. chloritidismutans*. Chlorate diffuses into the periplasm and may be transferred to the cytoplasm by means of an antiport exchanger (chlorite is transferred form cytoplasm to periplasm). Proton release into the periplasm occurs when the electrons are transferred from coenzyme (Q) to cytochromes (cyt; γ-subunit) and nonheme iron proteins (FeS; β-subunit) to the α-subunit, containing the molybdenum cofactor (MGD), which is the active site of the enzyme. This subunit donates electrons to chlorate which is then reduced to chlorite. Chlorite is converted into chloride an oxygen by a chlorite dismutase which is located in the periplasm. When protons move back to the cytoplasm via ATP synthase (not shown in the figure), ATP is synthesized from ADP and P_i. OM : outer membrane; PM : plasma membrane.

Antiport system

Chlorite dismutase

Chlorate reductase

This antiport is based on the antiport found in denitrifiers. The organization of chlorate reduction in *P. chloritidismutans* may resemble the organization of membrane-bound nitrate reductase. In the model, the chlorate reductase is associated with the membrane, but it was purified as a soluble enzyme. It is possible that during purification a specific component has been removed from the catalytically active chlorate reductase

(Per)chlorate-reducing bacteria contain a unique metabolism employing novel enzymes ((per)chlorate reductase, chlorite dismutase). Besides photosynthesis and catalase activity, the conversion of chlorite is the third biological process in which oxygen is produced.

Chapter 8

Samenvatting en afsluitende opmerkingen

In 1999, aan het begin van dit promotie onderzoek, was er weinig informatie beschikbaar over (per)chloraatreducerende bacteriën. Een aantal bacteriën was geïsoleerd uit reactoren waarin (per)chloraatreductie aangetoond was. Één van die bacteriën was stam GR-1. Met behulp van deze stam werd het (per)chloraatreductie proces nader bekeken. De reductie van perchloraat en chloraat bleek gekatalyseerd te worden door één enzym, het (per)chloraatreductase. Het chloriet dat bij deze reactie vrijkwam, werd door het chlorietdismutase omgezet tot chloride en zuurstof. Chloriet werd nooit waargenomen gedurende een groeiexperiment met (per)chloraat-reducerende bacteriën maar de aanwezigheid als intermediaire component werd aangetoond met de zuivering en karakterisering van het chlorietdismutase van stam GR-1. Het was echter onduidelijk of andere (per)chloraatreducerende bacteriën hetzelfde reductie/dismutase systeem gebruiken. Voordat de resultaten van de enzymen van GR-1 bekend werden, dacht men dat chloraatreductie activiteit veroorzaakt werd door de activiteit van nitraatreductases. In denitrificerende bacteriën was al aangetoond dat nitraatreductases, chloraatreductase activiteit vertoonden. Echter deze bacteriën vertoonden geen groei met chloraat.

Om meer inzicht te krijgen in het (per)chloraatreductieproces en om te kijken of deze reductie een veel voorkomend proces in de natuur is, werd de huidige studie geïnitieerd. In deze studie werden verschillende (per)chloraatreducerende bacteriën geïsoleerd en gekarakteriseerd. Door de isolatie en de karakterisering van de chloraatreducerende bacterie AW-1 uit een bioreactor, die een chloraat/bromaat vervuilde afvalstroom behandelde, was het mogelijk om chloraatreducerende bacteriën met denitrificerende bacteriën te vergelijken (Hoofdstuk 2). Ondanks de overéénkomende fylogenetische relatie tussen stam AW-1 en twee *Pseudomonas stutzeri* stammen, kon stam AW-1 geen nitraat als elektronenacceptor gebruiken en vertoonden de twee *P. stutzeri* stammen geen groei met chloraat. Deze resultaten bevestigden dat chloraat- en nitraatreductie twee aparte reductieprocessen zijn. Chlorietdismutase activiteit werd alleen aangetoond in stam AW-1. Door deze verschillen werd voorgesteld om stam AW-1 te benoemen als *Pseudomonas*

chloritidismutans sp. nov. Een onderscheid kon ook gemaakt worden met andere (per)chloraatreducerende bacteriën omdat stam AW-1 perchloraat niet als elektronenacceptor kon gebruiken.

Hoofdstuk 5 beschrijft de isolatie en karakterisering van stam MA-1, die perchloraat, chloraat, nitraat en zuurstof als elektronenacceptor kon gebruiken. Stam MA-1 werd geïsoleerd uit een bodemmonster, dat niet met perchloraat of chloraat verontreinigd was. 16S rDNA sequentie analyse toonde aan dat deze bacterie gelijkenis vertoonde met *Dechloromonas agitata* en *Ferribacterium limneticum*. De laatst genoemde bacterie is in staat om nitraat en Fe^{3+} te gebruiken als elektronenacceptor. Zowel stam MA-1 als *D. agitata* konden niet met Fe^{3+} groeien. Daarnaast kon *D. agitata* niet met nitraat groeien. DNA-DNA hybridisatie-experimenten toonden aan dat stam MA-1 niet gerelateerd kon worden tot het speciesniveau van *D. agitata*. Daarom werd voorgesteld om stam MA-1 te benoemen als *Dechloromonas hortensis* sp. nov.

Stam ASK-1 is een chloraatreducerende bacterie, die uit hetzelfde slibmonster als *P. chloritidismutans* is geïsoleerd (Hoofdstuk 5). 16S rDNA sequentieanalyse en DNA-DNA hybridisatie experimenten toonden aan dat stam ASK-1 gerelateerd is tot het speciesniveau van *P. chloritidismutans*. Stam ASK-1 en *P. chloritidismutans* zijn alleen in staat om chloraat en zuurstof als elektronenacceptor te gebruiken. Echter, er werden verschillen gevonden in het fenotype (morfologie) en de groeicondities.

Voor de recentelijk geïsoleerde stam GR-1 was nog geen taxonomische beschrijving uitgevoerd. In hoofdstuk 5 toonden 16S rDNA sequentie analyse en DNA-DNA hybridisatie experimenten aan dat stam GR-1 gerelateerd was aan *Dechlorosoma suillum*. Echter, *D. suillum* werd kort geleden hernoemd als *Azospira oryzae*.

Chloraat en perchloraat worden door de mens gemaakt en de (per)chloraat-reducerende bacteriën die tot op heden gekarakteriseerd en opgenomen zijn in cultuurcollecties, zijn allen geïsoleerd uit (per)chloraat vervuilde monsters. *D. hortensis* is echter geïsoleerd uit een grondmonster dat niet met (per)chloraat verontreinigd was en dat kan een indicatie zijn voor een mogelijke natuurlijke bron van chloraat. (A)biotische processen waarbij chloraat gevormd zou kunnen worden, staan beschreven in hoofdstuk 6. Ten eerste zou de vorming van chloraat gerelateerd kunnen worden aan de biologische productie van antibiotica. Tijdens de productie van antibiotica door verschillende micro-organismen, wordt HOCl gevormd dat resulteert

in de vorming van verschillende gechloreerde verbindingen, inclusief chloraat. Ten tweede is het mogelijk dat chloraat gevormd zou kunnen worden tijdens een onweersbui. Bliksemexperimenten hebben al aangetoond dat nitraat gevormd kan worden uit atmosferisch zuurstof en stikstof. De aanwezigheid van chloorgas en andere gechloreerde stoffen (ClO, HOCl, HCl) in de atmosfeer van kustgebieden, zou een aanwijzing kunnen zijn dat soortgelijke processen plaatsvinden voor chloor bevattende componenten. Voor beide (a)biotische processen zijn nog geen experimentele bewijzen gevonden. Het zou echter wel een verklaring kunnen zijn voor het wijd verspreid voorkomen van (per)chloraatreducerende bacteriën in het milieu.

Om meer inzicht te krijgen in het proces van chloraatreductie en hoe dit reductie-proces gekoppeld is aan de vorming van ATP, werd het chloraatreductase van *P. chloritidismutans* gezuiverd en gekarakteriseerd (Hoofdstuk 3). Het zuurstofgevoelige enzym vertoonde alleen chloraat- en bromaatreductase activiteit. Het (per)chloraat-reductase van stam GR-1 vertoonde daarnaast ook reductase-activiteit voor perchloraat, nitraat, en Mn(V). Het gezuiverde chloraatreductase van *Ideonella dechloratans* vertoonde reductase-activiteit met chloraat, bromaat, nitraat, iodaat en selenaat. De cytoplasmatische lokalisatie van het chloraatreductase van *P. chloritidismutans* verschilde van de periplasmatische lokalisatie van de enzymen van *I. dechloratans* en stam GR-1. Het moleculaire gewicht van het chloraatreductase van *P. chloritidismutans* was 167 kDa en bestond uit drie subunits van 97, 38 en 34 kDa. Dezelfde stoichiometrie ($\alpha_1\beta_1\gamma_1$) werd gevonden voor het chloraatreductase van *I. dechloratans* maar niet voor het (per)chloraatreductase van stam GR-1 ($\alpha_3\beta_3$) . De N-terminale sequentie van de β-subunit van het chloraatreductase van *P. chloritidismutans* vertoonde gelijkenis met N-terminale sequenties van β-subunits van andere reductases en hydrogenases. Er werd aangetoond dat het chloraatreductase ijzer en molybdeen bevatte zoals dat ook gevonden was voor het (per)chloraatreductase van stam GR-1. EPR-analyse bevestigde de aanwezigheid van molybdeen. De aanwezigheid van molybdeen is niet verwonderlijk, omdat de enzymen die in staat zijn om chloraat te reduceren (chloraatreductase van *P. chloritidismutans* en *I. dechloratans*; het (per)chloraatreductase van stam GR-1; nitraatreductases; het trimethylamine N-oxide (TMAO) reductase) allen molybdeen bevatten. Deze enzymen behoren tot de dimethylsulfoxide (DMSO) reductase familie.

In hoofdstuk 4 wordt het chlorietdismutase van *P. chloritidismutans* beschreven. Het enzym vertoonde overeenkomende karakteristieken in vergelijking met chlorietdismutases van stam GR-1, *I. dechloratans* en *Dechloromonas agitata*. Zo waren de lokalisatie (periplasma), compositie (homotetrameer), subunit grootte en spectrale karakteristieken (Soret piek, dat de aanwezigheid van een heemgroep aantoont) gelijk. Experimenten met gelabeld water ($H_2{}^{18}O$) toonden aan dat er geen $^{36}O_2$ of $^{34}O_2$ gevormd werd. Hiermee werd aangetoond dat water geen substraat is in de reactie en dat zuurstof geheel uit chloriet gevormd werd.

De N-terminale sequentie van het chlorietdismutase van *P. chloritdismutans* vertoonde geen overeenkomst met andere chlorietdismutases. Er werd wel gelijkenis gevonden met een interne gensequentie van een hypothetisch eiwit van *I. dechloratans*. De gensequentie van dit hypothetische eiwit werd gevonden naast de gensequentie van het chlorietdismutase. PCR reacties toonden aan dat de organisatie van het chlorietdismutasegen van *P. chloritidismutans* niet overeenkomt met dat van het chlorietdismutasegen en hypothetisch eiwit van *I. dechloratans*.

Het is nog niet duidelijk, hoe de ademhalingsketen in (per)chloraatreducerende bacteriën georganiseerd is. Hoewel, gebaseerd op de gegevens zoals die beschreven zijn in deze studie en in analogie met het nitraatreductieproces, kan een schematische model gegeven worden zoals in Fig. 1 te zien is. Het chloraatreductase en chlorietdismutase zijn in verschillende celfracties gelokaliseerd zodat het zuurstof gevoelige chloraatreductase gescheiden is van de plaats waar zuurstof gevormd wordt. Echter, in stam GR-1 en *I. dechloratans* zijn het zuurstofgevoelige (per)chloraat-reductase en chlorietdismutase wel in dezelfde celfractie (periplasma) te vinden. In Fig. 1 wordt gesuggereerd dat chloraat en chloriet middels een anti-port systeem over de cytoplasmatische membraan worden getransporteerd. Dit is gebaseerd op een zelfde situatie die gevonden is in denitrificerende bacteriën voor het transport van nitraat en nitriet. De structuur van het chloraatreductase van *P. chloritidismutans* komt overeen met de membraan gelokaliseerde nitraatreductases die een cytoplasmatische oriëntatie hebben. Het chloraatreductase van *P. chloritidismutans* was echter gezuiverd uit de oplosbare fractie.

(Per)chloraatreducerende bacteriën bevatten een uniek metabolisme, waarbij de reacties uitgevoerd worden door nieuwe enzymen (perchloraat-/chloraatreductase en chlorietdismutase). Naast fotosynthese en catalase activiteit, is chloraatreductie het derde biologische proces waarbij zuurstof wordt geproduceerd.

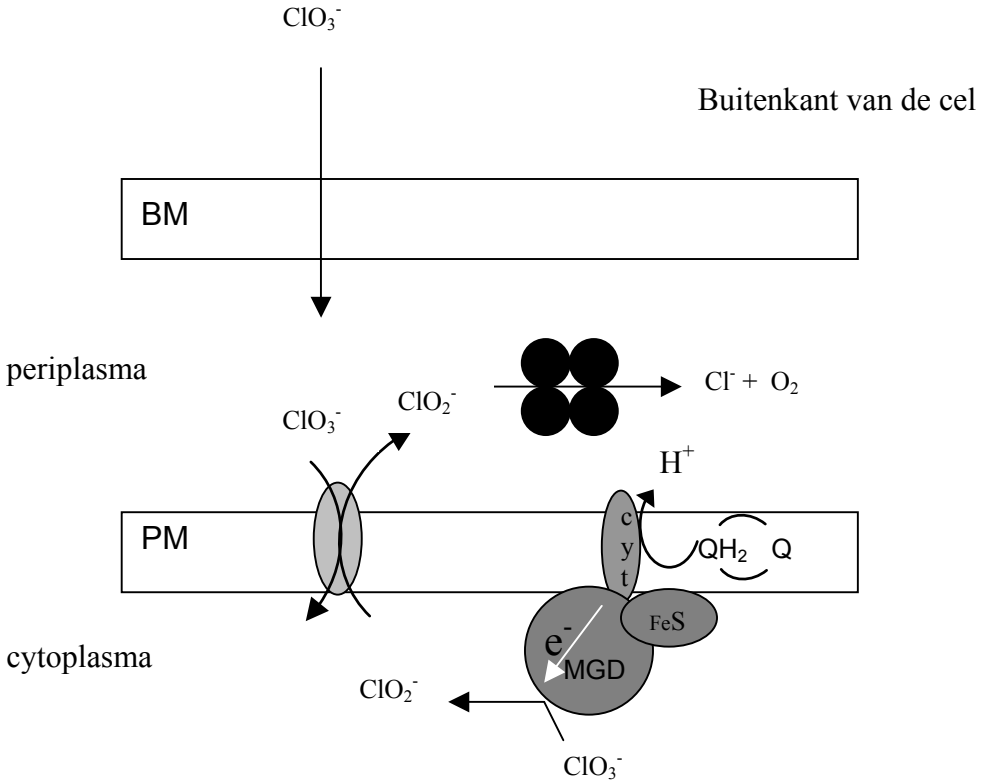

Fig. 1 Schematische presentatie van het chloraatreductie proces in *P. chloritidismutans*. Chloraat diffundeert naar het periplasma. Door middel van een antiport systeem wordt chloraat naar het cytoplasma getransporteerd. Tegelijkertijd wordt chloriet van cyto- naar periplasma overgebracht. Protonen worden afgegeven aan het periplasma wanneer elektronen worden overgebracht van het co-enzym (Q) aan cytochromen (cyt; γ-subunit), via een niet heem bevattende ijzer eiwitten (FeS; β-subunit) en vervolgens aan de α-subunit met de molybdeen co-factor (MGD), dat de actieve groep van het enzym is. Dit subunit doneert elektronen aan chloraat dat dan gereduceerd wordt tot chloriet. Chloriet wordt omgezet in chloride en zuurstof door het chlorietdismutase dat in het periplasma gelokaliseerd is. Wanneer protonen door een ATP syntase (niet in de figuur opgenomen) van het periplasma naar het cytoplasma worden getransporteerd, wordt ATP gevormd uit ADP en P_i.
BM : buiten membraan; PM : plasma membraan.

Antiport systeem

Chlorietdismutase

Chloraatreductase

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Curriculum vitae

Arthur Fredericus Wilhelmus Maria Wolterink werd geboren op 20 november 1970 te Lichtenvoorde. Het VWO-diploma werd in 1991 behaald op het Marianum te Groenlo. Vervolgens werd in datzelfde jaar begonnen met de studie Bioprocestechnologie aan de Landbouwuniversiteit te Wageningen. Afstudeervakken werden uitgevoerd bij de secties Proceskunde en Microbiologie waarna vervolgens een stage Microbiologie werd gedaan aan de Technische Universiteit te München, Duitsland. Het doctoraalexamen werd behaald in september 1996.

Het promotieonderzoek, waarvan de resultaten beschreven staan in dit proefschrift, werd uitgevoerd aan de Universiteit van Wageningen, bij de werkgroep microbiële fysiologie aan het Laboratorium voor Microbiologie (juni 1999 - juli 2003). Sinds 4 september 2003 is hij werkzaam bij de Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek (TNO-PG; Preventie en Gezondheid) in Leiden en vanaf 9 december 2003 in Rijswijk (TNO-PML; Prins Maurits Laboratorium).

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Wolterink, A.F.W.M., Vermeulen, A.J., Jiang, B., Schiltz, E., van der Oost, J., Hagedoorn, P.-L., Kengen, S.W.M. and Stams, A.J.M. Purification and characterization of a chlorite dismutase from *Pseudomonas chloritidismutans*. *Submitted for publication.*

Wolterink, A.F.W.M., Kengen, S.W.M. and Stams, A.J.M. Origin of chlorate. *Submitted for publication.*

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Pfffffff…….

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