Anaerobic microbial degradation of organic pollutants with chlorate as electron acceptor

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Dedicated to my Beloved Parents & Respected Teachers

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Chapter 1

General Introduction

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Pollution of the biosphere has increased strongly since the beginning of the industrial revolution. Organochlorine compounds and petroleum hydrocarbons are the most prevalent kinds of pollutants. The industrial application of chlorinated ethanes, chlorinated ethenes and chlorinated benzenes ranges from degreaser and solvent to biocide and precursor (Dijk 2005). They are of major concern for human and environmental health because some are known for their acute and chronic toxicity, persistence and bioaccumulation. Many organochlorines are present on the EPA list of priority pollutants, indicating their potential hazard for the environment (van Eekert 1999). The total amount of chlorinated aliphatic hydrocarbons and chlorinated aromatic hydrocarbons that reached the surface water in the Netherlands in 2001 was 11.23 and 1.89 ton, respectively (Dijk 2005). Besides an anthropogenic origin, more than 3500 organohalides are known to be produced naturally, comprising mainly chlorinated and brominated metabolites (Häggblom & Bossert 2003; Smidt & de Vos 2004).

Petroleum hydrocarbons are the most common environmental contaminants (Chayabutra & Ju, 2000). Major sources of petroleum hydrocarbon pollution are natural seepage and accidental spillage during transportation (www.eia.doe.gov). Petroleum hydrocarbons consist of four major components: saturates, aromatics, resins and asphaltenes (Head et al. 2006). On the average, saturated and aromatic hydrocarbons together make 80% of the crude oil (Widdel & Rabus 2001). Alkanes are the chemically least reactive, due to the lack of a functional group, presence of only sigma bonds, non-polar nature and low solubility in water.

Aromatic compounds comprise of a large variety of natural and synthetic compounds (Fuchs 2008). Various aromatic compounds like benzene, toluene, phenols, aniline, naphthalene and phenanthrene are of concern due to their persistence and toxicity (Harwood & Parales 1996: Cao et al. 2008).

Remediation of these various sorts of pollution are carried out with physical, chemical or biological processes. Bioremediation is an environmentally safe and cost effective method to eliminate the pollutants from the environment. Various microorganisms can metabolize these compounds either aerobically or anaerobically.

Aerobic degradation of organic pollutants

Oxygen is the most prevalent and most preferred electron acceptor in an oxic environment as it allows the highest growth yield to an organism (Lovley 1991). In the aerobic metabolism of organic pollutants oxygen not only acts as terminal electron acceptor but also as a co-substrate. The introduction of molecular oxygen in organic compounds is mediated through mono- and dioxygenases. Once the oxygen atom has been incorporated in a molecule it can be further degraded more easily, both aerobically and anaerobically.

Chlorinated aliphatics with low chlorine atom substitutions, such as vinyl chloride (VC) and monochlorobenzene (MCB), which are often found accumulated in anoxic ecosystems, can be degraded by aerobic bacteria (Davis & Carpenter 1990; Coleman 2002a). Similarly, dichloroethenes (DCE's) have been shown to be degraded aerobically (Coleman et al. 2002b; Chapelle & Bradely 2003). Epoxidation has been suggested to be the first step during this reaction (Coleman 2002b). An aerobic oxidative metabolism often involves the electrophilic attack by mono- and dioxygenases and ultimately yields carbon dioxide, chlorine and water as end products.

Among petroleum hydrocarbons *n*-alkanes are degraded faster than branched alkanes and aromatics (Leahy & Colwell 1990; Röling et al. 2003), yet quantitatively they are the major fraction of crude oil (Head et al. 2006). Alkanes may adsorb aromatics due to their hydrophobicity and their degradation may then increase the bioavailability of aromatics (Widdel & Rabus 2001). Moreover alkanes are involved in the co-oxidation of other components of hydrocarbons (Rontani et al. 1985; Leahy & Colwell, 1990). Degradation of alkanes by aerobic microorganisms goes fast (Berthe-Corti & Fetzner 2002; Wentzel et al. 2007). The physiology, biochemistry and genetics of aerobic alkane degraders has been studied extensively (Shanklin et al. 1997; Berthe-Corti & Fetzner 2002; Rojo et al. 2005; Head et al. 2006; van Beilen and Funhoff 2007; Wentzel et al. 2007). During aerobic degradation, oxygenases incorporate molecular oxygen into the *n*-alkanes to form the corresponding alcohols, which are further degraded by beta-oxidation (Wentzel et al. 2007). This initial step of oxygen incorporation is thought to be the rate-limiting step, and further degradation may occur without the involvement of oxygen (Chayabutra & Ju 2000). The oxygenases involved in the initial activation of alkanes are monoxygenases, but in one case the involvement of a dioxygenase has been reported (Maeng et al. 1996). The major types of monooxygenases involved are the rubredoxin dependent *alk B* and cytochrome *P450* type monoxygenases and their genes *alkB, alkM, AlmA and cyp153* family of *P450* monooxygenases have been studied extensively (Ratajczack et al. 1998; van Beilen et al. 2006; Throne-Holst 2007; van Beilen & Funhoff 2007).

Aromatic compounds are difficult to degrade due to the resonance energy of the aromatic ring (Harwood & Parales 1996). During the aerobic degradation of aromatic compounds the aromatic ring is activated by modification of the aromatic ring by incorporation of oxygen by mono- or dioxygenases. The catechol and to a lesser extent benzoate, are the central intermediates in the aromatics degradation pathway (Fig. 1).

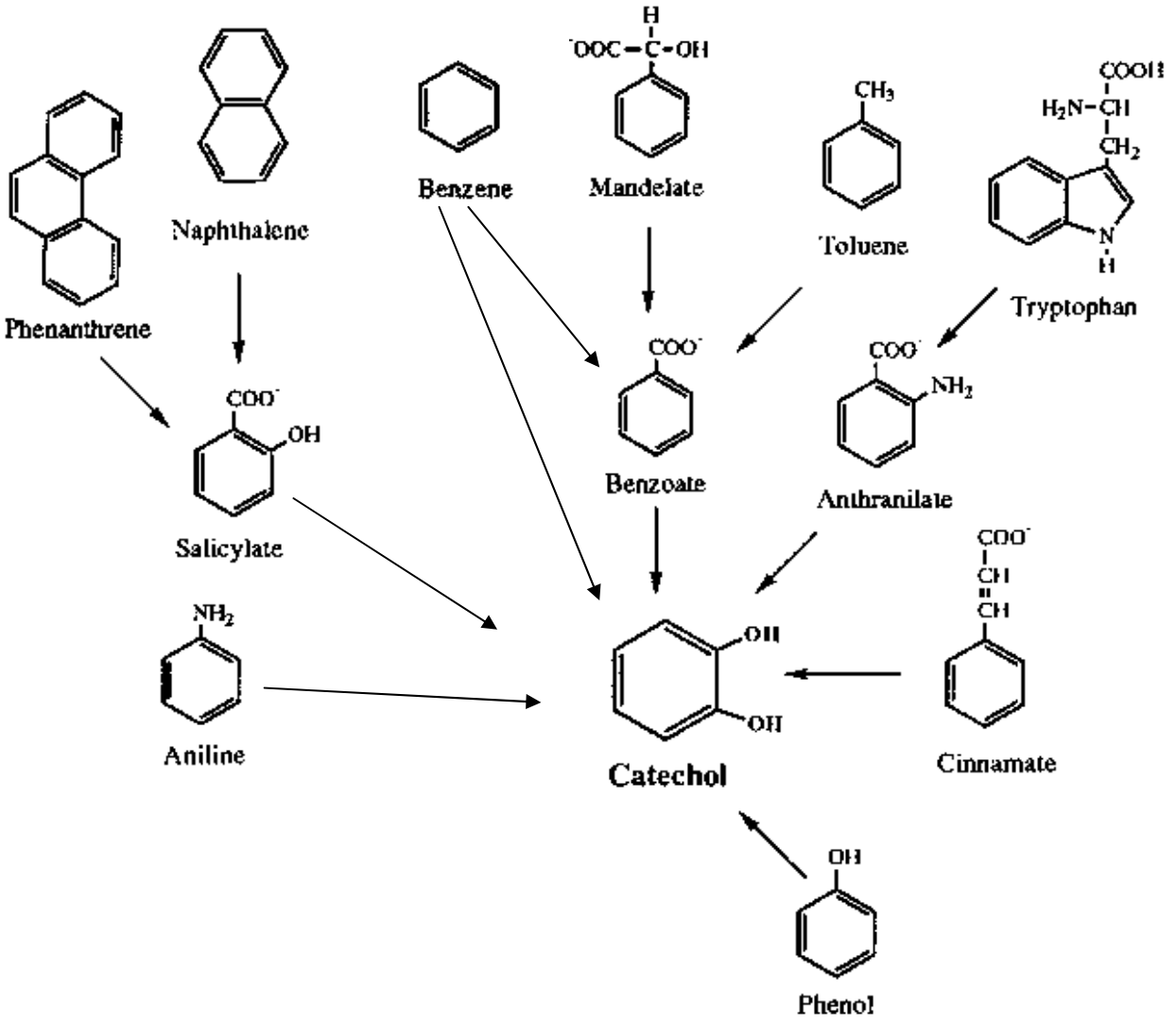

Fig. 1: Catechol and to a lesser extent benzoate as central intermediates in the aerobic degradation of aromatic hydrocarbons (Figure modified from Harwood & Parales 1996)

The next step is the cleavage of the aromatic ring, which is exclusively mediated by dioxygenases. Cleavage of the ring between two hydroxyl groups is called ortho-cleavage or intradiol cleavage and cleavage adjacent to one of the hydroxyl group is called meta-cleavage or extradiol cleavage. (Harwood & Parales 1996; Vaillancourt et al. 2006).

Anaerobic degradation of organic pollutants

Numerous mixed and pure culture studies of the past two decades have revealed that reductive dehalogenation is the main mechanism for the initial attack and degradation of a variety of aliphatic and aromatic organohalides in anoxic environments (El Fantroussi et al. 1998; Häggblom & Bossert 2003; Smidt & De Vos 2004). In anoxic environments the presence of an electron withdrawing group is favourable for an initial reductive attack (Bosma et al. 1988; Knackmuss 1992; Dolfing & Harrison 1993; Field et al. 1995). Molecules with more electron-

withdrawing substitutions such as highly chlorinated compounds are difficult to degrade aerobically (Okey & Bogan 1965; Knackmuss 1992; Field et al. 1995). Chlorinated aliphatics like tetrachloroethene (PCE) and trichloroethene (TCE) and chlorinated aromatics like hexachlorocyclohexane (HCH), which are aerobically recalcitrant are easily degraded via anaerobic degradation (van Doesburg et al. 2005; Cheng & He 2009). However, as these molecules are converted into less substituted chlorinated compounds it becomes more difficult to degrade them anaerobically. This may lead to persistence of less susbstituted chlorinated compounds in anoxic environments. For example, the reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) may lead to the formation of *cis*-dichloroethene (DCE), vinyl chloride (VC) or ethene (Davis & Carpenter 1990; Coleman et al. 2002b). Especially vinyl chloride has been found to accumulate in many polluted soils (Fennel 2001; Coleman 2002a). Similarly, the end products of the degradation of the beta isomer of hexachlorocyclohexane (*β*-HCH) are benzene and monochlorobenzene (MCB), which are very recalcitrant compounds in the absence of molecular oxygen (van Doesburg et al. 2005).

Little is known about the anaerobic degradation of *n*-alkanes. The first step in the anaerobic degradation of *n*-alkanes is thermodynamically difficult. It has been proposed to occur via carboxylation in the sulfate-reducing bacterium strain Hxd3 (So et al. 2003). Another proposed mechanism of alkane activation resembles the anaerobic toluene activation mechanism i.e. *n*-alkane activation through fumarate addition by an alkylsuccinate synthase (Rabus et al. 2001). Molecular evidence for this mechanism has recently been obtained (Callaghan et al. 2008; Grundmann et al. 2008). Anaerobic degradation of *n*-alkanes is slow compared to aerobic degradation and the number of isolates and range of substrate alkanes degraded is limited (So and Young 1999; Ehrenreich et al. 2000; Wentzel et al. 2007).

Aromatic compounds are quite recalcitrant in anoxic environments. The breaking of the aromatic ring which is quite easily mediated by the ring cleaving dioxygenases is energetically difficult in anoxic environments (Fuchs 2008). The mechanisms of anaerobic benzoate and toluene degradation are well understood. Facultative anaerobes use a so called “hybrid pathway” where benzoate is degraded via CoA-thioesters as intermediate and aromatic ring cleavage does not require oxygen (Zaar et al. 2001, Gescher et al. 2006). The activation of ring in the anoxic environment is mostly brought by carboxylation, phosphorylation or fumarate addition (Fuchs 2008). Anaerobic toluene degradation occurs via fumarate addition. A glycyl radical enzyme, benzylsuccinate synthase, which is highly oxygen sensitive, catalyzes this reaction. Benzylsuccinate is further oxidized to benzoyl-CoA, which is the central intermediate of anaerobic degradation of aromatic compounds (Leutwein & Heider 1999, Heider 2007, Fuchs

2008). Most of the aromatics like benzene and monochlorobenzene are anaerobically persistent. Similarly very little is known about the anaerobic degradation of the central intermediate of aromatics i.e. catechol. There are only a few pure culture studies (Szewzyk & Pfennig 1987; Schnell et al. 1989; Kuever et al. 1993, Gorny & Schink 1994; Ding et al 2008). This is in strong contrast with the aerobic pathway of catechol degradation, which is well characterized and completely understood (Vaillancourt et al 2006; Harwood & Parales 1996).

Aerobic versus anaerobic biodegradation

There are few anaerobic hydrocarbon degrading bacteria described upto now in comparison with aerobic bacteria. The rates of anaerobic hydrocarbon degradation in general are much lower than in the presence of oxygen. Aerobic hydrocarbon degrading bacteria can use a wider range of substrates than anaerobic ones (Wentzel et al. 2007; Chayabutra & Ju 2000). Compared to the large number of aerobic benzene degrading bacteria, only 5 bacterial isolates that can degrade benzene anaerobically, have been obtained (Coates et al. 2001; Kasai et al. 2006; Weelink et al. 2008). No bacterium has yet been isolated that can degrade MCB anaerobically, while several bacteria have been characterized that can degrade MCB aerobically (Reineke & Knackmuss 1984; Schraa et al. 1986; Kaschl et al. 2005; Blacke et al. 2008).

Moreover, aerobic pathways are well characterized, while we just have started to understand the (probably many different) mechanisms of anaerobic degradation of various compounds. Remediation strategies of polluted soils are mainly focused on the aerobic degradation of low substituted chlorinated compounds, alkanes and aromatics. Anoxic conditions may develop quickly upon soil pollution with hydrocarbons, due to the activity of aerobic bacteria (Logan and Wu 2002). As a consequence, the contaminated soil is often removed and treated elsewhere. As an alternative, oxygen may be injected under high pressure to stimulate aerobic hydrocarbon-degrading bacteria. The introduction of air/oxygen is not very effective due to the low solubility of oxygen. The introduction of oxygen in the form of hydrogen peroxide (H_2O_2) is a widely used technique, but hydrogen peroxide is toxic to many organisms and may instead inhibit the degradation of hydrocarbons (Morgen et al. 1993). The use of solid oxygen releasing compounds, like oxides of CaO_2 and MgO_2, is a costly process and an even distribution of the oxides is problematic (Weelink et al. 2008).

The problem may be solved by the addition of oxygen in a highly soluble form to the anoxic zone. (Per)chlorate is such a highly soluble compound e.g. sodium perchlorate has a solubility of 2 kg/l (Xu et al. 2003).

(Per)chlorate reduction and (per)chlorate-reducing microorganisms

(Per)chlorate is a term commonly used for perchlorate (ClO_4^-) and chlorate (ClO_3^-). Both have been found to be reduced by bacteria (Rikken et al. 1996; Wolterink et al. 2002). (Per)chlorate has a higher reduction potential than nitrate and oxygen (Table 1) and it has been suggested as an alternative electron acceptor in the oxidation of hydrocarbons (Coates et al. 1999a; Stams et al. 2004; Tan et al. 2006; Weelink et al. 2008). It was found to yield molecular oxygen upon reduction (Rikken et al. 1996; Wolterink et al. 2002; Dudley et al. 2008). The microbial reduction of perchlorate proceeds as depicted in Fig. 2.

Perchlorate (ClO_4^-) is reduced to chlorate (ClO_3^-) through a perchlorate reductase which in turn is reduced to chlorite (ClO_2^-) by a chlorate reductase. In perchlorate-reducing bacteria one enzyme may reduce both the perchlorate and chlorate (Kengen et al. 1999). Chlorite is then split into Cl^- and O_2 by a chlorite dismutase (Rikken et al. 1996; Wolterink 2002). This oxygen formation during anaerobic respiration is unique for (per)chlorate reducing bacteria (Coates et al. 1999a). Enzymes involved in the (per)chlorate reduction pathway have been isolated and characterized (van Ginkel et al. 1996; Kengen et al. 1999; Stenklo et al. 2001).

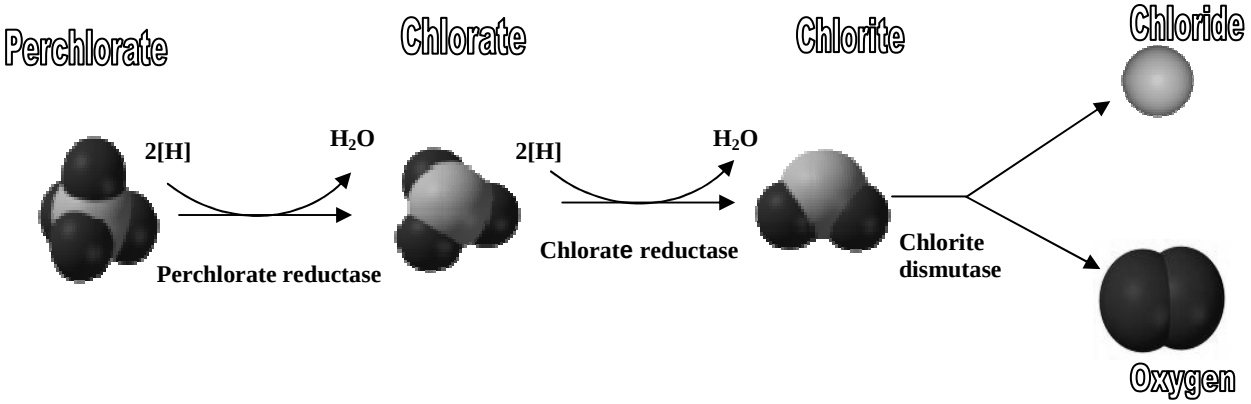

Fig. 2: (Per)chlorate reduction pathway.

(Per)chlorate was initially considered to be anthropogenic. It is used as solid rocket fuel, in road flares, fireworks, blasting agents, explosives, lubricating oils, nuclear reactors, air bags, making matches. (Urbansky 1998; Gullick et al. 2001; Aziz & Hatzinger 2008). In past (per)chlorate was used to treat hyperthyroidism (Urbansky, 1998). Chlorate is used as defoliant (Kengen et al. 1999). Chlorate is produced when chlorine dioxide is used as a bleaching agent in the paper and pulp industry (Kengen et al. 1999). Ammonium perchlorate represents approximately 90% of all perchlorate salts manufactured. Since 1950 more than 15.9 million kg of perchlorate salts have

been discharged into the environment (Motzer, 2001). Since perchlorate salts are highly soluble in water, they are readily transported through surface water and groundwater (Xu et al. 2003). Perchlorate has been detected in groundwater in 35 states of the USA (NAS 2005). In Henderson (Nevada), this perchlorate contamination ranged from 51.4 to 630 mg/l. Low levels of perchlorate have also been found in drinking wells in southern parts of Texas in an area of more than 30000 square miles (Urbansky 1998).

Table 1: Biologically relevant redox couples

			$E^{0'}$ (Volts)
$SO_4^{2-} + 3\ H^+ + 2\ e^-$	→	$HSO_3^- + H_2O$	-0.516
Ferredoxin (Fe^{3+}) + e^-	→	Ferredoxin (Fe^{2+})	-0.420
$NAD(P)^+ + H^+ + e^-$	→	NAD(P)H	-0.320
$S + 2\ H^+ + 2\ e^-$	→	H_2S	-0.274
$FAD + 2\ H^+ + 2\ e^-$	→	$FADH_2$	-0.180
$HSO_3^- + 6\ H^+ + 6\ e^-$	→	$HS^- + 3\ H_2O$	-0.110
$NO_3^- + 2\ H^+ + 2\ e^-$	→	$NO_2^- + H_2O$	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
$ClO_4^- + 2\ H^+ + 2\ e^-$	→	$ClO_3^- + H_2O$	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 and 25 °C.

From a microbiological point of view the anthropogenic origin of perchlorate was puzzling since (per)chlorate reducing bacteria have not only been found in contaminated environments but also in pristine environments. However, there is now evidence that (per)chlorate is also produced via natural processes and that it is ubiquitously present at low concentrations in arid and semi-arid areas in the world (Bao & Gu 2004; Plummer et al. 2006; Rajagopalan et al. 2006; Kang et al. 2008). Natural deposits of perchlorate are the mineral deposits in the hyper arid region of the Atacama desert of Chile (Orris et al. 2003). Isotopic analyses of the Atacama deposits showed a natural atmospheric origin (Bohlke et al. 1997; Bao & Gu 2004). Perchlorate is likely to be formed in the atmosphere by ozonation of aqueous solutions of chloride (aerosols) or ozonation of chloride coated sand and glass surface (Dasgupta et al. 2005; Kang et al. 2008). Homogenous photochemical reactions of aqueous oxyanions (hypochlorite, chlorite, and chlorate) precursors also yield perchlorate (Kang et al. 2006). Lightning may also cause the synthesis of atmospheric

perchlorate (Dasgupta et al. 2005). Natural occurrence of perchlorate has also been reported on Mars (Hect et al. 2009). Interestingly, Atacama desert soils have a strong resemblance with the Martian soil (Navarro-Gonzalez 2003).

Perchlorate is known to affect the thyroid gland in mammals by binding to the sodium iodide symporter. It inhibits competitively the uptake of iodide by thyroid gland which results in fatal bone marrow disease (Stanbury & Wyngaarden 1952; Achenbach et al. 2001). Several cases of aplastic anemia and renal damage were observed when perchlorate was administrated chemotherapeutically for the treatment of hyperthyroidism (Foye 1989; Urbansky 1998). Despite criticism, the US EPA has decided not to regulate the use perchlorate but instead has established a reference dose of 0.0007 mg/kg/day of perchlorate (Renner 2009).

Microbial (per)chlorate reduction has been reviewed by Logan et al. (2001), Xu et al. (2003), Coates and Achenbach (2004). A list of known (per)chlorate reducing bacterial isolates is given in table 2.

(Per)chlorate-reducing bacteria are spread among α, β, γ, and ε subclasses of proteobacteria (Korenkov et al. 1976; Stepanyuk et al. 1992; Wolterink et al. 2002; Kesterson et al. 2005). Recently, a firmicute has also been isolated (Balk et al. 2008). (Per)chlorate-reducing bacteria have been isolated from pristine as well as contaminated soils and sediments (Coates et al. 1999b; Wolterink et al. 2005).

The ubiquity of (per)chlorate reducing-bacteria was shown by most probable number count assays in samples from a variety of soils and sediments. The numbers of (per)chlorate-reducing bacteria ranged from 2×10^3 to 2×10^6 cells per gram (Coates et al. 1999b). Kesterson et al. (2005) showed that (per)chlorate reducing bacteria could even be two orders of magnitude higher at some locations.

Most of the (per)chlorate reducers are related at the species level with a non-(per)chlorate reducers. For instance, *Pseudomons chloritidismutans* AW-1^T, a chlorate reducer has 100% 16S rDNA similarity with *P. stutzeri* DSM 50227, but this *P. stutzeri* cannot reduce chlorate. Similarly, the chlorate reducer and benzene oxidizer *Alicycliphilus denitirificans* BC is 99.7% similar with a non-chlorate reducer *Alicycliphilus denitrificans* K601. Similar observations were made by Achenbach & Coates (2000). The majority of the perchlorate-reducing bacteria is facultative anaerobic, mesophiles and has a pH optimum close to neutral. However, some members of *Dechloromonas* and *Azospira* are capable of (per)chlorate reduction at pH 5 (Coates & Jackson 2008). Most of them are gram negative, but a gram positive thermophilic (per)chlorate reducer was isolated in our laboratory (Balk et al. 2008). Okeke et al. (2002) reported that *Citrobacter* strain IsoCock 1 reduced 35% perchlorate up to 7.5 % salinity, but

growth coupled to perchlorate reduction could not be demonstrated (Coates and Jackson 2008). *P. chloritidismutans* and *M. perchloritireducens* can grow in a medium containing up to 4 g/l of NaCl (Wolterink et al. 2002; Balk et al. 2008). (Per)chlorate-reducing bacteria require molybdenum as an essential trace element (Bruce et al. 1999; Chaudhuri et al. 2002). Growth rates and cell yields on (per)chlorate are comparable with aerobic ones (Logan et al. 2001). *Dechlorospirillum* WD can grow with 80 mM of (per)chlorate (Michaelidou et al. 2000).

Table 2: List of known (per)chlorate reducing isolates with references

Strain	Reference	Strain	Reference
Vibrio dechloraticans Cuznesove B-1168	Korenkov et al. 1976	*Pseudomonas chloritidismutans* AW1^T	Wolterink et al 2002
Acinetobacter thermotoleranticus	Stepanyuk 1992	*Dechloromonas* sp. PC1	Nerenberg et al. 2006
Ideonella dechloratans	Malmqvist et al. 1994	16 isolates (*Azospirillum & Dechloromonas)*	Waller et al. 2004
Azospira oryzae GR-1	Rikken et al. 1996	*Citrobacter* JB101 and JB109	Bardiya & Bae 2004
Wolinela succinogenes HAP-1 & W. succinogenes ATCC29543	Wallace et al. 1996	*Dechloromonas hortensis* MA-1^T & *P. chloritidismutans* ASK1	Wolterink et al. 2005
Azospira sp. Perc1ace	Herman & Frankenberger 1998	*Dechloromonas* sp. JDS5 & sp. JDS6	Shrout et al. 2005
Dechloromonas agitata CKB	Bruce et al. 1999	*Dechloromonas denitrificans* ED-1^T	Horn et al 2005
13 isolates (*Pseudomonas,Azospirillum & Dechloromonas*) *Dechloromonas* sp. SIUL, *Dechloromonas* sp. MissR, *Dechloromonas* sp. CL, *Dechloromonas* sp. NM *Azospira* sp. SDGM, *Azospira* sp. PS	Coates et al. 1999b	27 isolates Genus *Aeromonas, Azospira, Rahnella & Shewanella* isolates.	Kesterson et al. 2005
Dechloromonas sp. JM	Miller & Logan 2000	*Azospirillium lipoferum* DSM 1691^T	Peng et al. 2006
Dechlorospirillum WD & *Azospira suillus* PS	Michaelidou et al. 2000	*Dechlorospirillum* VDY	Thrash et al. 2007
10 isolates 2 in detail *Azospira* KJ, & PDX	Logan et al. 2001	*Moorella perchloratireducens*	Balk et al. 2008
Dechloromonas agitata	Achenbach et al. 2001	*Azospira* sp. HCAP-C (PCC)	Dudley et al. 2008
Dechloromonas aromatica RCB	Coates et al. 2001	*Dechlorospirillum anomalous* JB116	Bradiya & Bae 2008
Dechloromonas sp. HZ	Zhang et al. 2002	*Alicycliphilus denitrificans* BC	Weelink et al. 2008

All (per)chlorate reducers are chlorate reducers but vice versa is not always the case. Most bacteria can grow with acetate as the carbon and energy source. However, chemolithoautotrophic

perchlorate reducers are also known (Zhang et al. 2002; Shrout et al. 2005; Nerenberg et al. 2006). *Azospira* (*Dechlorosoma*) sp. HCAP-C is a unique perchlorate reducer which accumulates chlorate. The maximum observed chlorate accumulation is more than 20% of the initial perchlorate concentration (Dudley et al. 2008).

The *Dechloromonas* and *Azospira* genera are the two dominant genera of (per)chlorate reducers (Coates & Achenbach 2004). However, the genus *Azospirillum* may be more dominant at contaminated sites with low concentrations of (per)chlorate (Waller et al. 2004). Monod half saturation constants of two *Azospirillum* isolates were lower than those for the members of other genera, suggesting their effectiveness at low (per)chlorate concentrations.

Competition with other electron acceptors

Apart form chlorate and perchlorate, (per)chlorate reducing bacteria can utilize oxygen and nitrate as electron acceptor. Oxygen inhibits the (per)chlorate reduction (Rikken et al. 1996). The reason is the sensitivity of the enzyme (per)chlorate reductase for oxygen (Kengen et al. 1999; Wolterink et al. 2003). However, *Pseudomonas* sp. PDA performs perchlorate reduction in the presence of oxygen (Xu et al. 2004). Though the chlorate reductase of *P. chloritidismutans* is oxygen sensitive (Wolterink et al. 2003), the bacterium can simultaneously reduce chlorate and oxygen when oxygen is added to a chlorate-reducing culture (Wolterink et al. 2001). Recently, Shete et al. (2008) described aerobic perchlorate reducing bacteria, but neither growth coupled to (per)chlorate reduction nor complete reduction of (per)chlorate was demonstrated.

Historically, it was thought that perchlorate reduction is an ancillary activity of nitrate reducing organisms (Quastel et al. 1925; Hackenthal 1965). In addition, (per)chlorate reducing bacteria also use nitrate as electron acceptors. A competition for nitrate and chlorate may occur. The response to both electron acceptors is species specific (Sun et al. 2009). The (per)chlorate-reducing bacterium *Wolinella succinogenes* HAP-1 only reduces nitrate to nitrite (Wallace et al. 1996). *Dechloromonas agitata* strain CKB, is unable to grow on nitrate but can reduce the nitrate to nitrite while actively growing on perchlorate, suggesting a fortuitous reduction of nitrate (Chaudhuri et al. 2002). Strain *Azospira* sp. per1ace concomitantly utilizes perchlorate and nitrate without significantly affecting the reduction rates. It seems to have two separate reductases (Giblin & Frankenberger 2001). Nitrate reduction preceded (per)chlorate reduction when either (per)chlorate or nitrate pre-grown cells of *Azospira suillium* were inoculated in medium containing both nitrate and (per)chlorate (Chaudhuri et al. 2002). *Azospira suillium* has a unique perchlorate reductase which can distinguish between perchlorate and nitrate (Sun et al. 2009).

The (per)chlorate reductase and nitrate reductase are separate and inducible in *Azospira* sp. KJ (Xu et al. 2004). *Pseudomonas sp.* PDA is a chlorate reducer that is unable to degrade perchlorate or nitrate (Xu et al. 2004).

(Per)chlorate reduction for bioremediation of pollutants

Except for toluene, only a few bacterial strains have been isolated that are able to grow on BTEX anaerobically (Rabus and Widdel 1995; Kniemeyer et al. 2003). None of these purely anaerobic isolates is able to degrade all the BTEX compounds (Chakraborty et al. 2005). Two denitrifying, benzene degrading bacteria, *Dechloromonas aromatica* strain RCB and strain JJ have been described. Strain RCB was enriched on 4-chlorobenzoate and chlorate (Coates et al. 2001). In addition to benzene, this strain was able to grow on all the BTEX compounds i.e. toluene, all the isomers of xylene and ethylbenzene. The rates of benzene degradation were slightly higher when oxygen or (per)chlorate were used as electron acceptor, instead of nitrate (Chakraborty et al. 2005). Two more denitrifying bacteria, *Azoarcus sp*. strain DN11 and strain AN9, were reported to grow on benzene as sole source of carbon and energy (Kasai et al. 2006; 2007), but these strains have not been tested with (per)chlorate.

Weelink et al (2008) isolated *Alicycliphilus* strain BC from a chlorate-reducing community, that had 20-1650 times higher benzene degradation rates than reported till then for anaerobic benzene degradation. This bacterium is able to grow on benzene, toluene, phenol, and catechol with chlorate and oxygen, but not with nitrate. This suggests that during growth on chlorate, the oxygen that is formed during chlorite dismutation is incorporated by an oxygenase in the aromatic ring (Weelink et al. 2008). Some characteristics of pure cultures that degrade benzene anaerobically are given in table 3 and 4.

Another mechanism that may play a role in the enrichment obtained by Weelink et al. (2007) is interspecies oxygen transfer. A bacterium related to *Mesorhizobium sp*. WG dominates the enrichment when grown on benzene and oxygen. Another bacterium, related to *Stenotrophomonas acidaminophila,* is enriched when the culture is supplied with acetate and chlorate. It seems that the latter one is a chlorate reducer, which forms oxygen during chlorite dismutation, and the former one is an aerobic benzene degrader, which utilizes this oxygen to degrade benzene (Weelink et al. 2007). In another study, ^{14}C labeled naphthalene was quickly converted under anoxic conditions to $^{14}CO_2$, when chlorite was directly added to washed whole cell suspensions of a chlorate reducing, non-hydrocarbon degrading bacterium, *D. agitatus* strain CKB, and the hydrocarbon oxidizing *Pseudomonas* strain JS-150 (Coates et al. 1999a). They even found higher degradation rates under anoxic conditions compared to aerobic controls which was attributed to the limited diffusion rate of oxygen.

Table 3: Comparison of selected features reported for all the isolates (all belong to the subclass β-proteobacteria) described to be capable of anaerobic benzene degradation.

Name of strain	Isolated from	Isolated on	shape	Size (µm)	Opt. temp. (^oC)	Opt. pH	Doubling time	Highest conc. of benzene tested (µM)	Reference
Dechloromonas aromatica strain RCB	River sediment	4-chlorobenzoate + ClO_3^-	Rod	1.8 × 0.5	30	7.2	4 days[3] Benzene + ClO_3^-	160	Coates et al. 2001, Chakraborty et al. 2005
Dechloromonas strain JJ	Lake sediment	HDS[1] + NO_3^- acetate as C source	Rod	1.8 × 0.5	30	7.2	ND	ND	Coates et al. 2001
Alicycliphilus strain BC	Waste water treatment plant	Benzene + ClO_3^- with 0.125g/l FYE	Rod	1.2 × 0.6	30-37	7.3	1.4 days Benzene + ClO_3^-	1000	Weelink et al. 2008
Azoarcus strain DN11	Contaminated ground water	Benzene + NO_3^- on dCGY[2] medium	ND	ND	30	7	9 days[4] Benzene + NO_3^-	15	Kasai et al. 2006; 2007
Azoarcus strain DN9	Contaminated ground water	Benzene + O^2 on dGCY medium	ND	ND	25	ND	13 days[4] Benzene + NO_3^-	15	Kasai et al. 2006

[1] 2,6-anthrahydroquinone disulphonate; [2] dCGY medium (Bact. Casamino acid, Glycerol, Yeast extract, Agar); [3] Estimated from Fig. 2 (Chakraborty et al., 2005); [4] Estimated from Fig. 4 (Kasai et al., 2006); ND = not described/ not detected

Table 4: Comparison of degradation of hydrocarbons under different electron accepting conditions reported for isolates described to be capable of anaerobic benzene degradation.

	***Dechloromonas aromatica* RCB**			***Dechloromonas* strain JJ**			***Alicycliphilus* strain BC**			***Azoarcus* strain DN 11**			***Azoarcus* strain AN 9**		
	O_2	**NO_3^-**	**ClO_3^-**	**O_2**	**NO_3^-**	**ClO_3^-**	**O_2**	**NO_3^-**	**ClO_3^-**	**O_2**	**NO_3^-**	**ClO_3^-**	**O_2**	**NO_3^-**	**ClO_3^-**
Acetate	+	+	+	+	+	ND	+	+	+	ND	ND	ND	ND	ND	ND
Toluene	+	+	+	+	+	ND	+	-	+	ND	+	ND	ND	+	ND
Benzene	+	+	+	+	+	ND	+	-	+	+	+ [1]	ND	+ [1]	+	ND
Phenol	ND	+	ND	ND	ND	ND	+	-	+	-	-	ND	ND	ND	ND
Catechol	ND	ND	ND	ND	ND	ND	+	-	+	ND	ND	ND	ND	ND	ND
Benzoate	ND	+	ND	+	+	ND	-	-	-	+	+	ND	ND	ND	ND

ND = not described/ not detected; + = growth; - = no growth; [1] 20%-90% of amended substrate degraded. References for the organisms can be found in table 3

In a study by Logan and Wu (2002), toluene degradation rates were 1.36 fold enhanced when chlorate was amended to a sand column. This increase in toluene degradation was attributed to oxygen formation by chlorate-reducing organisms. The oxygen is thought to be taken up by toluene degrading bacteria to hydroxylate the toluene ring through oxygenases.

In another soil column study, high benzene degradation rates (31µmol/l/hr) coupled to chlorate reduction were observed. The results with batch cultures showed that only chlorate and not nitrate was used as an electron acceptor. This again points to an involvement of an oxygenase mediated mechanism of action (Tan et al. 2006).

To our knowledge, only one study has been published about the application of chlorate reduction in soil remediation. ^{14}C labelled benzene and chlorite were added to anoxic soil samples that were pre-treated with *Dechloromonas agitatus* strain CKB, a non hydrocarbon-degrading chlorate reducer. The indigenous hydrocarbon degrading population was rapidly stimulated and more than 90% of ^{14}C labeled benzene was recovered as $^{14}CO_2$. The rates were similar as in the aerobic controls (Coates et al. 1999a).

Bioremediation of soils contaminated with hydrocarbons depends on many factors, including availability of the hydrocarbons, presence of nutrients and electron acceptors, temperature, salinity, water activity and pH (Leahy and Colwell 1990). Since most of the (per)chlorate reducing organisms have a pH optimum close to neutral, the pH may have to be adjusted before the actual bioremediation starts. The addition of fertilizers along with the soluble electron acceptor, the (per)chlorate, may enhance the remediation process in soils lacking nutrients. The addition of (per)chlorate alone may not be sufficient. Therefore a dosage of a readily degradable substrate like acetate to enhance (per)chlorate reduction and production of oxygen may be essential (Tan et al. 2006).

Biochemistry of (per)chlorate reduction

(Per)chlorate reductase

(Per)chlorate reductases belong to the type II dimethyl sulfoxide reductase (DMSO) family of enzymes (Danielsson et al. 2003; Bender et al. 2005). Type II DMSO reductases have a common molybdenum cofactor known as bis(molybdopterin guanine dinucleotide)Mo (Moura et al. 2004). (Per)chlorate reductases are similar to other type II DMSO reductases like nitrate reductase and selenate reductase and ethyl benzene dehydrogenase. Most (per)chlorate reductases can also reduce nitrate.

The α subunit containing the molybdopterin is the catalytic subunit. The β subunit contains the Fe-S cluster and may be involved in electron transfer to the catalytic subunit. The γ subunit is a

cytochrome *C* moiety of chlorate reductase. The δ subunit is not a part of the mature enzyme and is proposed to be a chaperone involved in the assembly of the αβ complex (Bender et al. 2005, Danielsson et al. 2003). All (per)chlorate reductases are periplasmic. The chlorate reductase of *P. chloritidismutans* has 16 moles of iron, while (per)chlorate reductase of strain GR1 contains 11 moles of iron and the chlorate reductase of *Ideonella* 10 moles of iron (Kengen et al. 1999; Danielsson et al. 2003; Wolterink et al. 2003). Strain GR1 perchlorate reductase also contains selenium (Kengen et al. 1999). The γ subunit of chlorate reductase of *Ideonella* contains heme b (Karlson et al. 2005). Some of the other characteristics of the (per)chlorate reductases are presented in table 5.

Perchlorate reductase of GR-1 is the only enzyme of which kinetic parameters for both chlorate and perchlorate have been determined. It has a more than five times higher affinity for chlorate than for perchlorate and a V_{max} for chlorate that is about four times higher than for perchlorate. The chlorate accumulation in the *Azospira* sp. HCAP-C (Dudley et al. 2008) might be due to a different type of perchlorate reductase which has a higher affinity and a higher V_{max} for perchlorate than for chlorate. A unique type of chlorate reductase is the one of *Pseudomonas* sp PDA. It is constitutively expressed under both oxic and anoxic conditions (Xu et al. 2004). *Pseudomonas* sp. PDA is a chlorate reducer that is unable to degrade perchlorate and nitrate. The N-terminal amino acid sequence of 60 kDa protein of this enzyme did not show any similarity with that of other (per)chlorate reductases (Steinberg et al. 2005).

Chlorite dismutase

Chlorite dismutase is a key enzyme in the chlorate reduction pathway. The systematic name of this enzyme should be chloride:oxygen oxidoreductase (Hagedoorn et al. 2002). Chlorite dismutase is a heme-containing homotetrameric enzyme. It is periplasmic in location (van Ginkel et al. 1996; Stenklo et al. 2001). It is one of the few oxygen-generating enzymes in nature and the only one to form an O-O double bond besides photo system II (Streit & Dubious 2008). A detailed literature review and comparison of different chlorite dismutases is given in table 1 of chapter 4 of this thesis.

Table 5: Characteristics of (per)chlorate reductases

	***P.mirablis*[1]**	***Azospira oryzae* GR-1[2]**	***Pseudomonas chloritidismutans*[3]**	***Ideonella dechloratans*[4]**	***Azospira* sp. Per1ace[5]**	***Pseudomonas* sp. PDA[6]**	***Azospira* sp. KJ[6]**
Location	membrane	periplasmic	cytoplasmic	periplasmic	periplasmic	periplasmic	periplasmic
Electron acceptor	chlorate [a]	perchlorate, chlorate, nitrate, iodate & bromate	chlorate, bromate	chlorate, bromate, iodate, nitrate & selenate	perchlorate[b]	chlorate	chlorate, perchlorate
Size of subunit (kDa)	75, 63, 56	95, 40	97, 38, 34	94, 35.5, 27	35, 75	60, 48, 27	100, 40
Composition native enzyme	heterotrimer $\alpha_1 \beta_1 \gamma_1$	trimer of heterodimers $\alpha_{3-} \beta_3$	heterotrimer $\alpha_1 \beta_1 \gamma_1$	heterotrimer $\alpha_1 \beta_1 \gamma_1$	heterodimer $\alpha_1 \beta_1$	heterotrimer $\alpha_1 \beta_1 \gamma_1$	heterodimer $\alpha_1 \beta_1$
EPR parameters of Mo(V) (g values)	-	1.976 2.016 2.091	2.024 2.076	-	-	-	-
V_{max} (U/mg)	-	13.2 for ClO_3^- 3.8 for ClO_4^-	51	-	4.79	-	-
K_m (µM)	-	< 5 for ClO_3^- 27 for ClO_4^-	159	850	34.5	-	-

[a] only chlorate and nitrate were tested; [b] purified enzyme was not checked for chlorate or nitrate reductase activity; [1] Oltmann et al. (1976); [2] Kengen et al. (1999); [3] Wolterink et al. (2003); [4] Danielssonl et al. (2003); [5] Okeke & Frankenberger (2003); [6] Steinberg et al. (2005)

Molecular studies on (per)chlorate reducing bacteria

The genetics of chlorate reduction have not yet been studied in detail. There are only a few studies on the molecular aspects of chlorate reductases and chlorite dismutase.

The genes encoding the enzyme chlorate reductase in *Ideonella dechloratans* have been sequenced and are arranged as *clrABDC* which encode the α, β, γ and δ subunits, respectively. The order of genes of the perchlorate reductase of two *Dechloromonas* strains i.e. *D. aromatica* and *D. agitata* is *pcrABCD*. The analysis of chlorate reductase of *Ideonella dechloratans* predicted that its α subunit has a twin arginine motif for transport to the periplasm via Tat pathway. The β and δ subunit does not contain any signal peptide. It has been suggested that the β subunit first binds with the α subunit and is then transported outside. The γ subunit has a signal peptide and was also found to contain the heme b. The *Ideonella* perchlorate reductase has an upstream insertion sequence which is preceded by the chlorite dismutase gene oriented in opposite direction (Danielsson et al. 2003). *Pseudomonas* strain PK has a similar organization of chlorate reductase and chlorite dismutase genes. The chlorite dismutase genes in *D. aromatica* are downstream of the (per)chlorate reductase operon while in *D. agitata* they are upstream in the same orientation (Fig. 3 taken from Coates & Achenbach 2004). The clustering of chlorate reductase and chlorite dismutase suggests an evolution towards a functional succession.

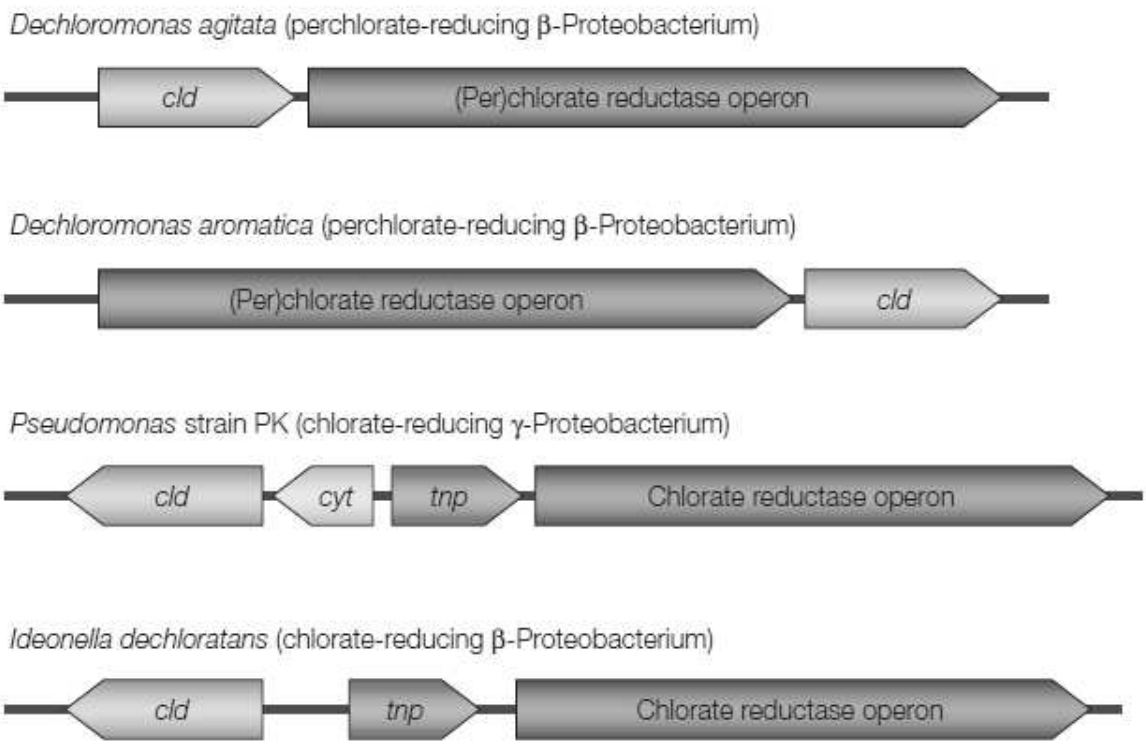

Fig. 3: Organization of chlorate reductase and chlorite dismutase genes in some isolates (figure taken from Coates & Achenbach 2004).

Molecular studies of (per)chlorate reductase genes show that the expression of the *pcrA* gene was completely inhibited by oxygen in *D. agitata*. The *D. aromatica* mutant lacking the *pcrA* gene has lost the ability to grow with perchlorate while still retaining the ability to grow with oxygen and nitrate (Bender et al. 2005). This characteristic together with the loss of a taxis response towards (per)chlorate by the *pcrA* gene mutant (Sun et al. 2009), suggests that *D. aromatica* has separate chlorate and nitrate reductases. Phylogenetic analyses of the *pcrA* gene showed that the perchlorate reductases of two *Dechloromonas* strains formed a separate monophyletic group from the chlorate reductase of *Ideonella dechloratans*. The analyses also indicated that perchlorate reductases are more closely related to nitrate reductases than to chlorate reductases (Bender et al. 2005). This can also been seen from the physiological properties of the known perchlorate reducers (*Azospira* GR1, *Dechloromonas aromatica*) that also reduce nitrate efficiently, while some of the chlorate-reducers (*Pseudomonas* sp. PDA, *P. chloritidismutans*) can not efficiently reduce nitrate. A qPCR assay targeting the catalytic unit of a (per)chlorate reductase gene showed 3.4×10^4 to 9.6×10^4 copies of the *pcrA* gene per gram of dry soil when treated with acetate or hydrogen as electron donor and perchlorate as electron acceptor (Nozawa-Inoue 2008).

Chlorite dismutase is key enzyme in (per)chlorate reduction. Chlorite dismutase (*cld*) genes are highly conserved among (per)chlorate-reducing bacteria. A highly specific immuno probe to target chlorite dismutase was developed and shown to bind to (per)chlorate-reducing bacteria. The probe did not bind to closely related non-(per)chlorate-reducing bacteria (O' Connor & Coates 2002). A certain level of the chlorite dismutase gene is constitutively expressed and transcriptionally up-regulated when grown on (per)chlorate (Bender et al. 2002; Coates & Achenbach 2004). In another study, the possible horizontal transfer of chlorite dismutase genes, based upon their gene phylogeny and the host taxonomy affiliation was suggested (Bender et al. 2004). A comparison of native and recombinant chlorite dismutase by MALDI-MS analysis showed a covalent cross-link between a histidine and a tyrosine side chain in the native chlorite dismutase. Such a cross-link was also found in other heme enzymes active under highly oxidizing conditions, and are suggested to increase the stability and electrophoretic mobility of the proteins (Danielsson et al. 2004). *The cld* gene from *Pseudomonas chloritidismutans* has been amplified, and its sequence analysis suggested a horizontal transfer from γ-proteobacteria (Cladera et al. 2006).

A catalytically active chlorite dismutase was found in the nitrite oxidizing "Candidatus *Nitrospira defluvii*". It was heterologously expressed in *E. coli* and has been suggested that nitrite oxidation might be coupled to chlorate reduction (Maixner et al. 2008). A large number of

genes similar to chlorite dismutase were found in sequenced genomes indicating their putative (per)chlorate reducing potential. Phylogenetic analysis of these chlorite dismutase genes has suggested that the enzyme appeared early in evolution and that it was inherited vertically as well as through horizontal gene transfer. The industrial contaminant was ruled out as the substrate of the first evolved chlorite dismutase. Functional diversification and still unknown functions of the enzyme has been suggested (Maixner et al. 2008). We searched for the presence of nitrate reductases in the putative (per)chlorate-reducing organisms from the study of Maixner et al. (2008) except for the members of the proteobacteria which are known chlorate reducers. Chlorate reductases in most of the genomes are annotated as nitrate reductases since both enzymes belong to the same dimethyl sulfoxide (DMSO) reductase family and can reduce each others substrate. Bacteria that contain a putative chlorite dismutase with the catalytic subunit of a putative nitrate reductase are presented in table 6.

The chlorate reducing bacterium, *D. aromatica* strain RCB, has been fully sequenced (http://genome.jgi-psf.org/finished_microbes/decar/decar.home.html) and has recently been published (Salinero et al. 2009). The analysis of its genome showed that genes involved in anaerobic aromatic degradation are missing, except for the phenylphosphate carboxylase enzyme which degrades phenol via 4-hydroxybenzoate. However, many enzymes responsible for aerobic aromatic degradation were found inside the genome. One of the two monoxygenase clusters is 76.5% identical to a toluene monoxygenase and 76.9% identical to a benzene monoxygenase. Six groups of oxygenase clusters are highly similar to enzymes involved in the aerobic degradation of phenol and phenylpropionate. Embedded in one of these clusters is another oxygenase cluster, showing a high identity to a dioxygenase capable of degrading bicyclic compounds like dibenzothiophene and naphthalene. However, this still has to be experimentally verified. Moreover, there is one benzoate dioxygenase cluster containing benzoate transport and catabolism genes which are similar to the xylene degradation cluster of *Pseudomonas* (Salinero et al. 2009). The absence of genes responsible for an anaerobic degradation pathway and the presence of genes for aerobic degradation pathways of aromatic compounds, suggests that this organism is capable of degrading aromatic compounds with oxygen or by utilizing the oxygen produced via chlorite dismutation.

Similarly, *Alicycliphilus* strain BC has a putative benzene monooxygenase (BC-BMOa) gene and a putative catechol 2,3-dioxygenase (BC-C23O) gene. The BC-BMOa gene sequence of *A. denitrificans* strain BC is 76% identical to the putative monoxygenase of the *D. aromatica* strain RCB sequence. Although these sequences are putative, all sequence features and physiological

data support the statement that these sequences are active and encode functional proteins (Weelink et al. 2008).

Moreover, a combined carbon and hydrogen isotopic fractionation has indicated the presence of a monoxygenase mediated pathway under chlorate reducing conditions in *Alicycliphilus* strain BC (Fischer et al. 2008).

Recently, Bäckland et al. (2009) were able to explain a part of the respiratory chain mechanism in the chlorate reducing bacterium *Ideonella dechloratans*. With MS analyses they identified a 6 kDa *c* cytochrome, which donates electrons to the chlorate reductase and to the cytochrome c oxidase.

Table 6: Putative chlorite dismutase containing bacteria that have a putative catalytic subunit of nitrate reductase.

Bacteria	**Nitrate reductase subunit**	**Bacteria**	**Nitrate reductase subunit**
Arthrobacteria sp	ABDG	*Bacillus thuringiensis*	ABDG
Mycobacterium avium	ABDG	*Bacillus coagulans 36D1*	ABDG
Mycobacterium smegmatis	ABG	*Geobacillus thermodenitrificans*	ABDG
Salinispora arenicola CNS 205	ABG	*Staphylococcus aureus sub. sp. aureus JH. 9*	ABDG
Mycobacterium bovis	ABDG	*Staphylococcus aureus*	ABDG
Mycobacterium tuberculosis	ABD	*Staphylococcus aureus sub. sp. aureus. JH-1*	ABDG
Nocardioides sp.	ABD	*Bacillus subtillus*	ABDG
Mycobacterium vanbaalenii	ABDG	*Bacillus weihenstephanensis KBAB4*	ABDG
Mycobacterium sp.	ABDG	*Staphylococcus aureus*	ABDG
Janibacter sp. HTCC 2649	ABDG	*Staphylococcus aureus*	ABDG
Saccharopolyspora erythraea	ABDG	*Staphylococcus aureus*	ABDG
Mycobacterium gilvum PYR-GCK	ABDG	*Staphylococcus haemolyticus*	ABD
Salinispora tropica CNB-440	ABDG	*Bacillus cereus G9241*	ABDG
Mycobacterium tuberculosis	ABD	*Staphylococcus aureus*	ABDG
Actinomyces odontolyticus	ABDG	*Bacillus clausii*	ABDG
Streptomyces coelicoler	ABDG	*Bacillus cereus*	ABDG
Rubrobacter xylanophilus	ABDG	*Bacillus licheniformis*	ABDG
Mycobacterium sp. KMS	ABDG	*Staphylococcus aureus*	ABDG
Nocardia farcinica	ABD	*Staphylococcus aureus*	ABDG
corynebacterium diphtheriae	ABD	*Staphylococcus aureus (N315)*	ABDG
Mycobacterium paratuberculosis	ABD	*Bacillus cereus*	ABG
Mycobacterium bovis	ABD	*Bacillus anthracis*	ABDG
Corynebacterium efficiens	ABD	*Staphylococcus epidermidis*	ABDG

The frequent distribution of chlorite dismutase genes and presence of many environmentally relevant oxygenases inside the genome of (per)chlorate-reducing bacteria indicate the unexploited power and the unexplored potential of chlorate reducers for bioremediation processes.

Outline of the thesis

There are only two major studies where the potential of (per)chlorate reducing bacteria in bioremediation of pollutants has been described (Coates et al. 2001; Weelink et al. 2008). Both studies describe the degradation of aromatics with (per)chlorate as electron acceptor. There is no knowledge about the degradation of saturated compounds like alkanes. Moreover none of these two bacteria were able to degrade a wide spectrum of aromatic and aliphatics together. Little is known about the genomics of (per)chlorate-reducing bacteria.

The aim of this thesis was to gain more insight in the bio-remediation potential of (per)chlorate-reducing bacteria. Keeping in view the unique ability of (per)chlorate reducers to yield the oxygen which might be used in the activation of recalcitrant compounds in anoxic zones, several possibilities were explored. The known (per)chlorate reducers were screened for their biodegradation potential and the physiology, biochemistry, genomics and proteomics of biodegradation pathways were studied.

The 2nd chapter of the thesis focuses on attempts to enrich for organochlorine-degrading chlorate oxidizing bacteria. Factors are described that may have led to the failing of enrichment for such bacteria. A strategy to enrich bacteria with these properties is proposed.

Chapter 3 describes the growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes (from C7-C12) with chlorate as electron acceptor. The bacterium also grows on intermediates of the aerobic pathway. i.e. on decanol and decanoate. The activities of the key enzymes in the pathway were measured. Growth with chlorate and oxygen, but not with nitrate, suggests an oxygenase mediated pathway.

In chapter 4 the isolation and characterization of a chlorite dismutase enzyme from *P. chloritidismutans* AW-1^T are described. The chlorite dismutase from this strain has been compared with other isolated chlorite dismutases. An ^{18}O labeled water experiment was conducted to show that both atoms of oxygen originate from chlorite.

Chapter 5 reports growth of *Pseudomonas chloritidismutans* AW-1^T on benzoate. Catechol was detected as intermediate. Growth on catechol was also observed. The activities of the enzymes involved were measured. The presence of benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase and other proteins involved in the benzoate degradation pathway were exclusively detected in the benzoate grown cells by MS/MS analysis.

In chapter 6, a proteogenomics approach was used to annotate the 454 sequenced genome of *Pseudomonas chloritidismutans* AW-1^T. A comparison was made with other closely related genomes. A whole proteome comparison with 5 different growth conditions was made to see the differential expression of the key proteins involved in various pathways.

Finally, in chapter 7 the main results of this thesis are concluded and discussed in a broader context. The key areas where research is needed are identified.

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Chapter 2

Bacterial degradation of chlorinated compounds coupled to chlorate reduction

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Abstract

Five different inoculum sources were tested for their ability to degrade chlorinated ethanes, chlorinated ethenes and chlorobenzene with different electron acceptors. Except for 1,1-dichloroethane all the compounds were degraded under at least one of the tested conditions. Chlorinated ethanes and trichloroethene (TCE) were degraded through reductive dechlorination, while chlorinated ethenes were degraded both aerobically and through reductive dechlorination. None of the compounds was degraded with chlorate as electron acceptor. Monochlorobenzene seemed to be degraded under all the tested conditions with activated sludge and Danube sediment, but conversion could only be sustained under aerobic conditions. Ebro sediment was the most efficient one in degrading the chlorinated ethanes, TCE and *trans*-1,2- dichloroethene (DCE). Eerbeek sludge was able to aerobically oxidize *cis*- and *trans*-1,2-DCE. The Rhine sediment was unable to degrade any of the tested chlorinated compounds.

Introduction

Chlorinated ethanes and chlorinated ethenes are widely used as solvents, degreasers, dry cleaning agents and precursors. Monochlorobenzene is used as solvent and grease remover, swelling agent and intermediate for the production of polymers, plastics, pharmaceuticals and some insecticides (Kaschl et al. 2005; Fung et al. 2009). The widespread use of organochlorine compounds has led to contamination of soil and groundwater (Bradley 2000; De Wildeman et al. 2003; Dinglassan-Panlilio et al. 2006; Nijenhuis et al. 2007).

The EPA list of priority pollutants includes 1,1–dichloroethane (1,1-DCA), 1,2-dichloroethane (1,2-DCA), trichloroethene (TCE), 1,1-dichloroethene (1,1-DCE), *trans*1,2-dichloroethene (*trans*-1,2–DCE) and monochlorobenzene (MCB) (http://www.epa.gov/waterscience/methods/pollutants.htm). Bioremediation is one of the cheapest and most cost effective ways to remove these pollutants from soil and water.

In anoxic environments a nucleophilic mechanism of attack is most common (Field et al. 1995). The compounds with a large number of chlorinated substituents undergo reduction and are converted to low chlorinated compounds (Bouwer 1994; Field et al. 1995). These low chlorinated compounds are quite persistent under anaerobic conditions. Though anaerobic oxidation may occur in anoxic zones, the conversion rates are generally low (Bouwer, 1994). So quite often degradation of highly chlorinated compounds yields anaerobically recalcitrant low chlorinated compounds (Coleman et al. 2002a; Coleman et al. 2002b; Fennel et al. 2001; van Doesburg et al. 2005). Chlorinated aliphatics with a low number of chlorine atoms are often found accumulated in the anaerobic zone, but are degraded in the aerobic zone (Davis & carpenter 1990; Coleman et al. 2002a; Coleman et al 2002b; Chapelle & Bradley 2003). Aerobic microorganisms utilize oxygenases to initiate an electrophilic attack (Field et al. 1995).

Introduction of oxygen in anoxic zones is costly and inefficient due to low solubility of oxygen. Introduction of oxygen in the form of hydrogen peroxide (H_2O_2) is a widely used technique, but H_2O_2 is toxic to many organisms and may instead inhibit the degradation of hydrocarbons (Morgen et al. 1993).

Microbial (per)chlorate reduction has recently been described as a novel way of bioremediation in anoxic environments, and bacteria have been isolated, which can degrade anaerobically persistent compounds (Tan et al. 2006; Weelink et al. 2008). Microbial (per)chlorate reduction is a unique way to introduce molecular oxygen in anoxic environments. The microbial reduction of perchlorate proceeds according to the following pathway; perchlorate (ClO_4^-) is reduced to chlorate (ClO_3^-) by a perchlorate reductase and then chlorate (ClO_3^-) is reduced to chlorite (ClO_2^-) by a chlorate reductase. Chlorite is converted to Cl^- and O_2 by a chlorite dismutase (Rikken et

al. 1996). The oxygen formed during the dismutation of chlorite can be used by the oxygenases to initiate the electrophilic attack. Moreover, energetically (per)chlorate is comparable to the electron acceptors like oxygen and nitrate. The possibility to degrade organic chlorinated compounds coupled to the reduction of (per)chlorate has been suggested (Stams et al. 2004). The aim of the present study is to enrich bacteria which can couple oxidation of low chlorinated compounds to chlorate reduction.

Materials and methods

Chemicals

All chemicals used were of analytical grade. TCE was obtained from Merck, Darmstadt, Germany while 1,1-DCA, 1,2-DCA, 1,1-Dichloroethene (1,1-DCE), *cis*-1,2-DCE, and *trans*-1,2-DCE were obtained from Aldrich Chemie N.V., Brussels, Belgium.

Inoculum

Inocula from five different sources were used in this study. Three of them were sediments, one from river Rhine (Wageningen, The Netherlands), one from river Ebro (Flix, Spain) and one from river Danube (Budapest, Hungary), while two were sludges, Eerbeek Sludge (industrial water, Eerbeek, The Netherlands) and activated sludge from the wastewater treatment plant, in Bennekom (The Netherlands).

Anaerobic media

The medium used was based on the medium described by Dorn et al. (1974). The composition of the medium (in grams per liter of anaerobic demineralized water) was as follows: $Na_2HPO_4.2H_2O$, 3.48; KH_2PO_4 1; resazurin, 0.005; $CaCl_2$, 0.009; ammonium iron (III) citrate, 0.01; NH_4SO_4, 1; $MgSO_4.7H_2O$, 0.04. Vitamins and trace elements were added as described by Holliger et al. (1993) supplemented with Na_2SeO_3, 0.06; $NaWO_4.2H_2O$ 0.0184. The final pH of the medium was 7.3.

Enrichment procedure

One gram of one type of sediment/sludge was added in 120-ml flasks containing 40 ml of media in an anaerobic glove box. The flasks were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminium crimp caps, and the head space was replaced with N_2 gas (140 kPa). Chlorinated compounds were added from anoxic stock solutions to give an end concentration of 50 µM. Chlorate was added in excess i.e. 10 mM in duplicate flasks (A & B). One flask was used as control without chlorate. One separate flask contained only the inoculum to see if there is any release of chloride from sediment or sludge. In addition, two flasks of one type of inoculum were kept without the chlorinated compounds, but with chlorate to estimate the

chlorate adsorbed on the sediment/sludge or utilized by the microorganisms in the absence of chlorinated compounds. More chlorate was added when it was about finished in these two flasks. Since enrichment appeared quite slow, 5 ml of oxygen was added in one of the duplicate flasks (B) on the 42nd day to determine the aerobic degradation potential of the inoculum.

Analytical methods

Chloride determination

Estimation of chlorate utilization was done by measuring the release of chloride. Chloride ion concentrations were determined with a Micro-chlor-o-counter (Marius, Utrecht, The Netherlands) with an NaCl solution as the standard as described by Schraa et al. (1986).

Analysis of chlorinated compounds

Chlorinated compounds were analyzed by injecting 0.4 ml of head space onto a Chrompack 436 gas chromatograph (Chrompack Bergen op Zoom, the Netherlands) equipped with a flame ionization detector and having a Sil 5 CB capillary column (25 m × 0.32 mm × 1.2 µm film) and a split injection (ratio 1:50). The temperature of the oven was kept at 70^{o}C. The temperature of the injector was 250^{o}C, while the detector temperature was 300^{o}C. A five point calibration was made by fortifying a known amount of the compound to 120-ml serum bottles containing 40 ml of medium to give the same liquid-to-headspace ratio as that for the cultures.

For equilibration, the time zero reading was taken after overnight incubation. The second reading was taken after 14 days and then every week for 2 months. Afterwards this interval was increased to 1 month.

Oxygen determination

Oxygen was analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) equipped with a packed column (Molsieve 13x 60/80 mesh, 2 m length, 2.4 mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature was 100°C and the injector and detector temperature was 90 and 150°C, respectively. Argon was used as carrier gas at a flow rate of 30 ml min^{-1}.

Results and Discussion

Degradation potential of the inocula

The Ebro sediment and Eerbeek sludge were found to have the highest degradation potential for chlorinated compounds. Ebro sediments and Eerbeek sludge were able to degrade all the tested chlorinated aliphatics except 1,1-DCE. Ebro sediment was able to degrade 1,1-DCA, TCE and trans 1,2-DCE through reductive dechlorination (Fig. 1, Table 1). This was expected as these sediments have a known history of organochlorine contamination (Lacorte et al. 2006). No

aerobic degradation was observed with Ebro sediment. The reason could be that the samples were taken from the anoxic zone and that the required aerobic bacteria were lacking.

Table 1: Degradation of compounds (50 µM) with different inocula

	Compounds Tested						
Source of inoculum	MCB	1,1-DCA	1,2-DCA	TCE	1,1-DCE	*cis*-1,2-DCE	*trans*-1,2-DCE
Activated sludge	+	-	-	-	-	-	-
Danube sediment	+	-	-	-	-	-	-
Ebro mixed sediment	-	+	-	+	-	-	+
Eerbeek sludge	-	-	+		-	+	+
Rhine sediment	-	-	-	-	-	-	-

+ Degradation; - No degradation

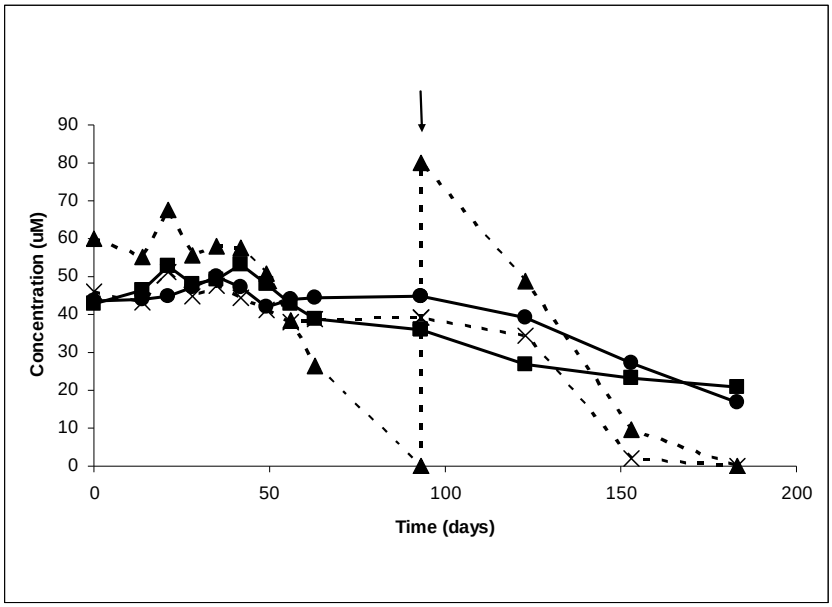

Fig. 1: Anaerobic degradation of chlorinated ethanes and chlorinated ethenes. Vertical line in the graph represents the time of re-addition of TCE. Curves with full lines represent chlorinated ethanes degradation i.e. ● represents 1,1-DCA degradation by Ebro sediment and ■ shows 1,2-DCA by Eerbeek sludge while curves with dashed line represents chlorinated ethenes degradation by Ebro sediment i.e. ▲ for TCE degradation and × for trans1,2-DCE degradation.

Eerbeek sludge was able to degrade 1,2-DCA from 50 µM to 20 µM (Fig. 1), with no external electron acceptor present. The chlorinated compound could have acted as electron acceptor for the oxidation of organic carbon. Eerbeek sludge was also able to oxidize both isomers of 1,2-DCE aerobically (Fig. 2).

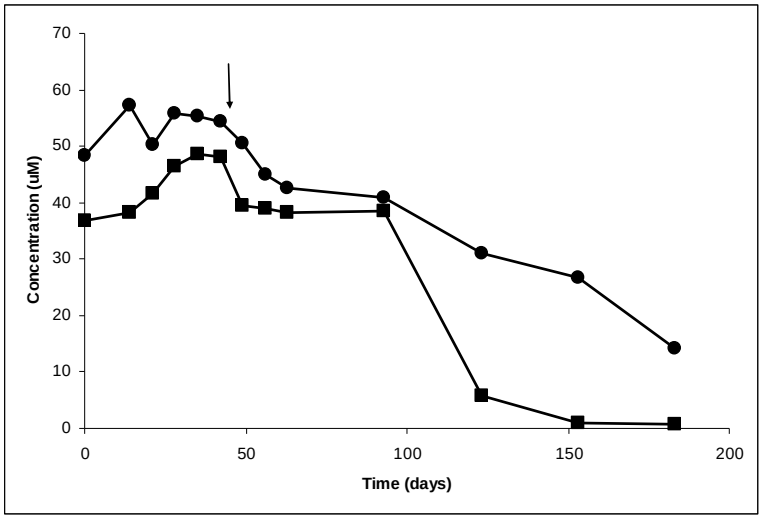

Fig. 2: Aerobic degradation of chlorinated ethenes in Eerbeek sludge sample. Arrow in the graph shows the time of oxygen introduction. ● represents *cis*-1,2-DCE and ■ represents *trans*-1,2-DCE.

Eerbeek sludge was the only inoculum that degraded the chlorinated aliphatics in the presence of oxygen. This suggests that except for the Eerbeek sludge none of the inocula has the aerobic degradation potential for chlorinated aliphatics. Eerbeek sludge is used for the treatment of industrial wastewater which may also contain chlorinated compounds. In the Ebro sediment with a known history of organochlorine contamination and Eerbeek sludge, bacteria may have evolved that degrade the chlorinated contaminants.

Danube sediment and activated sludge from the Bennekom wastewater treatment plant were able to degrade MCB (Fig. 3). However these samples lacked the ability to degrade any of the chlorinated aliphatics. The degradation of MCB by activated sludge suggests that the MCB is easily degraded in oxic environments while it is recalcitrant in the absence of molecular oxygen.

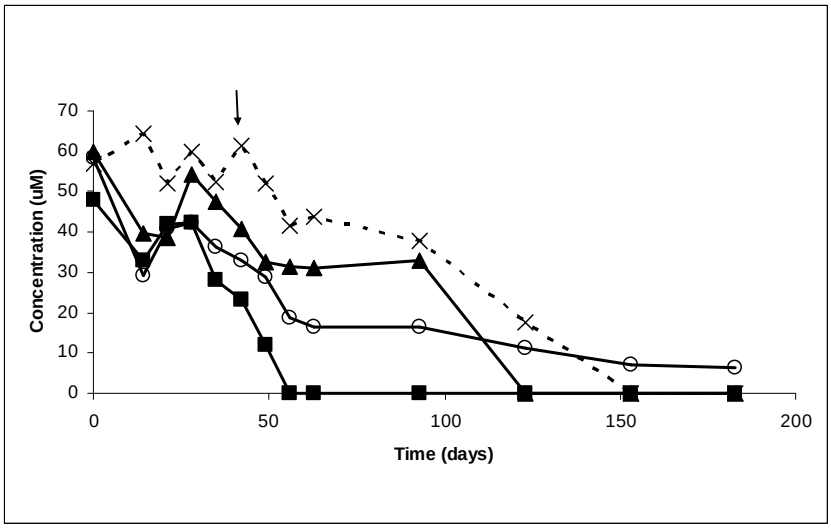

Fig. 3: Degradation of MCB by Danube sediment and activated sludge sediments under various redox conditions. Arrow in the graph shows the time of oxygen introduction. Continuous lines with ■ represents degradation by Danube sediment containing chlorate, ▲ represents aerobic degradation by Danube sediment and ○ represents the degradation in Danube sediment without any external electron acceptor added. The single discontinuous line with crosses (×) represents the aerobic degradation by activated sludge.

Rhine sediment was unable to degrade any of the compounds under the three different experimental conditions tested, which suggests that Rhine sediment is slightly polluted (Table 1). The microbes may not have developed biochemical mechanisms to deal with such compounds. However, the absence of reductive dechlorination may also be due to the low organic carbon content in Rhine sediment and in activated sludge samples.

Overall, none of the inocula was able to degrade the tested chlorinated compounds in the presence of chlorate (Table 2).

Table 2: Degradation of compounds (50 µM) with different electron acceptors

	Electron acceptor		
Compounds	ClO_3^-	O_2[1]	No electron acceptor[2]
MCB	-	+	-
1,1-DCA	-	-	+
1,2-DCA	-	-	+
TCE	-	-	+
1,1-DCE	-	-	-
cis-1,2-DCE	-	+	-
trans-1,2-DCE	-	+	+

[1] These flasks also contain chlorate; [2] Natural organic carbon is the only electron donor present; + Degradation; - No degradation

Persistence of the chlorinated compounds under chlorate-reducing conditions could be explained as most of the inocula have no aerobic degradation potential. Moreover, except for the Eerbeek sludge all other samples had a very low chlorate-reducing potential (Fig. 4). The Eerbeek sludge had reduced 19 mM of chlorate during the experiment (183 days). All other samples showed only a small amount of chlorate reduced, the highest one was a total of 3.5 mM with activated sludge (Fig. 4). Although chlorate reducing bacteria have been reported to vary from 2×10^3 to 2×10^6 cells /g in various soil and sediment samples (Coates et al. 1999; Kesterson et al. 2005), little chlorate reduction took place in our samples. One obvious reason is the toxicity and recalcitrance of substrates and the other possible reason could be the highly reduced nature of samples. They were obtained from strict anoxic zones and had a low potential to degrade chlorinated compounds with oxygen. Moreover the synergistic toxicity of chlorate/chlorite and chlorinated compounds may have led to the decreased chlorate reducing ability of bacteria.

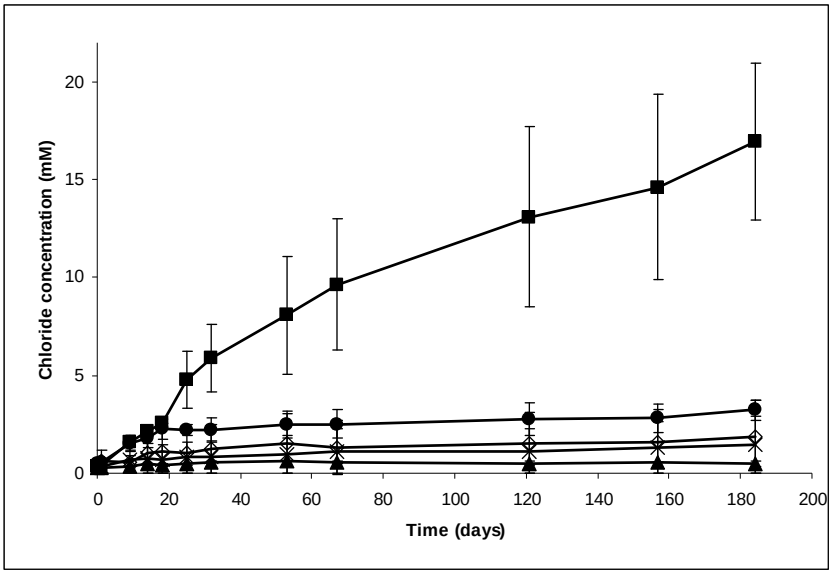

Fig. 4: Chloride formation as an indication of chlorate reduction by different inocula: Rhine sediment ▲, Eerbeek sludge ■, Danube sediment ×, Ebro sediment ◊, Activated sludge ●. Vertical bars represent the standard deviation between two flasks.

Degradation of chlorinated ethanes

Both chlorinated ethanes i.e. 1,1-DCA and 1,2–DCA were degraded under anoxic conditions (Table 2, Fig. 1). However, this only occurred in the anoxic controls where no electron acceptor was present. The most probable mechanism of degradation in this case is through reductive dechlorination. Natural organic carbon may have acted as electron donor in this case. Although 1,2-DCA is reported to be degradable both by aerobic (Hage & Hartmans 1999; Janssen et al. 1985; van den Wijngaard et al. 1993) and anaerobic microorganisms (De Wildeman et al. 2003; Dingslaan-Panlilio et al. 2006), in our study 1,2-DCA was only degraded under anoxic conditions and was persistent with all types of inocula in the presence of molecular oxygen. The degradation of 1,2-DCA with Eerbeek sludge under anoxic conditions started after 123 days (Fig. 1). It might be that bacteria took a long time to adjust to the conditions. Degradation of chlorinated ethanes under chlorate-reducing or oxic conditions was not observed in any of the tested inocula.

Degradation of chlorinated ethenes

Except for 1,1-DCE, the other chlorinated ethenes were degraded under any one of the tested conditions (Table 2). 1,1-DCE was recalcitrant under all the tested conditions (Table 2), although monooxygenase mediated 1,1-DCE oxidation has been reported (Chauhan et al. 1998; Doughty et al. 2005).

TCE was rapidly degraded in the Ebro sediment without chlorate as electron acceptor. It started to disappear after 49 days and was gone within 44 days. Upon re-addition of the TCE, it again

disappeared within approximately 60 days. This may point to an enrichment of TCE degrading bacteria (Fig. 1). No aerobic degradation of TCE was observed in any of the sediments (Table 2). Aerobic degradation of *cis*-1,2-DCE and *trans*-1,2-DCE was observed with the Eerbeek sludge sample. *Cis*-1,2-DCE degradation started at the 42nd day, but it was slow and even at the 183rd day still 14 mM of it was left in the flask. *Trans*-1,2-DCE degradation started at the 93rd day and the compound was completely degraded within the next 60 days (Fig. 2). *trans*-1,2-DCE was the only compound that was degraded both aerobically and anaerobically. Anaerobic degradation of *trans*-1,2-DCE was observed in Ebro sediment (Fig. 1). Reductive dechlorination seems to be the possible mechanism of degradation in this case. Unfortunately, the samples were not anlyzed for intermediates and products.

Degradation of Monochlorobenzene (MCB)

MCB was degraded with the Danube sediment under chlorate-reducing conditions within 56 days (Fig. 3). This was quite surprising since MCB was considered to be persistent in the absence of molecular oxygen. However, on day 123 of the experiment we also found MCB degradation in the aerobic flask (Fig. 3). Rapid degradation in the bottle with chlorate compared with the aerobic one suggests the micro-aerophilic nature of MCB degrading bacteria. On subsequent transfer, MCB degradation was no longer observed in the bottle containing chlorate.

MCB degradation in the bottle without any added electron acceptor was also observed (Fig. 3). By adding a small amount of a reducing agent (cysteine), the MCB degradation stopped completely. Hence, we concluded that the degradation of MCB observed under all the conditions was micro-aerophilic. Our results strongly support the conclusion that fully oxic environments are not essential to degrade monochlorbenzene and a very small amount of oxygen supplied continuously can still be effective to degrade MCB (Blacke et al. 2008). This microaerophilic degradation has been attributed to the extremely low affinity of chlorocatechol 1,2-dioxygenase for oxygen (K_m = 0.3 uM), which is comparable to lowest reported oxygen affinities of terminal oxidases (Blacke et al. 2008). Aerobic degradation of MCB was also observed in the activated sludge samples, where it completely disappeared within 153 days (Fig. 3).

In conclusion, the low aerobic degradation potential of the inocula, the low chlorate reducing ability, the long term storage of samples, the reduced nature of the samples, the synergistic toxicity of chlorate and the chlorinated compound may have contributed to the unsuccessful enrichment of chlorate-reducing, organochlorine-oxidizing bacteria. All the chlorinated aliphatics in this study have at least two substituted chlorine atoms. It is possible that these compounds cannot undergo microbial oxidation. For such compounds a mechanism of either reductive dechlorination or oxygenation which may occur at high oxygen concentration may

have developed in nature. In that case, the target compounds for such a study should be mono chloro-compounds. Moreover, the presumed synergistic toxicity of both chlorate/chlorite and chlorinated organic compound suggests a change in the enrichment strategy. We recommend to enrich for chlorate-reducing bacteria first. Then the bacteria should be enriched further to degrade the oraganochlorine compounds or vice versa. In that case, a stable community capable of degrading the chlorinated aliphatics coupled to chlorate reduction might be obtained. Our results also support the conclusion that completely oxic environments are not essential to degrade monochlorbenzene and tiny amounts of continuously supplied oxygen can still be effective to degrade monochlorobenzene (Blacke et al. 2008).

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Chapter 3

Growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes with chlorate as electron acceptor

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Abstract

Microbial (per)chlorate reduction is a unique process in which molecular oxygen is formed during the dismutation of chlorite. The oxygen thus formed may be used to degrade hydrocarbons by means of oxygenases under seemingly anoxic conditions. Up to now, no bacterium has been described that grows on aliphatic hydrocarbons with chlorate. Here, we report that *Pseudomonas chloritidismutans* AW-1^T grows on *n*-alkanes (ranging from C7 until C12) with chlorate as electron acceptor. Strain AW-1^T also grows on the intermediates of the presumed *n*-alkane degradation pathway. The specific growth rates on *n*-decane and chlorate and *n*-decane and oxygen were 0.5 $\pm$ 0.1 and 0.4 $\pm$ 0.02 day^{-1}, respectively. The key enzymes chlorate reductase and chlorite dismutase were assayed and found to be present. The oxygen dependent alkane oxidation was demonstrated in whole cell suspensions. The strain degrades *n*-alkanes with oxygen and chlorate, but not with nitrate, thus suggesting that the strain employs oxygenase-dependent pathways for the breakdown of *n*-alkanes

Introduction

Petroleum, a complex mixture of aromatic and aliphatic hydrocarbons, is one of the most common environmental contaminants. On average, saturated and aromatic hydrocarbons together make 80% of the oil constituents (Widdel & Rabus 2001). Since the saturated hydrocarbon fraction is the most abundant in crude oil, its biodegradation is quantitatively most important in oil bioremediation (Head et al. 2006). *n*-Alkanes are relatively stable due to lack of functional groups, presence of only sigma bonds, non-polar nature and low solubility in water.

Aerobic microbial degradation of *n*-alkanes is known since almost a century (Söhngen 1913), and the mechanisms of degradation, with the enzymes and genes involved, are rather well understood (Berthe-Corti & Fetzner 2002; Head et al. 2006; van Beilen & Funhoff 2007; Wentzel et al. 2007). During aerobic degradation, molecular oxygen acts as a co-substrate and as a terminal electron acceptor (Berthe-Corti & Fetzner 2002; Chayabutra & Ju 2000). Oxygenases incorporate molecular oxygen into the *n*-alkanes to form the corresponding alcohols, which are further degraded by beta oxidation (Wentzel et al. 2007). Since intermediates do not accumulate, the initial step of oxygen incorporation seems to be the rate limiting step (Chayabutra & Ju 2000).

Insight into anaerobic degradation of *n*-alkanes is limited. The first step of anaerobic degradation of *n*-alkanes is thermodynamically difficult, and has been proposed to occur in the sulfate-reducing bacterium strain Hxd3 via carboxylation (So et al. 2003). Molecular evidence for a mechanism of *n*-alkane activation through fumarate addition was obtained recently (Callaghan et al. 2008; Grundmann et al. 2008). Anaerobic degradation of *n*-alkanes is slow compared to aerobic degradation (Wentzel et al. 2007), and only a few denitrifying and sulfate-reducing bacteria have been isolated (Ehrenreich et al. 2000; So & Young 1999).

Microbial (per)chlorate reduction is a process that yields molecular oxygen, a property that has application possibilities in the bioremediation of polluted anoxic soils (Coates et al. 1998; Tan et al. 2006; Weelink et al. 2008). During chlorate reduction, chlorate (ClO_3^-) is reduced to chlorite (ClO_2^-) by the enzyme chlorate reductase. Chlorite is then split into Cl^- and O_2 by chlorite dismutase (Rikken et al. 1996; Wolterink et al. 2002). The oxygen released during chlorate reduction might be used to degrade *n*-alkanes by oxygenases.

Here we report the finding that *Pseudomonas chloritidismutans AW-1*^{T}, a chlorate reducing bacterium, that was previously isolated in our laboratory with acetate as carbon and energy source is able to grow on *n*-decane with oxygen or chlorate as electron acceptor. This finding suggests that an additional function of chlorite dismutation is to generate molecular oxygen to perform oxygenase-dependent reactions to support growth on *n*-alkane.

Materials and methods

Inoculum, media, cultivation and counting

P. chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink et al. 2002) and was kindly provided by Servé Kengen. For experiments with nitrate, it was adapted to nitrate by repeated sub-culturing on acetate and nitrate, while gradually decreasing the oxygen concentration according to Cladera et al. (2006).

The medium for *P. chloritidismutans* strain AW-1^T was based on the medium described by Dorn et al. (1974). The composition of the medium (in grams per liter of anaerobic demineralized water) was as follows: $Na_2HPO_4.2H_2O$, 3.48; KH_2PO_4 1; resazurin, 0.005; $CaCl_2$, 0.009; Ammonium Iron (III) citrate, 0.01; NH_4SO_4, 1; $MgSO_4.7H_2O$, 0.04. Vitamins and trace elements were added as described by Holliger et al. (1993) supplemented with Na_2SeO_3, 0.06; $NaWO_4.2H_2O$ 0.0184. The pH of the medium was 7.3.

P. chloritidismutans strain AW-1^T was cultivated in 120-ml flasks containing 40 ml of medium at 30^{o}C. The medium was made in anaerobic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminum crimp caps, and the head space was replaced by N_2 gas (140 kPa). All solutions that were added to the medium were made anaerobic and autoclaved at 121^{o}C for 20 minutes. The $CaCl_2$ was autoclaved separately to avoid precipitation and added aseptically to the already autoclaved salt solution. Vitamins and trace elements were filter sterilized. Chlorate and nitrate were supplied from a 0.4-M stock solution to get a final concentration of 10 mM. Pure oxygen was added from a sterilized gas stock. To prepare a stock solution of *n*-decane, a flask was made anaerobic by flushing with N_2 and then autoclaved. 99 % pure *n*-decane (Merck) was added to this flask through a 0.2-µm membrane filter. For mass balance analyses 1 mM of *n*-decane was added from a 50 mM stock solution of *n*-decane in acetone. The inoculum size for cultivation was 10% (v/v). Other *n*-alkanes tested were *n*-propane, *n*-butane, *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, *n*-nonane, *n*-undecane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. 1-decanol was added from an anaerobic filter sterilized solution, while sodium decanoate was added from an anaerobic, autoclaved 0.4-M stock solution.

Cell numbers were enumerated by phase contrast microscopy using a Bürker-Türk counting chamber at 1,000X magnification.

Three aerobic alkane utilizing bacteria, *Alcanivorax borkumensis* SK2 (DSM 11573), *Acinetobacter* sp. strain (DSM 17874) and *Acinetobacter baylyi* (DSM 14961), and the non-alkane degrading *Pseudomonas putida* KT2440 (DSM 6125) were obtained from the DSMZ, Braunschweig, Germany.

Preparation of cell free extracts

Cell free extracts of strain AW-1^T, grown in anaerobic medium with *n*-decane as sole carbon and energy source and chlorate as electron acceptor, were prepared anaerobically as previously described by Wolterink et al. (2002). The only modification was the centrifugation of whole cells at 13,000 rpm for 10 minutes at 4 °C. Cell free extracts were stored under a N_2 gas phase at 4 °C in 12 ml serum vials.

The protein content of the cell free extract fraction was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Enzyme activity measurements

Chlorate reductase and chlorite dismutase activities were determined with cell free extracts. Chlorate reductase activity was determined spectrophotometrically as described by Kengen et al. (1999), by monitoring the oxidation of reduced methyl viologen at 578 nm and 30 °C. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of chlorate per minute.

Chlorite dismutase activity was determined by measuring oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH, USA) as described by Wolterink et al. (2002). One unit of activity is defined as the amount of enzyme required to convert 1 μmol of chlorite per minute.

Alkane oxidation activity was determined by measuring the decrease in *n*-decane concentration in time by gas chromatography with whole cell suspensions. Cells were harvested by centrifugation at 13,000 rpm for 10 min. at 4 °C. Cells were washed and suspended in the buffer and then starved for 2 days to decrease the endogenous activity. After starvation, cells were suspended in a 15-mM phosphate buffer containing 2.5-mM *n*-decane added from a 50-mM stock solution in acetone. The reaction mixture was placed in a shaker set at 180 rpm at 30 °C. Two-milliter samples were taken in duplicate periodically after 0, 5, 10, 15, and 30 minutes. The *n*-decane was extracted as described by Staijen et al. (2000). Cells treated for 10 minutes at 100 °C were used as controls. One unit of activity is defined as the amount of enzyme required to convert 1 μmol of *n*-decane per minute. Starved cells resuspended in the anoxic buffer, flushed with the nitrogen, and reduced with 3 mM cystein, were used as anaerobic control.

Alcohol dehydrogenase activity was determined spectrophotometrically in the reductive direction at 30 °C using a spectrophotometer (U-2010, Hitachi). The activities were assayed in 1-ml reaction mixtures containing 15-mM sodium phosphate buffer with 0.3 mM NADH, and 0.5 mM aldehyde. The decrease in A_{340} was monitored to assess the activity. One unit of enzyme activity is defined as the amount enzyme required to oxidize 1 μmol of NADH. We also tried to

determine the alcohol oxygenase activity by the above mentioned spectrophotometric method using NADH and air flushed reaction mixtures.

Analytical techniques

Chlorate, chloride, nitrate, nitrite were measured, as described by Scholten & Stams (1995) after separation on a Dionex column (Ionpac AS9-SC) (Breda, The Netherlands), with a conductivity detector. Potassium fluoride (2 mM) was used as internal standard.

Oxygen was analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) equipped with a packed column (Molsieve 13x 60/80 mesh, 2-m length, 2.4-mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature used was 100 °C and the injector and detector temperatures were 90 and 150 °C respectively. Argon was used as the carrier gas at a flow rate of 30 ml min^{-1}.

CO_2 was analyzed by gas chromatography on a Chrompack CP9001 gas chromatograph fitted with a thermal conductivity detector (Henstra & Stams 2004). pH was measured by a glass microelectrode connected to a pH meter (Radiometer, Copenhagen). Total amount of bicarbonate present inside the flask was calculated by using the Henderson-Hesselbach equation. At 30 °C, the α and pK' are 0.665 and 6.348, respectively (Breznak & Costilow 1994).

n-Decane was extracted from 40-ml culture with 20 ml of *n*-hexane by shaking for 3 h and then separating the two phases through a separating funnel. *n*-Decane was analyzed in the hexane phase after adding octane as internal standard. One microliter was injected with a CP9010 autosampler in a CP9001 gas chromatograph (Chrompack), equipped with flam ionization detector and having a Chrompack Sil 5 CB capillary column (length, 25 m; diameter, 0.32 mm; *df*, 1.2 um) with nitrogen, 50- kPa inlet pressure, as carrier gas. The temperature of the injector, column and the detector was 250 °C, 100 °C and 300 °C, respectively.

Detection of alkane oxygenase genes

For the detection of putative genes encoding alkane oxygenase, the genomic DNA was extracted from cultures of strain AW-1^T grown on *n*-decane and chlorate using a FastDNA SPIN kit for Soil (Qbiogene). The extracted DNA was precipitated with isopropanol and vacuum dried. The primers developed by Whyte et al. (2002), Heiss-Blanquet et al. (2005), Kloos et al. (2006) and van Beilen et al. (2006) were used for the detection of the alkane oxygenases *alkB, alkM, almA, P450*. In addition, we developed some degenerated primers targeting *alkB, almA* and Acyl CoA dehydrogenase. Polymerase chain reaction (PCR) was carried out under the following conditions: final volume of 50 µl of 1X PCR Buffer (Promega) supplemented with $MgCl_2$, (2.5 mM), 200 µM dNTP´s, 0.5 µM of each primer and 0.3 U/µl Taq DNA Polymerase (Promega). The amplification conditions used were as follows: an initial denaturation step at 94 °C for 3

min, 35 times of a three steps cycle of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 minute, a final elongation step of 72 °C for 8 min. Polymerization reactions were stopped by cooling the samples at 4 °C. In addition, a gradient PCR using a temperature gradient of 40-65 °C was done with the *alkB* and *Cyt P450* primers used above and also with the primers designed by Smits et al. (1999, 2002), Kohno et al. (2002), and the degenerate primers used by Kubota et al. (2005). Sequences of the primers used in this study are given in Table 1. The positive controls of amplification were obtained in reactions with primers targeting *alkB*, and CYP153 genes using genomic DNA extracted from *Alcanivorax borkumensis* SK2, in reactions with primers targeting *almA*, with *Acinetobacter* sp. strain DSM 17874 genomic DNA and with *Acinetobacter baylyi* DSM 14961 in reactions targeting *alkM*.

Nucleotide sequence accession numbers

The DNA sequence of a putative acyl-CoA dehydrogenase gene of *P. chloritidismutans* AW-1^T was deposited in the GenBank/EMBL/DDBJ under accession number FJ477383.

Table 1: Different primers sets used in this study targeting conserved regions of *alkB, almA, Cytochrome P450 Subfamily CYP153*, and *Acyl CoA dehydrogenase* genes.

Primer Name	Sequence (5'→ 3')	Reference
	alkB	
TS2Sf TS2Smodf TS2Smod2f	AAYAGAGCTCAYGARYTRGGTCAYAAG AAYAGAGCTCAYGARITIGGICAYAAR AAYAGAGCTCAYGARITITCICAYAAR	Smits et al., 1999
DEG1RE DEG1RE2	GTRAGICTRGTRGTRCGCTTAAGGTG GTRTCRCTRGTRGTRCGCTTAAGGTG	
alkMUp alkMDn	CGGGGTAAGCATGAATAGCT CGTACAGCTACTTGGTGGAC	Tani et al., 2001
Alk-1F Alk -1R	CATAATAAAGGGCATCACCGT *GATTTCATTCTCGAAACTCCAAAC*	Kohno et al., 2002
Alk-3F Alk-3R	*CCGTAGTGCTCGACGTAGTT* *CAGGCGTTCTTCGGGTTGCGCTGCTCGA*	
AlkBpaFwd AlkBpaRv2	AACTGGAATTCACGATGTTTGA CTGCCCGAAGCTTGAGCTAT	Smits et al., 2002
AlkBpaBfw AlkBpaBrv	GGAGAATTCTCAGACAATCT GAGGCGAATCTAGAAAAAACTG	
B5 –Eco B3-Hind	GGAGAATTCCAAATGCTTGAG TTTGTGAAAGCTTTCAACGCC	
Pp alkB-F Pp alkB-R	TGGCCGGCTACTCCGATGATCGGAATCTGG CGCGTGGTGATCCGAGTGCCGCTGAAGGTG	Whyte et al., 2002
Rhose Rhoas	ACGGSCAYTTCTACRTCG CCGTARTGYTCGAGRTAG	Heiss-Blanquit et al., 2005
Pseuse1 Pseuas1	GARCATAATAARGGBCATC AGCARWCCGTARTGYTCA	
Pseuse2 Pseuas2	AYGTSCGYGGCCACCATGT CGACGTAGTTGAYGAYYTCC	
Acinse Acinas	ACWCCTGAAGATCCRGCWTC TRTTCCATCTAGCTCWGGC	
alkB-1f alkB-1r	AAYACNGCNCAYGARCTNGGNCAYAA GCRTGRTGRTCNGARTGNCGYTG	Kloos et al., 2006
alkBF alkBR	GSNCAYGARYTSRKBCAYAA GCRTGRTGRTCNSWRTGNCGYTG	This study
	Cytochrome P450 Subfamily CYP153	
CF CR	ATGTTYATHGCNATGGAYCCNC NARNCKRTTNCCCATRCANCKRTG	Kubota et al., 2005
P450fw1 P450rv3	GTSGGCGGCAACGACACSAC GCASCGGTGGATGCCGAAGCCRAA	Van Beilen et al., 2006
	almA	
almA-F1 almA-R1 almA-R2	CCBGGBATYCGBTCNGAYTCNGAYATGT GGHGADCGYTGYARCATVGTNACGTNACBATGYTRCARCGH TCDCC CANAVVCGYTSRTCCCANGGVTTRTATAYAABCCNTGGGAYS ARCGBBTNTG	This study
	Acyl-Co A dehydrogenase	
Acyl-F1 Acyl-R1 Acyl-R2	GGYTCNATYGARCABAARATGGG CCCCAYTCRCGRATRWARCCRTGVCCRCCRAA TGRAYRCCRTTRGTRCCTTCRTARAT	This study

Results

n-Alkane degradation

P. chloritidismutans AW-1^T uses *n*-decane as a sole source of carbon and energy. Growth on *n*-decane and chlorate was indicated by the increase in optical density (Fig. 1a). An OD of 0.34 corresponds to a bacterial count of 1.31×10^9. Growth followed *n*-decane degradation as indicated by CO_2 formation, chlorate reduction and chloride production (Fig. 1a). No growth was observed in controls without inoculum or without *n*-decane or controls without chlorate (results not shown). The specific growth rate on *n*-decane and chlorate was 0.5 $\pm$ 0.1 per day (doubling time 1.4 $\pm$ 0.2 day). After 7 days, 87% of the 1 mM of the added *n*-decane was oxidized. The oxidation of 1 mM of *n*-decane led to a reduction of 9.2 $\pm$ 0.7 mM of chlorate and yielded 7.7 $\pm$ 0.6 mM of bicarbonate and 8.3 $\pm$ 0.8 mM of chloride. The balance fits relatively well with the theoretical stoichiometry of complete oxidation of *n*-decane coupled to chlorate reduction:

$$C_{10}H_{22} + 10.33\ ClO_3^- \rightarrow 10\ HCO_3^- + 10.33\ Cl^- + H_2O + 10\ H^+ \quad (1)$$

The bacterium also grows aerobically on *n*-decane (Fig. 1b). The specific growth rate on *n*-decane and molecular oxygen was 0.4 $\pm$ 0.02 per day (doubling time 1.7 $\pm$ 0.1). Growth and CO_2 production were not observed in the presence of *n*-decane and nitrate, using *P. chloritidismutans* adapted to growth on nitrate and acetate (results not shown).

Other substrates utilized

Apart from *n*-decane, other *n*-alkanes were also screened as possible substrates with chlorate as electron acceptor. Strain AW-1^T grew with C7 to C12 *n*-alkanes, but not with smaller *n*-alkanes. It grew equally well on odd and even chain *n*-alkanes. Strain AW-1^T grew equally well on C8 till C11, while growth on C7 and C12 was slower.

Strain AW-1^T also grew on the possible intermediates of the aerobic *n*-alkane degradation pathway, namely, 1-decanol and decanoate. Table 2 shows the amount of bicarbonate formed with various substrates using different electron acceptors. With *n*-decane and 1-decanol as substrates, bicarbonate was formed with chlorate and oxygen, but not with nitrate as electron acceptor. With decanoate and nitrate, bicarbonate was also formed.

Fig. 1a:

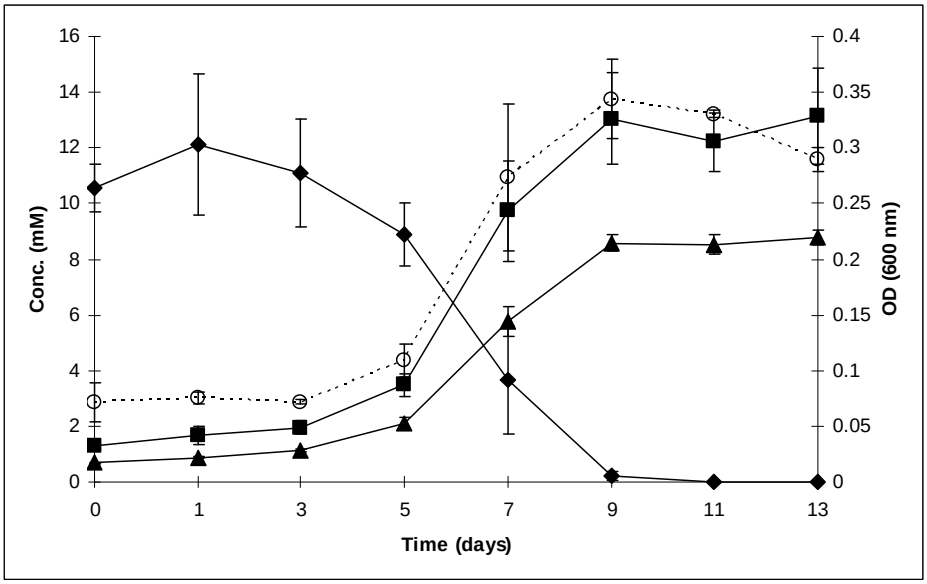

Fig.1b:

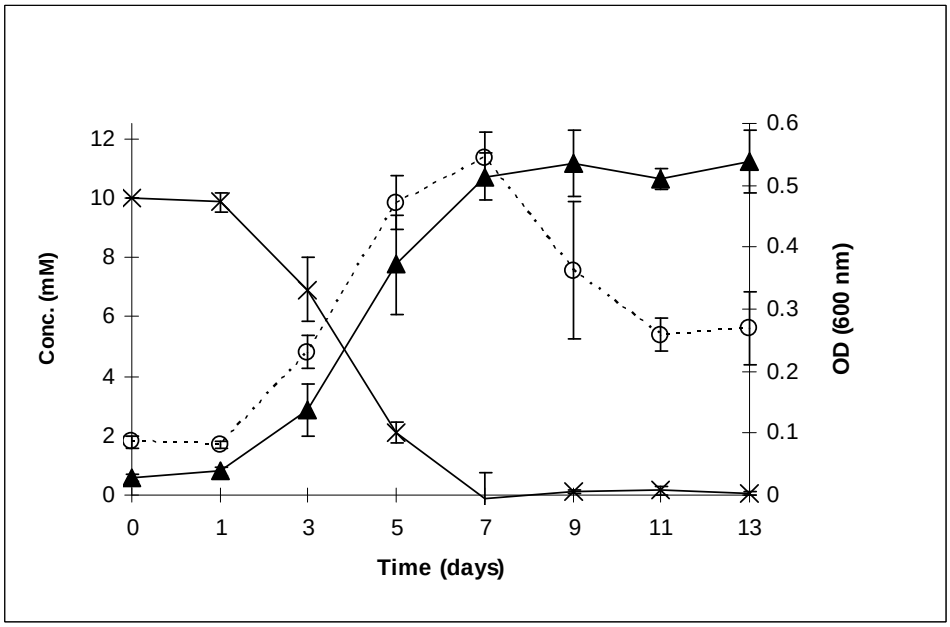

Fig. 1: Growth of strain AW-1^T (a) with decane and chlorate and (b) with decane and oxygen. Values are means of three replicates. The bars represent standard deviation. Dotted line with open circles (Θ) represents the OD at 600nm. The continuous lines represent ♦ chlorate utilized; ⁎ O_2 utilization; ■ chloride produced and ▲ bicarbonate formed.

Table 2: Formation of bicarbonate (in mM) by strain AW-1^T during growth on different substrates.

	Electron acceptor		
Substrate	**O_2**	**ClO_3^-**	**NO_3^-**
n-Decane	10.6 ± 1.1	7.9 ± 0.3	0.7 ± 0.2
1-Decanol	9.8 ±1.3	9.8 ± 0.3	0.2 ± 0.1
n-Decanoate	8.3 ± 0.4	8.6 ± 0.4	8.0 ± 0.9

Values for decane are after 9 days, while for decanol and decanoate the samples were analyzed after 7 days

Enzyme assays

Extracts of cells grown on *n*-decane and chlorate and on acetate and chlorate showed chlorate reductase and chlorite dismutase activity (Table 3). The specific chlorite dismutase activity is dependent on the amount of cell extract and the chlorite concentration (Mehboob et al. 2009). The chlorite dismutase presented in Table 3 is the activity under optimal conditions.

Alkane oxidation activity could be measured with starved cells grown on *n*-decane and chlorate. A relatively small amount of activity was also observed with starved whole cells grown on acetate and chlorate (Table 3).

Table 3: Activities of chlorate reductase, chlorite dismutase and alkane oxygenase of strain AW-1^T grown on acetate and chlorate or on *n*-decane and chlorate.

	Specific enzyme activity (U/mg of protein)	
	Acetate + ClO_3^-	***n*-Decane + ClO_3^-**
Chlorate reductase	11.4 ± 0.3	26.6 ± 1
Chlorite dismutase	7.7 ± 1.4	2.8 ± 0.7
Alkane oxygenase*	26 ± 9	93 ± 31

The alkane oxidation activity was measured with whole cells and is average of the activities at four different time points i.e. after 5, 10, 15 & 30 min.

No alkane oxidation was observed with the anoxic control. Cell free extract of alkane grown cells did not show alcohol oxygenase activity, but we found an activity of 0.06 U/mg of protein of NAD^+-dependent decanol dehydrogenase. This was quite surprising as during the growth experiment strain AW-1^T was unable to grow with decanol and nitrate. Our strain is known to grow with ethanol and chlorate (Wolterink et al. 2002). We checked and found that strain AW-1^T is able to grow with ethanol using oxygen, chlorate and nitrate as electron acceptors. Even ethanol and nitrate adapted cells could not grow on the decanol and nitrate. Cell free extract of decane grown culture showed twofold higher activity of 0.15 U/mg of protein with acetaldehyde.

Detection of alkane oxygenase genes

Various primers at different annealing temperatures were used to detect the following alkane oxygenase genes: *alkB, alkM, almA*, and cytochrome P450 subfamily CYP153. Though we got

the expected results in positive controls, with the available specific primers sets we were not able to detect any kind of known alkane oxygenase genes in our strains. We were able to amplify a sequence which was 51% and 57% similar to two acyl-CoA dehydrogenases involved in the degradation of *n*-alkane in *Acinetobacter* strain M-1 (Tani et al. 2002).

Discussion

Pseudomonas chloritidismutans AW-1^T is a gram-negative, facultative, anaerobic and chlorate reducing bacterium, which has been isolated on acetate and chlorate in our laboratory (Wolterink et al. 2002). We tested its ability to grow on *n*-alkanes and found that strain AW-1^T grows on *n*-alkanes with oxygen and chlorate as electron acceptor. Many pseudomonades have the ability to grow aerobically on *n*-alkanes (Söhngen 1913; Wentzel et al. 2007), but strain AW-1^T is the only known bacterium that grows on *n*-alkanes by supplying molecular oxygen formed by chlorite dismutation. The doubling time with *n*-decane and chlorate is 1.4 $\pm$ 0.2 days. Except for strain HxN1, which has a doubling time of 11 hours (Ehrenreich et al. 2000), all other anaerobic *n*-alkane degraders grow slower, e.g., strains Hxd3 and Pnd3 have doubling times of 9 days (Aeckersberg et al. 1998) and strain AK-01 has a doubling time of 3 days (So & Young 1999). In contrast, doubling times of aerobic alkane degrading bacteria are approximately 1 h for *Pseudomonas aeruginosa* (Ertola et al. 1965) and 5 hours for *Rhodococcus* species (Bredholt et al. 1998).

Strain AW-1^T grows on n-decane with chlorate and oxygen, but not with nitrate, suggesting the involvement of oxygenases. Oxygen is incorporated in *n*-decane through an oxygenase to form decanol. When chlorate is used as electron acceptor, oxygen is formed by dismutation of chlorite. This is supported by the similar specific growth rates on *n*-decane with oxygen (0.4 $\pm$ 0.02 day^{-1}) or chlorate (0.5 $\pm$ 0.1 day^{-1}) as electron acceptor. Strain AW-1^T also grows on possible aerobic intermediates, like 1-decanol and decanoate with oxygen and chlorate as electron acceptor. It was unable to utilize 1-decanol when nitrate was used as electron acceptor. This also suggests that, in the conversion of decanol, an oxygenase as found by Buhler et al. (2000) and Katopodis et al. (1984) is involved. Growth on decanoate with nitrate suggests that no oxygenases are required for decanoate degradation. Growth of strain AW-1^T with oxygen or chlorate was observed to be the fastest on decanoate followed by decanol and then *n*-decane.

We faced a problem in detecting alkane oxygenase activity with cell free extracts. This has also been observed by others (Katopodis et al. 1984; Tani et al. 2001) and was attributed to the poor solubility of the substrate (Smits et al. 2002; Tani et al. 2001), the instable nature of the alkane oxygenase complex (Katopodis et al. 1984; McKenna & Coon 1970; Ruettinger et al. 1974) and the involvement of unknown factors (Tani et al. 2001), like some unique electron transfer

proteins (van Beilen et al. 2006). However, alkane oxidation activity could be demonstrated with whole cells grown on *n*-decane and chlorate. Almost 3.5-fold more activity was observed with the cells grown on decane and chlorate as compared with the cells grown on acetate and chlorate showing the induction of alkane oxygenase when grown on *n*-decane**.**

We were unable to detect an alcohol oxygenase in decane-grown cell-free extracts. However, we found an alcohol dehydrogenase that has a more than twofold higher activity for acetaldehyde than for decanal. Since the strain AW-1^T grows with ethanol with oxygen, chlorate and nitrate but is unable to grow with decanol and nitrate, it is unlikely that this alcohol dehydrogenase is involved in long-chain alcohol oxidation. Instead the detected alcohol dehydrogenase only seems to be involved in growth with short-chain alcohols.

We were unable to amplify an alkane oxygenase gene from our strain with new and known primers designed to detect different classes of alkane oxygenases. However, we amplified a sequence, similar to an acyl CoA dehydrogenase from strain AW-1^T. This acyl-CoA dehydrogenase enzyme has been reported to be involved in *n*-alkane degradation in *Acinetobacter* strain M-1 (Tani et al. 2002). The same group proposed that a dioxygenase is involved in the initial oxidation (Finnerty pathway) of *n*-alkanes (Maeng et al. 1996). However, we have observed that the N-terminal sequence of this dioxygenase and the above mentioned acyl-CoA dehydrogenase is similar. This sequence seems highly conserved in all *Pseudomonas* genomes. Therefore, we also did a growth test on *n*-alkanes as carbon and energy source with *Pseudomonas* sp. KT2440, for which the genome sequence is available. The genome contains acyl-CoA dehydrogenase genes, but evidence for the presence of a conventional alkane hydroxylase system is lacking. No obvious aerobic growth of this strain on *n*-alkanes was found. Hence, we assume that the acyl-CoA dehydrogenase is not involved in the first step of activation of *n*-alkane, as reported by Maeng et al. (1996), but is important in a later reaction step of *n*-alkane degradation.

A reason why we were not able to detect alkane oxygenase genes could be that the alkane oxygenases have a very high sequence diversity (i.e. the protein sequence similarity between reported *alkB* types can be as low as 35%), especially among the *Pseudomonas* group. The *Pseudomonas* alkane oxygenases are as distantly related to each other as to the alkane oxygenases from phylogenetically unrelated bacteria (Smits et al. 2002). This may have led to similar false negative results as reported by others (Chandler & Brockman 1996; Heiss-Blanquet et al. 2005; van Beilen et al. 2006). It may also be that a novel type of alkane oxygenase is involved in this process, of which the genes are not known yet and which may be specific for *n*-alkane degradation at low oxygen concentrations, as is evidently the case for growth under

chlorate-reducing conditions. The extent of diversity of alkane oxygenases became apparent in recent research by Kuhn et al. (2009). They found that only one out of the 76 clones of the putative *alk* genes had a significant sequence similarity with previously known *alk* genes.

Based on all the physiological features, enzyme measurements and the amplification of an acyl-coA dehydrogenase gene, we propose a hypothetical *n*-alkanes (C7-C12) degradation pathway as depicted for *n*-decane in Fig. 2. We suggest that oxygen formed in the dismutation of chlorite is used to convert *n*-decane to decanol and decanol to decanal by means of oxygenases. Decanal is further oxidized to decanoate, which upon activation is degraded by β-oxidation.

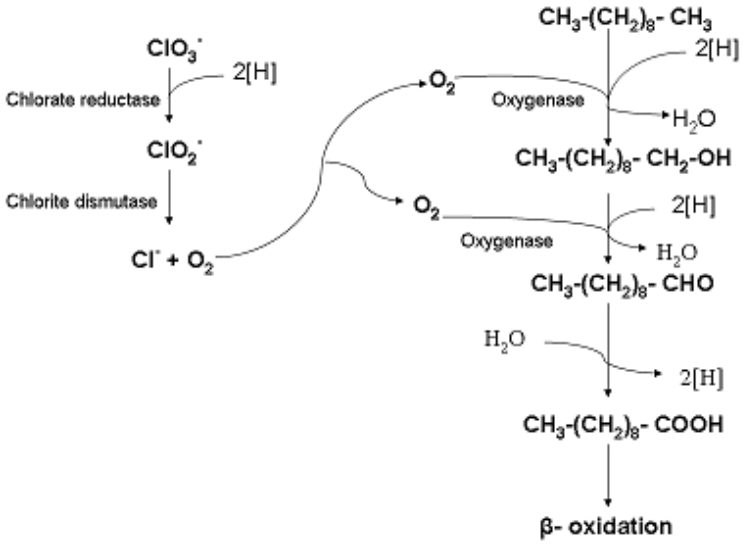

Fig. 2: Hypothetical pathway of degradation of n-decane coupled to chlorate reduction. Oxygen released from chlorite dismutation is used by a presumed oxygenase to incorporate in the n-alkane molecule to form an alcohol and later on an aldehyde. Further degradation may occur in the absence of oxygen.

This is the first report of the degradation of aliphatic hydrocarbons with chlorate, both as electron acceptor and as source of oxygen needed for the oxygenase activity. The degradation of aromatic hydrocarbons with chlorate has recently been described (Tan et al. 2006; Weelink et al. 2008). This study adds to the possibility to apply microorganisms with oxygenase-dependent pathways for the bioremediation of anoxic soils polluted with compounds that are difficult to degrade in the absence of molecular oxygen.

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Chapter 4

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

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Abstract

The chlorite dismutase (Cld) of *Pseudomonas chloritidismutans* was purified from the periplasmic fraction in one step by hydroxyapatite chromatography. The enzyme has a molecular mass of 110 kDa and consists of four 31-kDa subunits. Enzyme catalysis followed Michaelis Menten kinetics, with V_{max} and K_m values of 443 U/mg and 84 µM, respectively. A pyridine-NaOH-dithionite-reduced Cld revealed a Soret peak at 418 nm, indicative for protoheme IX. The spectral data indicates the presence of 1.5 mol of protoheme IX per mol of tetrameric enzyme while metal analysis revealed 2.2 moles of iron per mole of tetrameric enzyme. High concentrations of chlorite resulted in disappearance of the Soret peak which coincided with loss in activity. Electron paramagnetic resonance analyses showed an axial high-spin ferric iron signal. Cld was inhibited by cyanide, azide, but not by hydroxylamine or 3-amino-1,2,3-triazole. Remarkably, the activity was drastically enhanced by kosmotropic salts, and chaotropic salts decreased the activity, in accordance with the Hofmeister series. Chlorite conversion in the presence of ^{18}O-labeled water, did not result in the formation of oxygen with a mass of 34 (^{16}O - ^{18}O) or a mass of 36 (^{18}O - ^{18}O), indicating that water is not a substrate in the reaction and that both oxygen atoms originate from chlorite.

Introduction

Chlorite dismutase (Cld) (EC 1.13.11.49) catalyzes the splitting of chlorite (ClO_2^-) into chloride and molecular oxygen ($ClO_2^- \rightarrow Cl^- + O_2$), which is one of the central reactions in the dissimilatory reduction of (per)chlorate. The name 'chlorite dismutase' is in fact not correct, because the reaction is not a dismutation but an intramolecular redox reaction. The systematic name for this enzyme should be chloride:oxygen oxidoreductase or chlorite O_2-lyase (EC 1.13.11.49) as suggested by Hagedoorn et al. (2002).

During dissimilatory perchlorate reduction, perchlorate (ClO_4^-) is reduced to chlorate (ClO_3^-) and chlorate is subsequently reduced to chlorite (ClO_2^-), through the action of a (per)chlorate reductase. Next, the 'chlorite dismutase' converts the toxic chlorite to chloride and molecular oxygen. Because of this production of oxygen, microbial (per)chlorate reduction has been suggested as a novel strategy for bioremediation of hydrocarbons in anoxic zones (Coates et al. 1998; Tan et al. 2006; Weelink et al. 2007).

So far, Cld has been purified from only four chlorate-reducing strains, i.e. *Azospira oryzae* GR-1 (van Ginkel et al. 1996), *Dechloromonas agitata* (Coates et al. 1999), *Ideonella dechloratans* (Stenklo et al. 2001) and *Dechloromonas aromatica* RCB (Streit & DuBois 2008). The Cld from *D. agitata* has not been described in detail and the one from *D. aromatica* RCB has been purified after cloning and heterologous expression in *Escherichia coli.* Cld from *I. dechloratans* has also been heterologously expressed and compared with the native enzyme (Danielsson-Thorell et al. 2004). All dismutases described thus far have a periplasmic location, a homotetrameric composition and absorption spectra characteristic for protoheme IX.

Apart from the known (per)chlorate reducers, Cld-like proteins from non-(per)chlorate-reducing microorganisms have been studied, like *Thermus thermophilus* HB8 (Ebihara et al. 2005), *Haloferax volcanii* (Bab-Dinitz et al. 2006) and *Candidatus Nitrospira defluvii* (Maixner et al. 2008). The Cld from *N. defluvii* is so far the only Cld-like protein with high catalytic activity from a bacterium that is not known for (per)chlorate reduction ability (Maixner et al. 2008). Recently Cld from *A. oryzae* GR-1 has been cloned, expressed, crystallized and subjected to X-ray diffraction analysis (de Geus et al. 2008). The mechanism of chlorite "dismutation" has been proposed to occur via the formation of high valent oxo intermediates compound I and compound II (Hagedoorn et al. 2002; Lee et al. 2008).

An immuno probe specific to Cld was shown to bind only (per)chlorate-reducing bacteria indicating that the gene is highly conserved (O' Connor & Coates 2002). Based on the primers developed by Bender et al. (2004) the Cld gene from *Pseudomonas chloritidismutans* has been

amplified, and its sequence analysis suggests a horizontal transfer from gamma-proteobacteria (Cladera et al. 2006).

In the present study, the purification and characterization of the Cld from *P. chloritidismutans* is described. The chlorate reductase of *P. chloritidismutans* has already been purified, and characterized and found to be quite different from the (per)chlorate reductase of strain GR-1 (Kengen *et al*. 1999). The present report describes a simple one-step purification procedure for Cld from the periplasmic fraction, the effect of various salts on the Cld activity and a comparison with the previously described Cld's

Materials and Methods

Bacterial strain

Pseudomonas chloritidismutans strain AW-1^T (DSMZ 13952; ATCC BAA-443) was isolated from sludge of an anaerobic bioreactor treating a chlorate and bromate polluted waste stream. The bacterium was grown in strictly anoxic medium with acetate and chlorate, as described before (Wolterink et al. 2002).

Enzyme purification

For purification of the Cld 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 g at 4°C. The cell pellet was checked for the presence of whole cells by light microscopy. The supernatant was subjected to ultracentrifugation at 110,000 g for 1 h at 4ºC and the red supernatant (periplasmic fraction) 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* (GE Healthcare). Four milliliters of the diluted supernatant was loaded on a hydroxyapatite column (CHT5-I; 10 x 64 mm, Bio-Rad Lab. Inc.) equilibrated with 10 mM Tris-HCl, pH 7.2. The Cld 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 Cld were kept at 4ºC for further analysis or stored at -20ºC

Determination of the molecular mass

Protein fractions were checked by sodium dodecyl sulfate-polyacrylamide gel 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 Cld on a Superdex 200 column (16 x 705 mm, GE Healthcare) 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 RNAse (14).

Activity assay

Cld activity was measured with a Clark-type Electrode (YSI, Yellow Springs, OH) as done by van Ginkel et al. (1996). Under a standard condition, 2.8 ml of buffer (100 mM sodium phosphate buffer, pH 6) was added to the sample chamber together with 10 µl of a stock solution of sodium chlorite, to yield 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 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 100 mM sodium phosphate buffer, pH 7.2).

At the optimal temperature of 25ºC, the activity was measured at different pH values, ranging from 5 to 8.5 using sodium phosphate 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. The protein content was determined according to Bradford (1976) with bovine serum albumin as standard.

Spectroscopy and metal analysis

UV-visible 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 before the measurement in a stoppered cuvette, flushed with N_2 gas. The heme content was calculated using the molar extinction coefficient ε_{418} = 191,500 M^{-1} cm^{-1} (Falk 1964). X-band electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER-200D spectrometer with peripheral equipment and data handling as described before (Pierik & Hagen 1991). EPR spectra were recorded of 0.91 mM Cld in 10 and 500 mM potassium phosphate buffer (pH 6). The iron content was determined by inductively coupled plasma (ICP)-MS (Elan 6000, Perkin-Elmer) (Jarvis et al. 1992).

Inhibition and stabilization of chlorite dismutase

The effect of cyanide, azide, hydroxylamine, EDTA, H_2O_2 or 3-amino-1,2,4-triazole as inhibitors was tested by adding a final concentration of 70 or 700 µM. Substrate inhibition of Cld was analyzed by adding 56 mM chlorite (final concentration) to the enzyme solution. After 1 min, the

activity was measured and a UV-visible spectrum of the protoheme was generated. After 10 min, again 56 mM chlorite (final concentration) was added and the measurements were repeated.

The amount of enzyme present in the assay mixture was found to influence the total amount of oxygen produced (µM), therefore, undiluted and a 10-fold diluted solution (0.64 and 0.064 mg protein, respectively) of Cld was added to the assay.

The effect of Hofmeister salts on the activity of Cld was determined. The following salts were added to the assay mixture at concentrations between 0 to 2 M: sodium phosphate; potassium phosphate; $NaClO_3$, $NaNO_3$, NaCl, NH_4Cl, $(NH_4)_2SO_4$, Na_2SO_4, $NaHCO_3$. The pH of the solutions 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 at. % ^{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 $H_2{}^{18}O$ or 100 µl $H_2{}^{16}O$ (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 $H_2{}^{18}O$ (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 $^{16}O_2$, $^{17}O_2$ and $^{18}O_2$ using a GC/MS equipped with a capillary column (Innowax 30 m x 0.25 mm, split ratio 25:1, Hewlett-Packard). Helium gas was used as carrier gas, the inlet pressure was 1 kPa and the column temperature was 40ºC.

Results and Discussion

Enzyme purification

Localization experiments performed previously, had shown that the Cld has a periplasmic orientation (Wolterink et al. 2003). A periplasmic location was also suggested for the Cld of strain GR-1 and *I. dechloratans*. Therefore, the first purification step concerned the isolation of the periplasmic fraction (after Sebban et al. 1995). After removing the protoplasts by centrifugation and membrane debris by ultracentrifugation, a red supernatant was obtained (lane 2, Fig. 1).

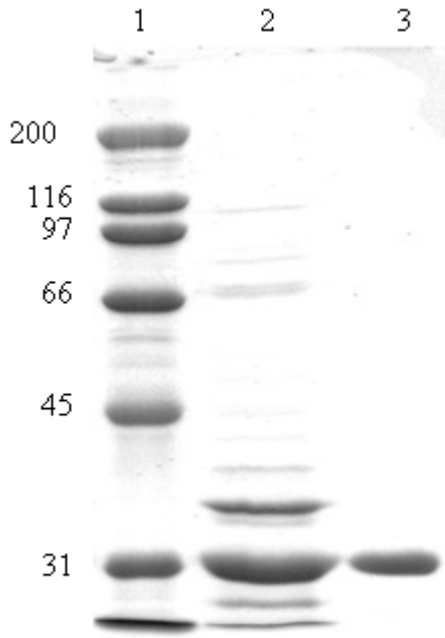

Fig. 1: SDS-PAGE of chlorite dismutase from *P. chloritidismutans*. Lane 1, marker proteins with indicated molecular masses; lane 2, periplasmic fraction; lane 3, hydroxyapatite fraction.

This periplasmic fraction was diluted (1:1) with 10 mM Tris-HCl buffer (pH 7.2) and subsequently loaded on a hydroxyapatite column. The Cld, 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. According to the visible spectrum (not shown) this fraction is most likely another periplasmic heme protein. The Cld preparation was > 95% pure as assessed by SDS-PAGE (Fig. 1). The hydroxyapatite step resulted in an increase of the specific activity from 21 to 208 U/mg, with an overall yield of 69%, which is higher than that reported (43%) for strain *A. oryzae* GR-1 (van Ginkel et al. 1996). The purification by a factor 10 indicates that the Cld is an abundant protein in the periplasm. A similar yield (63%) and an eight-fold purification was found for Cld from *I. dechloratans* (Stenklo et al. 2001).

Gel electrophoresis and determination of the molecular mass

The purified Cld was loaded on a SDS-PAGE. This resulted in a single band at 31 kDa (Fig. 1, 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 Cld from A. oryzae GR-1, *I. dechloratans, D. agitata* and *D. aromatica* RCB. Table 1 shows a comparison of all known Cld's. Except for the Cld-like protein from *T. thermophilus,* which has a very low catalytic activity (Ebihara et al. 2005), all are tetramers. Their molecular mass varies from 110-140 kDa and subunits size ranges from 25 to 32 kDa.

Spectral characteristics

The pyridine-NaOH-dithionite-reduced Cld showed peaks indicative of protoheme IX (Soret, 418 nm; β max, 526; α max, 557) (Falk 1964). The native Cld showed a Soret peak at 411 nm, which shifted to 433 nm on addition of dithionite. In the presence of cyanide, the Soret peak shifted to 420 nm. Similar characteristic Soret peaks have been observed in all other Cld's (Table 1). The formation of a cyanide complex, indicative of ferric heme in the protein, was also observed for the Cld of *A. oryzae* GR-1.

The heme content was determined to be 1.5 mol of heme per mol of tetrameric Cld, based on a molar extinction coefficient of 191,500 M^{-1} cm^{-1}. Similarly, *A. oryzae* GR-1 Cld contains 1.7 mol heme/mol Cld (van Ginkel et al. 1996) while the one from *I. dechloratans* has 2.4 mol of protoheme IX (Stenklo et al. 2001). The slightly higher heme content in *I. dechloratans* could be a result of the use of a different extinction coefficient (24,000 M^{-1} cm^{-1} at 558 nm) compared with the one used for *A. oryzae* GR-1 and *P. chloritidismutans* (191,000 M^{-1} cm^{-1} at 418 nm).

On the other hand, differences in growth condition may also affect the heme content. Supplementation of the medium with hemin resulted in near-stoichiometric heme incorporation in heterologously produced Cld from *D. aromatica* RCB (Streit & DuBois 2008). Nevertheless, often the heme content of the enzyme is lower than expected for a homotetrameric enzyme.

The exposure to chlorite also causes a bleaching of the heme and simultaneous loss of activity (Stenklo et al. 2001). This was also observed by van Ginkel et al. (1996), where the decrease of Cld activity at high chlorite concentrations was attributed to the oxidation of the heme. The Soret peak disappeared upon addition of 500 mM of chlorite in strain GR-1. These findings were confirmed here by adding 56 mM (final concentration) chlorite to the Cld of *P. chloritidismutans* two times. The Soret peak disappeared, which coincided with an activity loss of 80% after the first addition and 90% (of the starting activity) after the second addition of chlorite. Thus, the Cld is bleached and inactivated by high concentrations of substrate.

Metal analysis by ICP-MS revealed the presence of 2.2 mol Fe per mol of tetrameric enzyme. This is slightly higher compared to the calculated heme content (1.5 mol/ mol of tetrameric enzyme). The cld of strain GR-1 contained 2.8 atoms of iron per tetramer. For the hemin supplemented enzyme of *D. aromatica* RCB a 1:1 ratio was found for the Fe and heme content (Streit & DuBois 2008).

Table 1: Characteristics of chlorite dismutases

	P. chloritidismutans **AW-1**[Ta]	***Strain*** **GR-1**[b]	***I. dechloratans*** [c]	***D. aromatica*** **RCB** [d]	**Candidatus *Nitrospira defluvii*** [e]	***Thermus thermophilus*** **HB8**[f]
Subunit size (kDa)	31	32	25	27	30	26
Relative molecular mass (kDa)	110	140	115	116	ND	130
T- optimum (ºC)	25	30	ND	ND	25	ND
pH optimum	6	6	ND	ND	6	ND
V_{max} (U/mg)	0.44×10^3	2.2×10^3	4.3×10^3	4.7×10^3	1.9×10^3	1.6[e]
K_m (mM)	0.08	0.17	0.26	0.22	15.8	0.01
k_{cat} (s^{-1})	0.23×10^3	1.2×10^3	1.8×10^3	1.88×10^3	0.96×10^3	0.77
k_{cat}/K_m ($M^{-1}sec^{-1}$)	2.7×10^6	7.1×10^6	6.9×10^6	35.4×10^6	6.1×10^4	59
Heme content	1.5	1.7	2.4	3.7	ND	0.6
Soret band (Fe^{3+})	411	394[g]	392	388	415	403
Soret band (Fe^{2+})	433	432[g]	434	434	433	429

[a] :Present study; [b] : van Ginkel et al., 1996; [c]: Stenklo et al., 2001; [d] : Streit & DuBois, 2008; [e] : Maixner et al., 2008; [f] : Ebihara et al., 2005; [g] : Hagedoorn et al., 2002; ND: not described

EPR measurements

For the isolated Cld of *P. chloritidismutans* an EPR-signal with $g_{\perp} = 6.0$ and $g_{//} = 2.0$ was found, which can be attributed to axial high-spin ferric iron (Fig. 2).

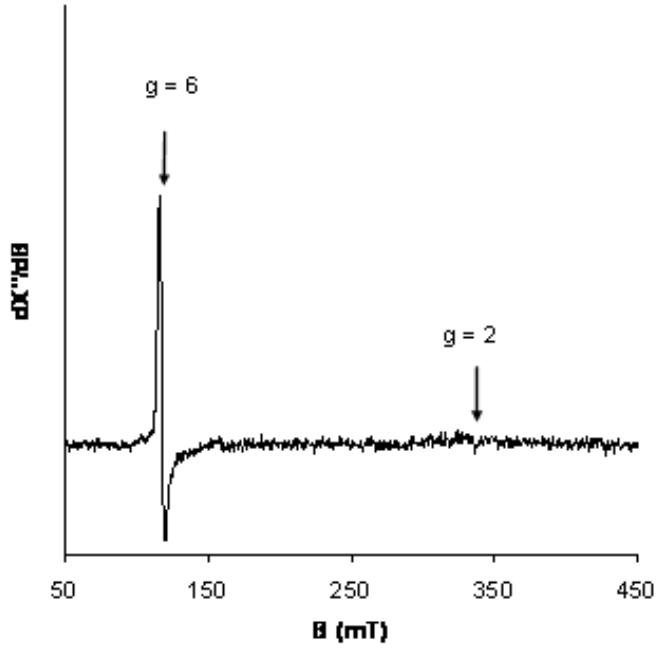

Fig. 2: Electron paramagnetic resonance spectra of *P. chloritidismutans* chlorite dismutase as isolated (in 10 mM sodium phosphate). EPR conditions: microwave frequency, 9.432 GHz; Microwave power, 50 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 10.2 K.

This result is similar to previous observations of the ferric ion in Cld from *A. oryzae* GR-1 (Hagedoorn *et al*., 2002), which showed a mixture of two high-spin signals with close to axial rhombicity (E/D values of 0.01-0.02 and 0.03-0.04). *Pseudomonas chloritidismutans* Cld exhibits only a single axial high-spin ferric species, which indicates a slightly different micro-environment around the active site. No low-spin ferric species was observed at pH 6, which is consistent with the fact that the low-spin ferric species observed in isolated Cld from other organisms originates from the hydroxide adduct with a pKa of 8.2 (Hagedoorn *et al*. 2002).

Kinetic characterization

The total amount of oxygen produced by Cld in the assay was affected by the amount of enzyme present. In Table 2, the results are shown of enzyme assays using undiluted and 10 times diluted purified cld. In the presence of the same concentration of chlorite, but with less enzyme, the total amount of oxygen produced was less. These results suggest that the enzyme is inactivated by chlorite at low enzyme concentrations before all chlorite is converted. These results are also supported by the disappearance of the Soret peak upon addition of excess chlorite as mentioned

before. Therefore, in all kinetic analyses care was taken to ensure that sufficient enzyme was present in the assay mixture.

Table 2 Effect of enzyme concentration on oxygen production

Amount of ClO_2^- added (µM)	O_2 produced by undiluted chlorite dismutase (0.64 mg/l) (µM)	O_2 produced by 10 times diluted chlorite dismutase (0.064 mg/l) (µM)
100	98	34
200	205	69
300	300	96

O_2 Production measured in 1M phosphate buffer at 25ºC. All values are average of duplicates and standard deviation is less than 10%.

The enzyme was active between pH 5 and pH 8.5, with an optimum at pH 6, and 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 (Table 1). The V_{max} value is slightly lower than other reported values, while its K_m value indicates a higher affinity for chlorite. As a result, the catalytic efficiency k_{cat}/K_m (M^{-1} sec^{-1}) of 2.7×10^6 is in the same range as that found for all other Cld's (Table 1).

Effect of salts

The activity of Cld was strongly influenced by the type and concentration of salts present in the assay mixture. The results are shown in Fig. 3a and Fig. 3b. When phosphate or sulfate was used, activity increased at higher salt concentrations up to 1M and then decreased again. When chlorate or nitrate was used, the activity decreased with increasing salt concentration (Fig. 3a). Activity was not influenced by the bicarbonate salt and similarly chloride salts up to a concentration of 1.5 M showed no significant effect (Fig. 3b).

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 $PO_4^{3-} > F^- > SO_4^{2-} > Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > BrO_3^- > ClO_4^-$. Increase of chaotropic salts like nitrate and chlorate reduced the activity. The increase of the concentration of kosmotropic salts resulted in higher V_{max} as well as higher K_m values (for 1 M phosphate, the V_{max} and K_m values were 4650 U/mg and 833 µM, respectively).

Fig. 3a:

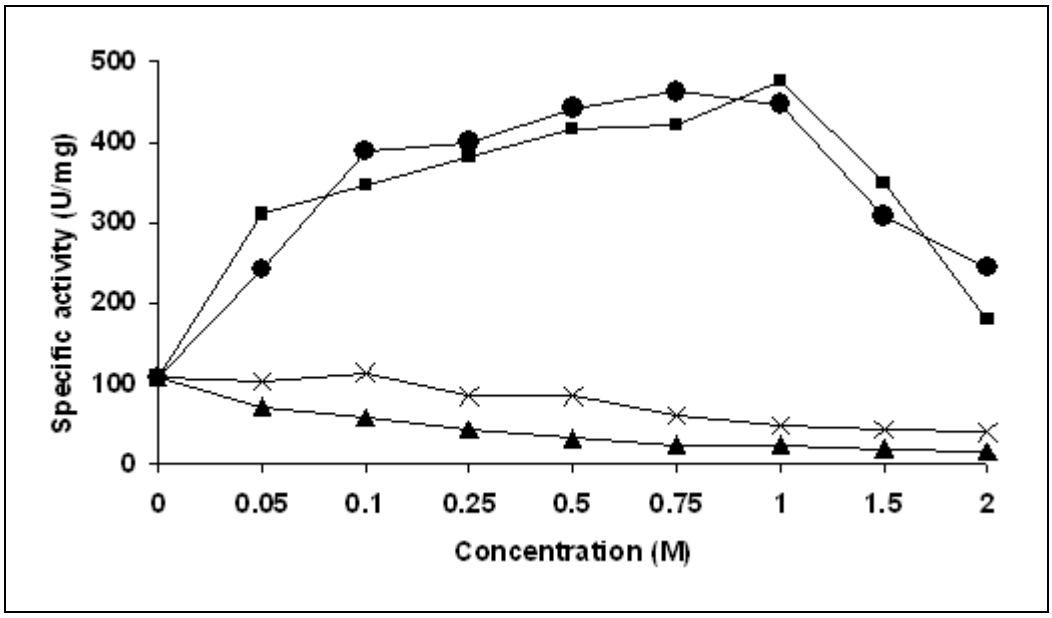

Fig. 3b:

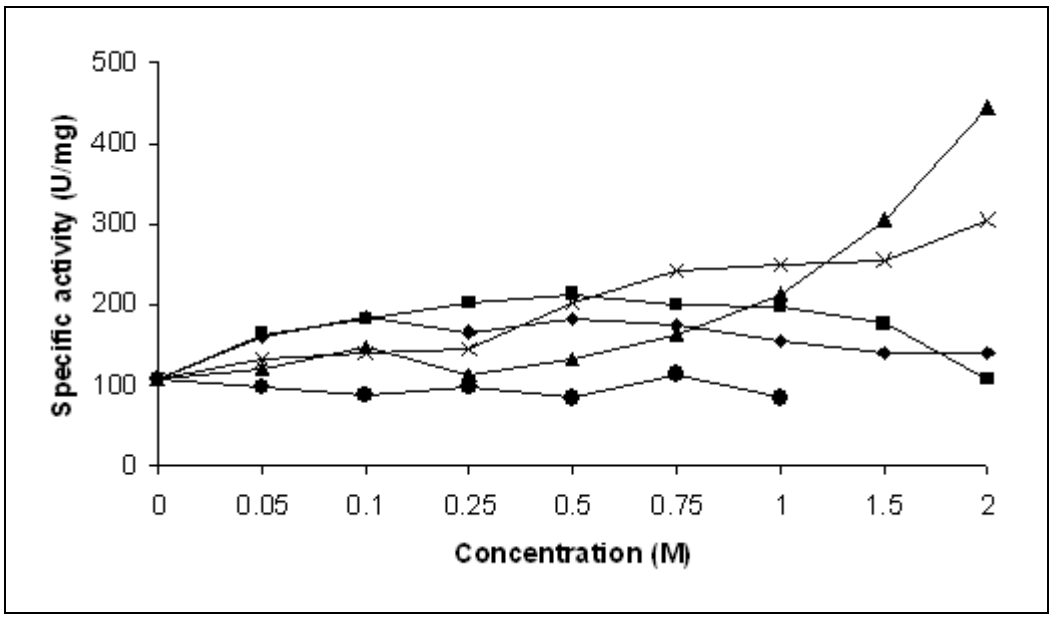

Fig. 3 (a& b): Effect of different salts on activity of chlorite dismutase isolated from *P. chloritidismutans*. Experiments were done at 25ºC and pH 6. The conversion of chlorite into chloride and oxygen did not alter the pH of the buffer solution. In Fig. 3A ■ represents specific activity with K_2HPO_4/ KH_2PO_4, ● is specific activity with Na_2HPO_4/ NaH_2PO_4, ▲ is specific activity with $NaClO_3$ while ✳ is specific activity with $NaNO_3$. While in Fig. 3B, ♦ represent specific activity with NaCl, ■ is specific activity with NH_4Cl, ▲ is specific activity with $(NH_4)_2SO_4$, ✕ is specific activity with Na_2SO_4 and ● represent the specific activity with $NaHCO_3$. All points are average of two and standard deviation at all the points is less than 10%.

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 performed with Cld in 10 mM and 0.5 M phosphate solution (both at pH 6). Both samples exhibited

identical axial high-spin ferric EPR signals, and no evidence of a low-spin ferric species was observed (not shown). Activity increase by phosphate therefore seems to be due to water structuring effects on the protein. Whether this effect of kosmotropic salts also holds for previously characterized Cld's is not known, but it may very well explain observed differences in V_{max} and K_m values.

No significant inhibition was observed in the presence of 1.5 M concentrations of sodium and ammonium salts of chloride (Fig. 3b), which is in contrast to the results for the cld of *D. aromatica* RCB where chloride has been found to be a mixed inhibitor (Streit & Dubois 2008).

Inhibition studies

Inhibition studies showed that cyanide and azide were effective inhibitors (Table 3). The Cld of *A. oryzae* GR-1 was, however, not inhibited by even 20 mM of azide (van Ginkel *et al*. 1996). In contrast, the Cld of *P. chloritidismutans* was not inhibited by hydroxylamine, while the Cld of strain GR-1 was. These differences cannot yet be explained. With respect to 3-amino-1,2,4-triazole, the Cld of strain GR-1 and *P. chloritidismutans* behaved similarly; both were not inhibited, whereas it is an effective inhibitor of catalases (Diaz & Wayne 1974; Jouve et al. 1983).

Involvement of water

In order to elucidate the mechanism of chlorite dismutation, the involvement of water in the dismutation reaction was investigated. ^{18}O labeled water was used and the evolution of oxygen with a mass of 32 (^{16}O - ^{16}O), or a mass of 34 (^{16}O - ^{18}O) or a mass of 36 (^{18}O - ^{18}O) was monitored using GC-MS. During all conditions tested, only a mass peak of *m/z* = 32 (^{16}O - ^{16}O) was measured, indicating that despite the presence of different concentrations of $H_2{}^{18}O$, water did not take part in the reaction. Hence, both oxygen atoms in molecular oxygen originate only from chlorite. These data correspond with the recent mechanistic analysis described by Lee et al. (2008).

In conclusion, *P. chloritidismutans* contains a heme-containing Cld, which is similar to other characterized Clds with respect to oligomeric structure, cell localization, and kinetic data. However, differences exist concerning the deviant inhibition pattern by cyanide, azide and chloride. For the first time we described the strong effect of kosmotropic and chaotropic salts on Cld kinetics. The data contribute to the understanding of this remarkable enzyme, being one of the few oxygen-generating enzymes in nature and the only one to form an O-O double bond next to photosystem II. The number of Cld-like sequences in various databases is increasing rapidly. Further purification and characterization of the corresponding enzymes is necessary to define the

boundary between catalytically active and substantially less-active 'pseudo'-Clds from non-chlorate-reducing bacteria.

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Chapter 5

Growth of *Pseudomonas chloritidismutans* AW-1^T on benzoate and chlorate via catechol and characterization of the pathway by proteogenome analysis

Farrakh Mehboob, Gosse Schraa, Sjef Boeren, Peter J Schaap, Alfons JM Stams

Abstract

Microbial chlorate reduction is a unique process, which releases molecular oxygen during the dismutation of chlorite. The oxygen thus formed may be used to degrade hydrocarbons by means of oxygenases. *Pseudomonas chloritidismutans* AW-1^T was found to grow on benzoate with chlorate or oxygen, but not with nitrate. The specific growth rates on benzoate and chlorate, and benzoate and oxygen were 0.2 $\pm$ 0.01 and 1.1 $\pm$ 0.04 day^{-1}, respectively. Catechol was detected as intermediate of benzoate degradation and the bacterium also grows with catechol. The key enzymes chlorate reductase, chlorite dismutase, benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase were detected. Benzoate1,2-dioxygenase, catechol 1,2-dioxygenase and other enzymes involved in benzoate degradation were exclusively detected in benzoate grown cells in comparison with acetate grown cells by LC-MS/MS analysis. The degradation of benzoate with oxygen and chlorate, but not with nitrate, detection of catechol as intermediate, biochemical activities of enzymes involved and confirmation of oxygenases in the benzoate grown cells proteome show that the strain employs oxygenase-dependent pathways for the breakdown of benzoate.

Introduction

A large variety of natural and anthropogenic compounds are aromatic (Fuchs 2008). The aerobic degradation of aromatic compounds is well understood (Harwood & Parales 1996; Vaillancourt et al. 2006). During aerobic degradation of aromatic compounds the aromatic ring is first modified by mono or dioxygenases. The dihydroxylated benzene ring containing compounds formed are central intermediates in the aromatics degradation pathway. The next step, the cleavage of the aromatic ring, is mediated by dioxygenases. The ring is either cleaved between the two hydroxyl groups, called ortho-cleavage (or intradiol cleavage), or cleaved adjacent to one of the hydroxyl groups, called meta-cleavage or extradiol cleavage (Harwood & Parales 1996; Vaillancourt et al. 2006). The breaking of the aromatic ring is energetically quite difficult in anoxic environments (Fuchs 2008). A common way of activation of the aromatic ring in an anoxic environment is often done through fumarate addition by a glycyl radical enzyme (Selmer et al. 2005; Fogt 2008). There are a few other mechanisms of anaerobic degradation, but they have been poorly characterized (Fuchs 2008).

Benzoate is an intermediate in the aerobic degradation of aromatic compounds like mandelate, toluene and benzene (Harwood & Parales 1996; Yerushalmi 2001). Benzoate has also been detected as intermediate during anaerobic benzene degradation under sulphate-reducing, methanogenic and iron-reducing conditions (Caldwell & Suflita 2000; Kunapuli et al. 2008). Moreover, benzoate is often used as a model aromatic compound for biodegradation studies (Cao et al. 2008).

Many soils that are polluted with hydrocarbons are anoxic. Introducing oxygen in such zones might help to destabilize the stable benzene ring, thus allowing the degradation of resulting intermediates anaerobically (Wilson & Bouwer 1997). The introduction of air or pure oxygen is not effective due to the low solubility of oxygen. Introduction of hydrogen peroxide (H_2O_2) might be toxic for some microorganisms (Morgen et al. 1993). Solid oxygen releasing compounds like oxides of CaO_2 and MgO_2, are costly and their even distribution is difficult (Weelink et al. 2008).

(Per)chlorate is a unique compound, which upon microbial reduction yields molecular oxygen (Rikken et al. 1996; Dudley et al. 2008). (Per)chlorate also has a reduction potential comparable with that of nitrate and oxygen (Coates & Achenbach 2004; Stams et al. 2004). It has been proposed as an alternative electron acceptor for the oxidation of hydrocarbons (Coates et al. 1999; Tan et al. 2006; Weelink et al. 2008).

A catechol 1,2-dioxygenase has been amplified from *Pseudomonas chloritidismutans* via PCR (Cladera et al. 2006). We screened the degradation capacity of this bacterium for various

aromatics and found that *Pseudomonas chloritidismutans AW-1*^{T}, a chlorate reducing bacterium, is capable of growth on benzoate with oxygen or chlorate as electron acceptor. This could be important for bioremediation applications, since this strain is also capable of alkane oxidation with oxygen and chlorate as electron acceptor (Mehboob et al. 2009a). To get insight in the degradation pathway(s) of aromatic compounds, we studied the differential expression of oxygenases involved in the aromatic degradation pathway in the whole proteome.

Materials and Methods

Inoculum, media and cultivation

Pseudomonas chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink et al. 2002). For the degradation experiments with nitrate, it was adapted to nitrate as described by Mehboob et al. (2009a).

The *P. chloritidismutans* strain AW-1^T was grown in the medium described by Mehboob et al. (2009a). The composition of the medium (in grams per liter of anaerobic demineralized water) was as follows: $Na_2HPO_4.2H_2O$, 3.48; KH_2PO_4 1; resazurin, 0.005; $CaCl_2$, 0.009; ammonium iron (III) citrate, 0.01; NH_4SO_4, 1; $MgSO_4.7H_2O$, 0.04. Vitamins and trace elements were added as described by Holliger et al. (1993) but supplemented with Na_2SeO_3, 0.06; $NaWO_4.2H_2O$ 0.0184. The pH of the medium was 7.3.

P. chloritidismutans strain AW-1^T was cultivated in 120-ml flasks containing 40 ml of medium at 30^{o}C. The medium was made in anaerobic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with butyl rubber stoppers and aluminum crimp caps, and the head space was replaced by N_2 gas (140 kPa). All solutions that were added to the medium were anaerobic and autoclaved at 121^{o}C for 20 minutes. Chlorate and nitrate were supplied from a 0.4 M stock solution to get a final concentration of 10 mM. The end concentration of benzoate and catechol was 2 mM. The aerobic degradation experiments were done in aerobic medium and no headspace flushing was done. The bottles with catechol were wrapped in aluminum foil to avoid photo-oxidation (Borraccino et al. 2001). For proteome analysis the bacteria were grown in 1-liter bottles with 600 ml of medium.

Preparation of cell free extracts

Cell free extracts of strain AW-1^T, grown in anaerobic medium with benzoate as sole carbon and energy source and chlorate as electron acceptor, were prepared anaerobically as previously described (Mehboob et al. 2009a). Cell free extracts were freezed in 12-ml serum vials until used.

The protein content of the cell free extract fraction was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Enzyme activity measurements

Chlorate reductase and chlorite dismutase activities were determined at 30°C with cell free extracts. Chlorate reductase activity was determined spectrophotometrically as described by Kengen et al. (1999), by monitoring the oxidation of reduced methyl viologen at 578 nm. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of chlorate per minute.

Chlorite dismutase activity was determined by measuring oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA) as described by Wolterink et al. (2002). One unit (U) of activity is defined as the amount of enzyme required to convert 1 μmol of chlorite per minute.

Benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase activity were measured spectrophotometrically. Benzoate 1,2-dioxygenase activity was determined as described by Yamaguchi & Fujisawa (1980) by measuring the decrease in absorbance of NADH at 340 nm. NADH oxidase activity without the benzoate addition was subtracted from the given activity. One unit (U) of activity is defined as the amount of enzyme required to convert 1 μmol of substrate per minute. Catechol 1,2-dioxygenase activity was determined as described by Cao et al. (2008) by measuring the increase in absorbance at 260 nm due to the formation of cis, cis-muconate. One unit (U) of activity is defined as the amount of enzyme required to form 1 μmol of *cis,cis*-muconate per minute.

Analytical techniques

Chlorate, chloride, nitrate and nitrite were measured, as described by Scholten and Stams (1995) after separation on a Dionex column (Ionpac AS9-SC) (Breda, The Netherlands), with a conductivity detector. Potassium fluoride (2 mM) was used as internal standard.

Oxygen was analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) as described previously (Mehboob et al. 2009a).

Total amount of inorganic carbon present inside the flask was calculated by using the Henderson-Hesselbach equation as described previously (Mehboob et al. 2009a). CO_2 was analyzed by gas chromatography on GC2014 gas chromatograph fitted with a TCD detector. The injector and detector temperatures were 60 and 130°C, respectively. CO2 was analyzed with a Poraplot Q column (Chrompack; 25 m length, 0.53 mm internal diameter; film thickness, 20 μm). Helium was the carrier gas at a flow rate of 15 ml min^{-1}, and the oven temperature was 33°C.

Benzoate and catechol were analyzed by HPLC as described by Weelink et al. (2008).

DNA extraction and sequencing

The DNA of *P. chloritidismutans* strain AW-1^T was extracted by the standard CTAB method of JGI. The 454 sequencing was done as described by Droege & Hill (2008).

Computational and statistical analysis

A six frame translation was used for analysis of proteomics data. Details of the proteomics analysis pipeline are described in chapter 6. Spectral counting was used for comparative quantification (Liu et al. 2004). A G-test was applied to see the significant differential expression (Sokal & Rohlf 1994). The minimum requirement for the G-test was that at least 5 peptides match for one protein under one condition.

Differential expression of the whole proteome

Sample preparation for tandem-MS

Cultures were analyzed for comparative analysis of the whole proteome. The bacterium was grown aerobically on acetate and benzoate. Equal amounts of each sample (250 µg) were separated on 12% SDS polyacrylamide gels, and gels were stained according to the manufacturer's protocol using Colloidal Blue Staining (Invitrogen, Carlsbad, CA, USA). Each of the two gel lanes was cut into five slices, and slices were cut into smaller pieces. After washing twice with ultra-pure water, gel samples were treated with 50 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 (pH 8.0) for 1h at 60°C. DTT solution was decanted and samples were alkylated with 100 mM iodoacetamide in NH_4HCO_3 (pH 8.0) for 1h at room temperature in the dark with occasional mixing. The iodoacetamide solution was decanted and samples were washed with NH_4HCO_3 (pH 8.0). Gel pieces were rehydrated in 10 ng/µl trypsin (Sequencing grade modified trypsin, Promega, Madison, WI, USA) and digested overnight at 37°C. To maximize peptide extraction, the solution from trypsin digest was transferred to new tubes, and gel pieces were subjected to two rounds of 1 min sonication, the first round with 5% trifluoroacetic acid (TFA) and the second round with 15% acetonitrile and 1% TFA. After each of these two rounds solutions were removed and added to the original trypsin digests.

Liquid chromatography tandem mass spectrometric analysis

Samples were analyzed on LC-MS/MS as described previously (van Esse et al. 2008).

Mass spectrometry database searching

The resulting spectra from the MS analysis were submitted to a local implementation of the OMSSA search engine (8). MS/MS spectra were searched against a peptide database derived

from a six frame translation of *Pseudomonas chloritidismutans* strain *AW-1*^{T} and a decoy reverse database constructed from the reverse of all the six frame translation output.

All OMSSA searches used the following parameters: a precursor ion tolerance of 0.2 Da, fragment ion tolerance of 0.3 Da, a missed cleavage allowance of up to and including 2, fixed carbamide methylation, variable oxidation of methionine and deamination of glutamine and asparagine.

The Expect value threshold was determined iteratively from the false discovery rate (FDR) and was set to 0.01. With this setting an FDR of < 5% is expected.

The FDR calculation was calculated as follows: peptide-spectrum matches (PSM) with each individual peptide database were ranked by their E-value for each identified spectrum with a threshold E-value < 0.01 and the top hit identified peptide sequence was selected. For FDR calculation, top hit spectral matches to peptides in the reversed database were taken and the number of false positives was divided by the number of total positives.

Nucleotide sequence accession numbers

The protein sequences obtained in this study will be deposited in the GenBank/EMBL/DDBJ.

Results

Benzoate degradation

Pseudomonas chloritidismutans AW-1^T used benzoate as a sole source of carbon and energy. Growth on benzoate and chlorate was shown by an increase in optical density (Fig. 1a). Benzoate degradation coincided with inorganic carbon formation, chlorate reduction and chloride formation (Fig. 1a, 1b). No growth was observed in controls without inoculum, without benzoate or without chlorate (results not shown). In bottles without an electron acceptor added a little bit of benzoate (less than 0.2 mM) was oxidized probably due to residual oxygen. Catechol was detected as intermediate in the benzoate degradation. During growth, the medium turned brownish. The specific growth rate on benzoate and chlorate was 0.2 $\pm$ 0.01 per day (doubling time 2.9 $\pm$ 0.2 days). The oxidation of 1.0 $\pm$ 0.1 mM of benzoate led to a reduction of 4.8 $\pm$ 0.4 mM of chlorate, the production of 6.3 $\pm$ 0.9 mM of bicarbonate and 3.8 $\pm$ 0.7 mM of chloride. The balance fits rather well with the theoretical stoichiometry of a complete oxidation of benzoate coupled to chlorate reduction:

$$C_7 H_5 O_2 Na + 5\ ClO_3^- + 4\ H_2O \rightarrow 7\ HCO_3^- + 5\ Cl^- + Na^+ + 6\ H^+ \quad (1)$$

Fig. 1a:

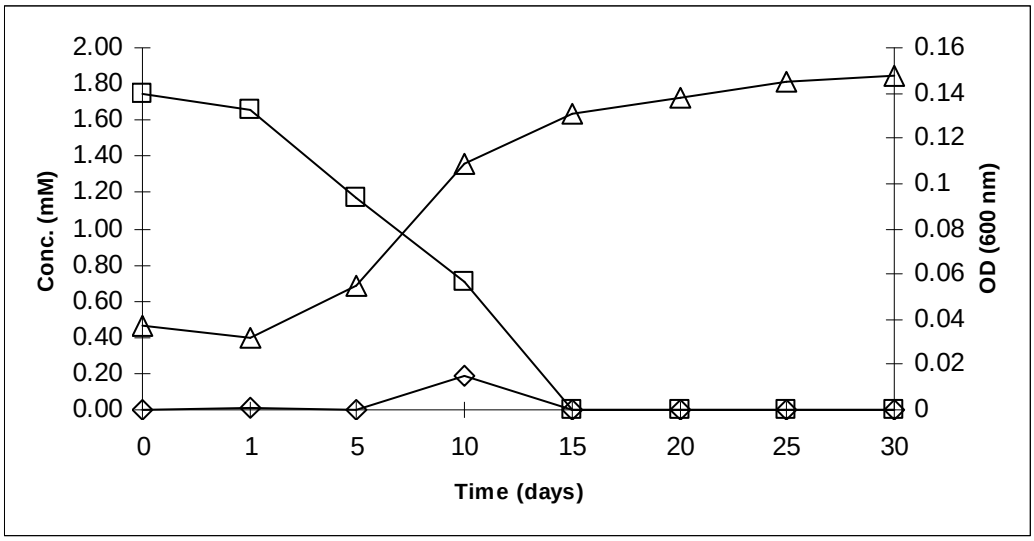


Fig. 1b:

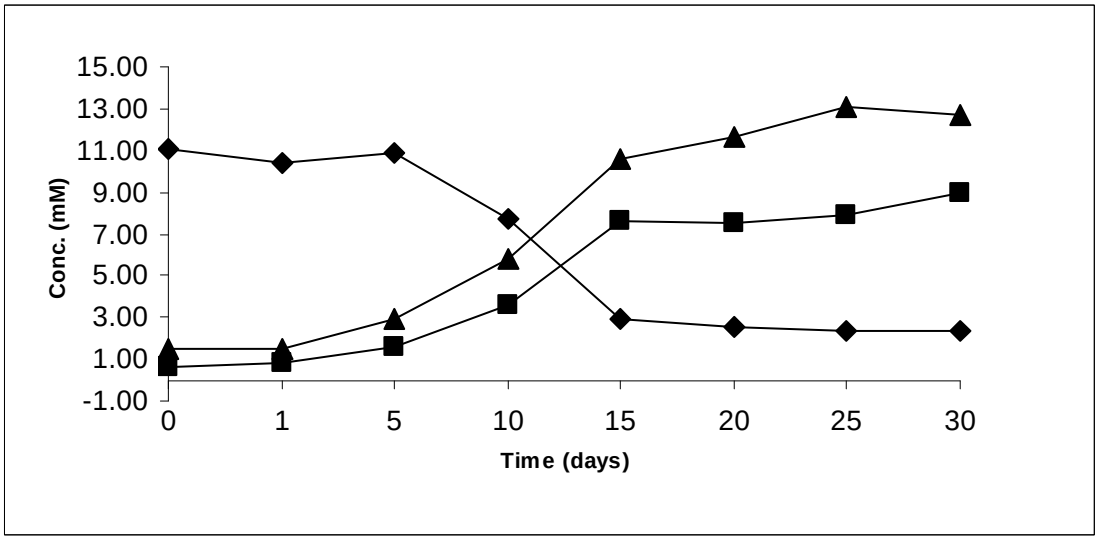


Fig 1: Growth of strain AW-1^T with benzoate and chlorate. In **(a):** ▫ benzoate, ◊ catechol and △ optical density at 600 nm. In **(b):** ♦ chlorate, ■ chloride and ▲ total inorganic carbon.

The bacterium also grew aerobically on benzoate (Fig. 2a). The increase in OD followed benzoate degradation. Catechol was also detected as an intermediate (Fig. 2a). Oxygen consumption and total inorganic carbon formation are shown in Fig. 2b. An unknown peak was seen which elutes before catechol. This highly polar compound could be dihydrodihydroxybenzoate or *cis,cis*-muconate (Reineke, personal communication). Since this peak stayed even after the catechol was degraded, it is likely *cis,cis*-muconate. The specific growth rate on benzoate and oxygen was 1.1 $\pm$ 0.04 per day (doubling time 0.6 $\pm$ 0.02 days).

Neither growth nor increase in total inorganic carbon was observed with benzoate and nitrate, using *P. chloritidismutans* inoculum adapted to grow on nitrate and acetate (results not shown).

Fig. 2a:

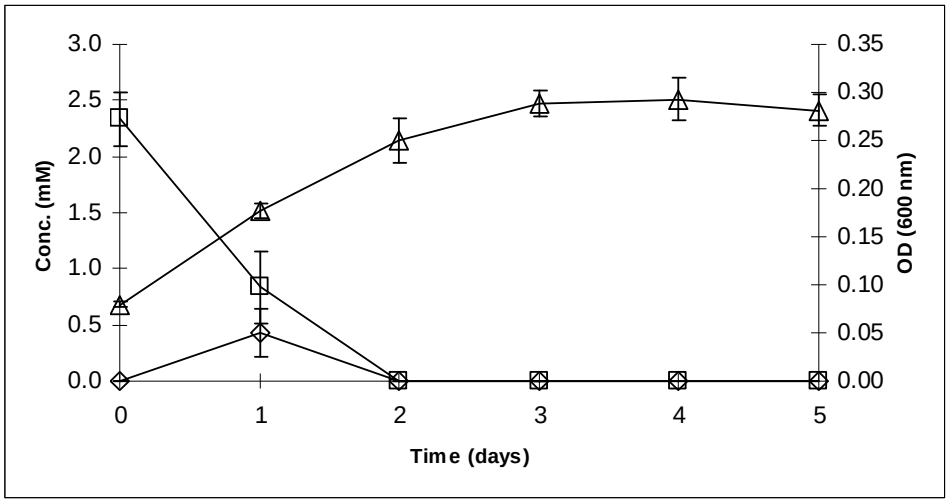


Fig. 2b:

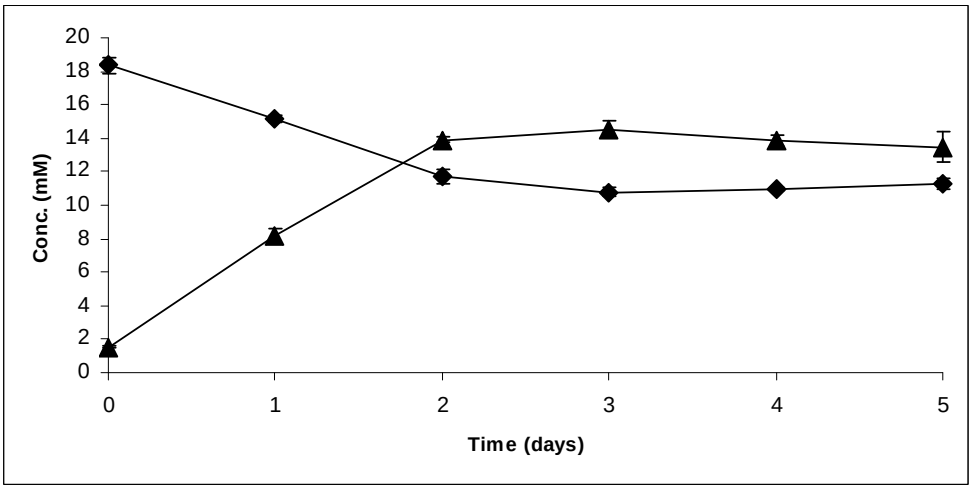


Fig. 2: Growth of strain AW-1^T with benzoate and oxygen. In **(a):** ▫ benzoate, ◊ catechol and △ optical density at 600 nm. In **(b):** ♦ oxygen consumed and ▲ total inorganic carbon.

Catechol degradation and screening of other aromatics

Apart from benzoate, *Pseudomonas chloritidismutans* AW-1^T used catechol with chlorate and oxygen, but not with nitrate. However, growth on catechol with chlorate was slower than growth on benzoate both aerobically and anaerobically. A small amount of CO_2 formation was also observed in the aerobic controls without inoculum, showing a slight abiotic oxidation of catechol as was also observed by Majcher et al. (2000).

Other substrates tested were benzene, toluene, aniline, phenol, xylenes, cresols, chloro-phenols, chloro-benzoates, aminobenzoates, benzaldehyde and mandelate. Except for benzaldehyde, no growth was found with these substrates. Among aromatic amino acids, growth was found with tyrosine and phenylalanine, but not with tryptophan.

Enzyme assays

Cell free extracts of cells grown on benzoate and chlorate showed chlorate reductase, chlorite dismutase, catechol 1,2-dioxygenase and benzoate 1,2-dioxygenase activity. The chlorate reductase activity was 11.3 $\pm$ 1.4 U/mg of protein, while a chlorite dismutase activity of 0.07 + 0.05 U/mg of protein was obtained. The benzoate grown cell free extract also showed 0.5 $\pm$ 0.1 U/mg of protein of benzoate1,2-dioxygenase and 0.01 $\pm$ 0.0 U/mg of protein of catechol 1,2-dioxygenase activity.

The low activity of catechol 1,2-dioxygenase was due to the accumulation of cis, cis-muconate. Due to its high absorbance at 260 nm it was not possible to add more cell free extract.

Differential expression of the whole proteome

The whole proteome of acetate grown cells was compared with that of benzoate grown cells. Overall, 91 proteins were significantly ($P<0.05$) up-regulated on benzoate while 33 were down-regulated. The number of peptides for each protein involved in benzoate and catechol degradation and which were only expressed during growth on benzoate are listed in Table 1.

Table 1: Number of peptides found for each protein, involved in benzoate catabolism and expressed only during growth on benzoate.

Protein	Number of peptides found
Benzoate dioxygenase, alpha subunit (*BenA*)	80
Benzoate dioxygenase, beta subunit (*BenB*)	11
Benzoate dioxygenase ferredoxin reductase component (*BenC*)	13
Benzoate-specific porin (*BenF*)	27
Benzoate MFS transporter (*BenK*)	10
1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (*BenD*)	33
Catechol 1,2-dioxygenase (CatA)	59
cis,*cis*-muconate cycloisomerase (CatB)	34
3-oxoadipate:succinyl-CoA transferase (*CatE*) subunit A	26
3-oxoadipate:succinyl-CoA transferase (*CatE*)subunit B	16
beta-ketoadipyl CoA thiolase (*CatF*)	38

Apart from those proteins some other proteins like two zinc dependent alcohol dehydrogenases, a TRAP transporter and a Ton-B dependent receptor were also induced during growth on benzoate.

Among the up-regulated proteins the multifunctional fatty acid oxidation complex, two other alcohol dehydrogenase, a long chain fatty acid transporter and outer membrane protein might be involved in the benzoate degradation. Amino acid metabolism genes also seem to be up-regulated. The expression of two alkyhydroperoxide reductases, a catalase and nitrous oxide reductase suggest a higher level of oxidative stress.

Discussion

Pseudomonas chloritidismutans AW-1^T is a gram-negative, facultative anaerobic, chlorate-reducing bacterium, which has been isolated on acetate and chlorate (Wolterink et al. 2002). It can also grow on alkanes with oxygen and chlorate (Mehboob et al. 2009a). A catechol 1,2-dioxygenase has been amplified through PCR from its genome (Caldera et al. 2006). However, the bacterium was never tested for growth on catechol. We tested its ability to grow on aromatics and found that, apart from using some aromatic amino acids (phenylalanine and tyrosine), strain AW-1^T is able to grow with benzoate and catechol with oxygen and chlorate, but not with nitrate as electron acceptor. This suggested the involvement of an oxygenase dependent pathway. Strain AW-1^T has the ability to grow in the absence of external oxygen on simple aromatics and their intermediates with chlorate as electron acceptor and source of oxygen. This might be of significance for bioremediation purposes.

The two (per)chlorate reducing strains *Dechloromonas aromatica* RCB and *Alicycliphilus denitrificans* BC are known to grow on aromatics with chlorate. *A. denitrificans* BC can grow on catechol, but not on benzoate. Though the *D. aromatica* RCB genome has a benzoate dioxygenase (Salinero et al. 2009), the bacterium was only shown to grow on benzoate with nitrate (Coates et al. 2001). Chlorate was not tested. Growth on the central intermediate of aromatics degradation (catechol) in the absence of external oxygen is important since there are only a few pure culture studies about anaerobic catechol degradation (Szewzyk and Pfennig 1987; Schnell et al. 1989; Kuever et al. 1993, Gorny and Schink 1994; Ding et al 2008). Growth on benzoate was slower with chlorate than with oxygen. The doubling time with benzoate and chlorate is 2.9 $\pm$ 0.2 days, which is much slower than the doubling time on benzoate and oxygen i.e. 0.6 $\pm$ 0.02 days. The reason can be the low activity of the chlorite dismutase enzyme.

During growth on benzoate and chlorate the colour of the medium turned brownish indicating the formation of catechol as intermediate. During benzoate degradation, another unknown highly polar compound eluted from the HPLC column. Because of the high absorbance of CFE at 260 nm, it is likely that it is *cis,cis*-muconate. *Cis,cis*-muconate accumulation has also been observed by other researchers and has been attributed to delayed expression of muconate cycloisomerase (Schmidt & Knackmuss 1984).

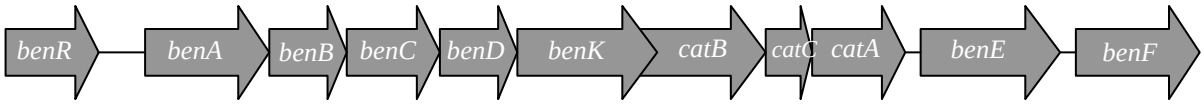
The measured chlorite dismutase activity in benzoate grown cells (0.07 U/mg of protein) is lower than measured for acetate grown cells (7.7 U/mg of protein). However, different salts and solvents may have an effect on the chlorite dismutase activity, as was found before (Mehboob et al. 2009b).

A comparison of the whole proteome of acetate and benzoate grown cells showed the induction of a whole operon containing benzoate and catechol catabolism genes (Table 1, Fig. 3). The clustering of benzoate and catechol metabolism genes points towards their functional coherence. The operon contains the three subunits of benzoate di-oxygenase (*benABC*), a benzoate specific porin (*benF*) and two benzoate specific transporters (*benE* and *benK*). The operon also encompasses a putative transcriptional activator (*benR*). In the same operon we found the dihydrodihydroxybenzoate dehydrogenase (*benD*) enzyme that forms catechol. The catechol 1,2-dioxygenase and two enzymes of beta-ketoadapiate pathway i.e. *cis,cis*-muconate lactonizing enzyme (*catB*) and muconolactone isomerase (*catC*) were also found in the same operon. With the help of mapping of proteins on the genomic contig we found the gene order presented in Fig. 3a.

Based on all the physiological features, enzyme measurements and induction of benzoate catabolism proteome, we propose a benzoate degradation pathway as shown in Fig. 3b. The enzymes, which were found to be induced in benzoate grown cells are shown in bold. We were unable to detect the muconolactone isomerase (*catC*) and beta-ketoadipate succinyl CoA transferase (*catD*) in the proteome but encoded by the genome. The reason might be that they have either a very short half life or they are required in relative low abundance. We suggest that oxygen formed in the dismutation of chlorite is used to convert benzoate to catechol and then catechol to *cis,cis*-muconate by the enzyme benzoate 1,2-dioxygenase and catechol 1,2 dioxygenase, respectively.

This is the first report about a bacterium that is able to degrade alkanes and aromatics in the absence of external oxygen, while generating oxygen via chlorite dismutation. For the degradation of each mole of benzoate only two moles of oxygen are needed by the oxygenases while the remaining five moles of oxygen can be used in respiration. Further research is needed to determine the exact fate of the extra oxygen produced.

3a:



3b:

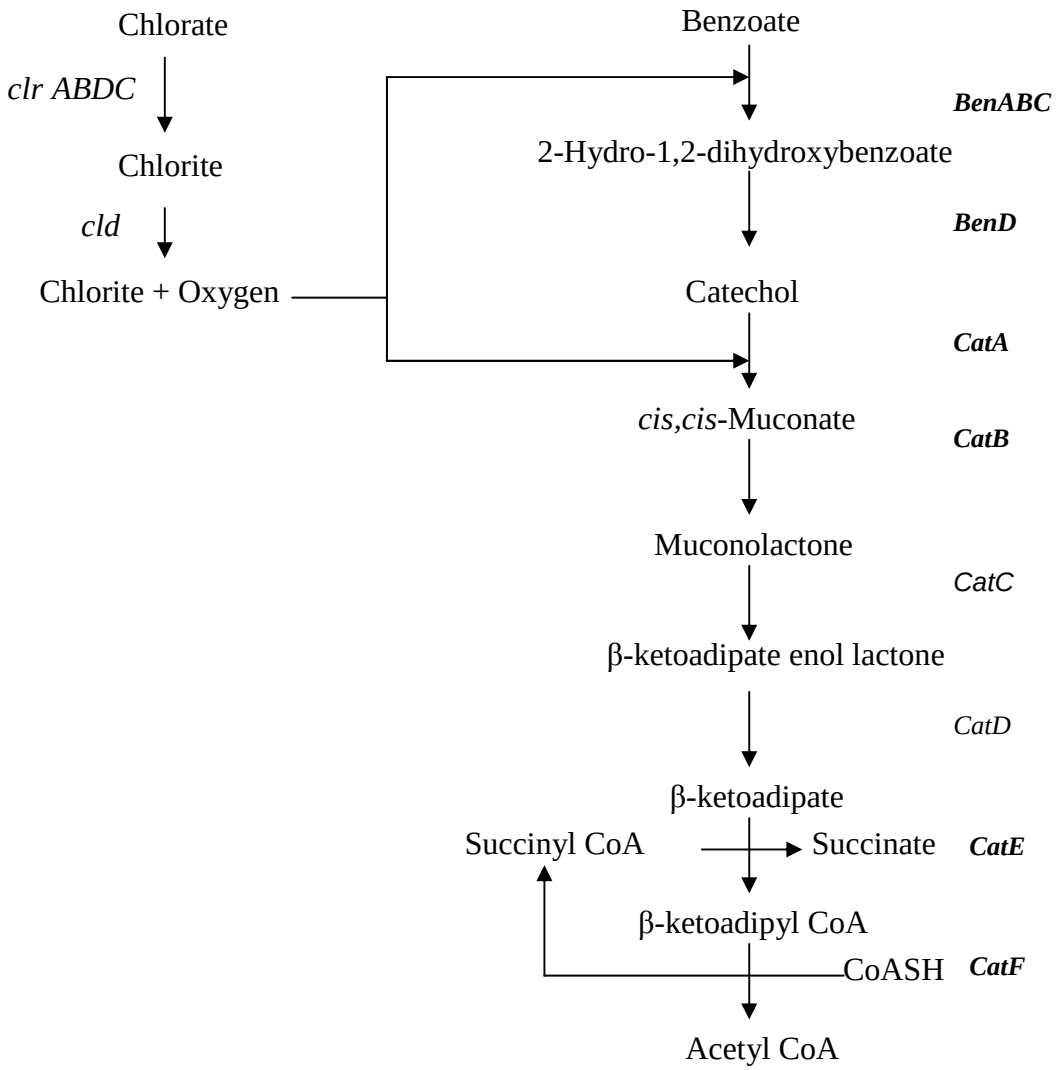


Fig. 3: (a) Benzoate and catechol catabolism operon. (**b**) **Degradation pathway of benzoate coupled to chlorate reduction**. The proteins, which were found to be expressed only in benzoate grown cells are shown in bold. *benABC/ BenABC*: benzoate 1,2- dioxygenase, *benD*/ B*enD* : dihydroxybenzoate dehydrogenase, *benE* and *benK:* benzoate specific transporters, *benF:* benzoate specific porin, benR: a putative transcriptional activator, *catA*/ *CatA*: catechol 1,2-dioxygenase, *catB*/ *CatB: cis,cis*-muconate lactonizing enzyme, *catC/ CatC:* muconolactone isomerase, *catD/ CatD:* beta-ketoadipate succinyl CoA transferase, *CatE*: 3-oxoadipate:succinyl-CoA transferase, *CatF:* beta-ketoadipyl CoA thiolase, *clrABDC*: chlorate reductase, *cld*: chlorite dismutase.

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Chapter 6

Proteogenomics of the alkane-oxidizing, chlorate-reducing bacterium *Pseudomonas chloritidismutans* AW-1^T under five different growth conditions

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Abstract

Pseudomonas chloritidismutans AW-1^T is a bacterium which is able to oxidize alkanes in the absence of externally supplied oxygen by using chlorate as a source of oxygen and as electron acceptor. The proteogenomic analysis of *Pseudomonas chloritidismutans* AW-1^T showed the versatility of this bacterium to adapt to different growth conditions. The enzymes involved in the alkane oxidation pathway were identified. Alkane monooxygenase was exclusively detected in alkane-oxygen-grown cells as well as alkane-chlorate-grown cells, indicating that under chlorate-reducing conditions an oxygenase mediated pathway is employed for degradation of alkanes. Up-regulation of *cbb3* type cytochrome oxidase in chlorate-grown cells shows the adaptive nature of the bacterium to low oxygen concentrations. Proteomic and biochemical data also showed that both chlorate reductase and chlorite dismutase are constitutively expressed and are up-regulated under chlorate-reducing conditions and down-regulated when grown with oxygen.

Introduction

Pseudomonas chloritidismutans AW-1^T, a gram-negative, facultative anaerobe was originally isolated from chlorate and bromate polluted wastewater (Wolterink et al. 2002). It is the only known microorganism that can degrade medium chain *n*-alkanes (C7-C12) in the absence of externally supplied oxygen by generating its own oxygen via chlorite dismutation. However, using primers that target different classes of oxygenases, the alkane oxygenase genes responsible for alkane oxidation could not be found (Mehboob et al. 2009).

With the advent of next generation sequencing technologies it is quite economical to sequence the genomes of microorganisms. Experimental validation of predicted genes via proteomics is currently the best option to identify protein coding genes (Ansong et al. 2008). MS-based proteomics corrects the genome annotation errors by discovering un-annotated novel genes, reversal of reading frames, determining the protein start and termination sites or programmed frame shifts, and validating the gene functions, and hypothetical open reading frames (Ansong et al. 2008, Armengaud 2009, Kyrpides 2009).

Insight into the biochemical mechanism of alkane degradation in two alkane degrading bacteria was obtained by two-dimensional gel electrophoresis (2-DE) combined with MALDI-TOF MS analysis (Sabirova et al. 2006, Feng et al. 2007). However, due to the hydrophobic nature of membrane proteins from alkane grown cells, a clear 2-DE image was difficult to obtain (Sabirova et al. 2006). One dimensional gel electrophoresis combined with nanoLC-MS/MS is more suitable for the analysis of membrane proteins (Bindschedler et al. 2009). In addition, it led to an increased number of detected proteins (Kim et al. 2009).

In this study we analyzed the genome and proteome of *Pseudomonas chloritidismutans AW-1^T*. The genome is compared with the most closely related *P. stutzeri* A1501. The bacterium was grown under 5 different growth conditions and a comparison of the whole proteome of cells grown on *n*-decane and acetate with different electron acceptors (oxygen, chlorate and nitrate) was analyzed. The results from 1 DE combined with nanoLC-MS/MS analysis are compared with biochemical data.

Materials and Methods

Strain, media and cultivation

Pseudomonas chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink et al. 2002). For experiments with nitrate, it was adapted to nitrate as described (Mehboob et al. 2009). *Pseudomonas stutzeri* strain A1501 (accession number 109869) was obtained from the Collection of Institute Pasteur (CIP) in Paris (France).

P. chloritidismutans strain AW-1^T was grown in the medium described by Mehboob et al. (2009). *P. chloritidismutans* strain AW-1^T was cultivated at 30^{o}C in 1-L flasks containing 600 ml of medium. The medium was made in anaerobic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with butyl rubber stoppers and aluminum caps, and the head space was replaced by N_2 gas (140 kPa). All solutions that were added to the medium were anaerobic and autoclaved at 121^{o}C for 20 minutes. Sodium salts of chlorate and nitrate were supplied from a 0.4-M stock solution to get a final concentration of 10 mM. The aerobic degradation experiments were done in aerobic medium and no head space flushing was done.

Preparation of cell free extracts

Cell free extracts of strain AW-1^T were prepared anaerobically as previously described by Mehboob et al. (2009). Cell free extracts were stored in 12-ml serum vials at -80^{o}C for proteomic analysis and anaerobically at -20^{o}C for biochemical analysis.

The protein content of the cell free extract fraction was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Enzyme activity measurements

Chlorate reductase, nitrate reductase and nitrite reductase activities were determined spectrophotometrically as described by Kengen et al. (1999). The substrate-dependent oxidation of reduced methyl viologen was monitored at 578 nm and 30°C. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of substrate per minute.

Chlorite dismutase activity was determined by measuring chlorite-dependent oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA) as described by Wolterink et al. (2002). One unit (U) of activity is defined as the amount of enzyme required to convert 1 μmol of chlorite per minute.

DNA extraction and sequencing

DNA of *P. chloritidismutans* strain AW-1^T was extracted by standard CTAB method of JGI. The 454 sequencing was done as described by Droege & Hill (2008).

Computational and statistical analysis

A six frame translation was used for analysis of the proteomics data. Spectral counting was used for comparative quantification (Liu et al. 2004). A G-test was applied to see the differential expression (Sokal & Rohlf 1994). The minimum requirement for the G-test was that at least 5 peptides match for one protein under one condition. Signal peptides were determined by SignalP 3.0 server (Bendtsen et al. 2004).

Differential expression of the whole proteome

The whole proteogenomic analysis flow sheet is summarized in Fig. 1.

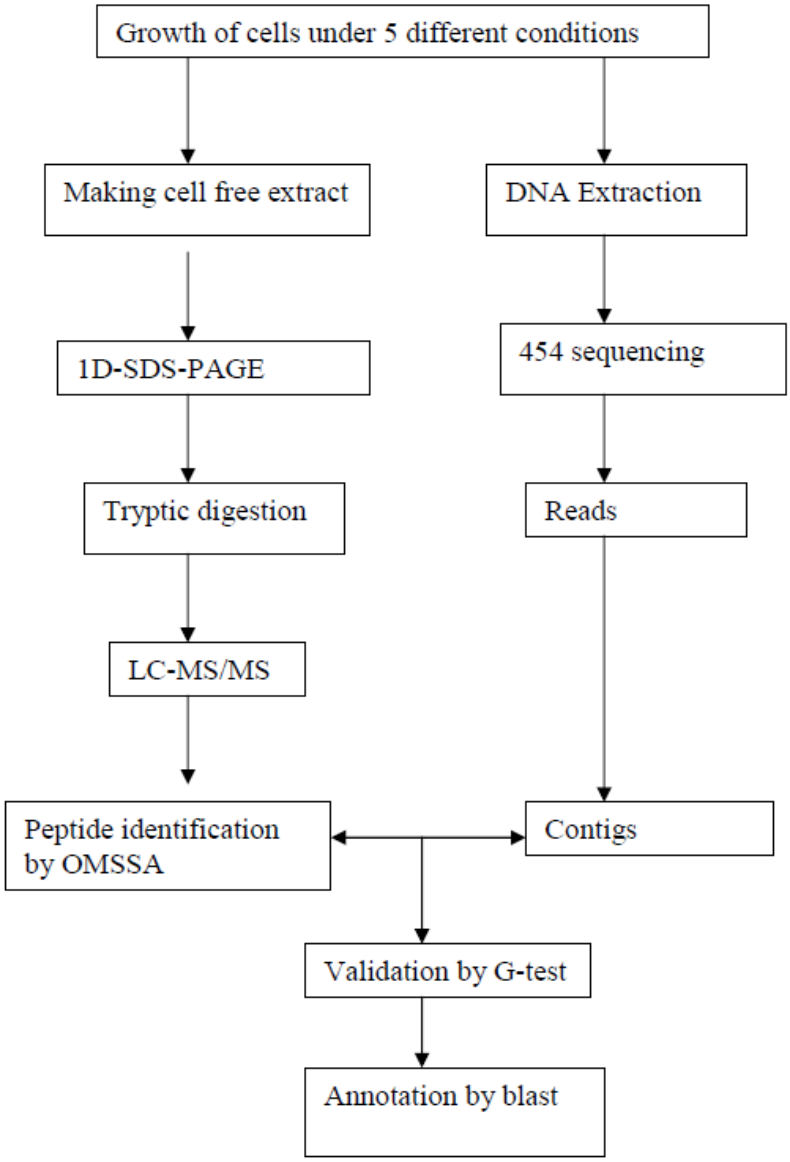

Fig. 1: Flow sheet diagram of proteogenomic analysis of *P. chloritidismutans*.

Sample preparation for tandem-MS

A comparative analysis of the proteome of cells grown under the 5 different conditions were made. Equal amounts of each sample (250 µg) were separated on 12%-SDS polyacrylamide gels, and gels were stained according to the manufacturer's protocol using Colloidal Blue Staining (Invitrogen, Carlsbad, CA, USA). Each of the two gel lanes was cut into five slices, and slices were cut into smaller pieces. After washing twice with ultra-pure water, gel samples were treated

with 50 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 (pH 8.0) for 1h at 60°C. DTT solution was decanted and samples were alkylated with 100 mM iodoacetamide in NH_4HCO_3 (pH 8.0) for 1h at room temperature in the dark with occasional mixing. The iodoacetamide solution was decanted and samples were washed with NH_4HCO_3 (pH 8.0). Gel pieces were rehydrated in 10 ng/µl trypsin (sequencing grade modified trypsin, Promega, Madison, WI, USA) and digested overnight at 37°C. To maximize peptide extraction, the solution from trypsin digest was transferred to new tubes, and gel pieces were subjected to two rounds of 1 min sonication, the first round with 5% trifluoroacetic acid (TFA) and the second round with 15% acetonitrile and 1% TFA. After each of these two rounds, solutions were removed and added to the original trypsin digests.

Liquid chromatography tandem mass spectrometric analysis

Samples were analyzed on LC-MS/MS as described previously (van Esse et al. 2008).

Mass spectrometry database searching

The resulting spectra from the MS analysis were submitted to a local implementation of the OMSSA search engine (Geer et al. 2004). MS/MS spectra were searched against a peptide database derived from a six frame translation of *Pseudomonas chloritidismutans* strain *AW-1*^{T} and a decoy reverse database constructed from the reverse of all the six frame translation output.

All OMSSA searches used the following parameters: a precursor ion tolerance of 0.2 Da, fragment ion tolerance of 0.3 Da, a missed cleavage allowance of up to and including 2, fixed carbamide methylation, variable oxidation of methionine and deamination of glutamine and asparagine.

The Expect value threshold was determined iteratively from the false discovery rate (FDR) and was set at 0.01. With this setting an FDR of < 5% is expected.

The FDR was calculated as follows: peptide-spectrum matches (PSM) with each individual peptide database were ranked by their E-value for each identified spectrum with a threshold E-value < 0.01 and the top hit identified peptide sequence was selected. For FDR calculation, top hit spectral matches to peptides in the reversed database were taken and the number of false positives was divided by the number of total positives.

Nucleotide sequence accession numbers

The nucleotide and protein sequences obtained in this study will be deposited in the GenBank/EMBL/DDBJ.

Results

Whole genome shotgun sequencing of the *P. chloritidismutans* AW-1^T resulted in 6.7 Mbp assembled sequence data. Out of this 6.7 Mbp, 4.4 Mbp was assembled in 115 contigs larger than 10 kbp. A six frame naive translation of the sequenced genome of *P. chloritidismutans* AW-1^T gave 14142 putative ORFs, which were used for further proteomic analysis. Overall the GC contents of the sequenced genome was 63.1%. Genome annotation on GeneMark.hmm (Lukashin & Borodovsky 1998) detected 6,762 putative ORFs larger than 100 amino acids, which are more than the 4,146 annotated genes of *P. stutzeri* A1501. This suggests that *P. chloritidismutans* has one of the biggest *Pseudomonas* genomes characterized so far. A comparison with the most closely related fully sequenced genome of *P. stutzeri* A1501 (Yan et al. 2008) shows that in *P. chloritidismutans* AW-1^T the complete operons for nitrogen fixation, putative arsenate reduction, cellulose synthesis, type IV fimbrial biogenesis, nitrate dependent formate dehydrogenase, chromate resistance and transport genes and CRISPR associated family proteins are missing. On the other hand, *P. stutzeri* A1501 lacks the genes coding for all the subunits of chlorate reductase, chlorite dismutase and alkane-1-monooxygenase. We tested and found that indeed *P. stutzeri* A1501 is unable to grow on *n*-decane and also cannot reduce chlorate or perchlorate.

One dimensional gel electrophoresis of cell free extracts of *P. chloritidismutans* grown under 5 different conditions was conducted (Fig. 2). Proteomic analysis revealed the proteins that were expressed at each condition. Overall out of 118,802 spectra obtained, 46,581 peptides were identified over the 5 conditions with a peptide spetrum match efficiency of 39.2%. The number of spectra obtained and the number of peptides identified for each growth condition are listed in Table 1.

Table 1: The number of spectra obtained and the number of peptides identified for each growth condition.

	Acetate + Oxygen	**Acetate + Chlorate**	**Decane + Oxygen**	**Decane + Chlorate**	**Acetate + Nitrate**	**Total**
Spectra	21988	19580	25214	26792	25228	118802
Number of peptides identified	8650	7796	8973	10760	10353	46533

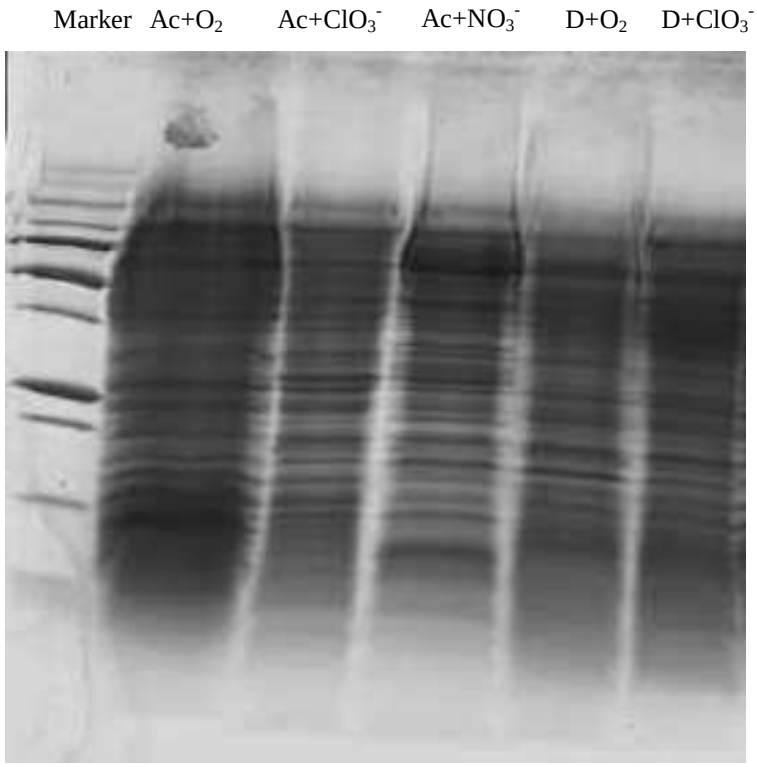

Fig. 2: Crude cell free extract of *P. chloritidismutans* AW-1^T 1-DE under 5 different growth conditions. Gels were overloaded to cover the maximum proteome

Differential expression of proteins involved in *n*-alkane degradation

A total of 177 proteins were differentially expressed in *n*-decane and chlorate versus acetate and chlorate, grown cells. Out of these 177 proteins, 32 proteins were only expressed in decane-grown cells while 83 proteins were significantly ($P<0.05$) up-regulated when grown with decane. While comparing alkane and oxygen- and acetate and oxygen-grown cells a total of 259 proteins were differentially expressed. 46 proteins were exclusively present in alkane-grown cells and another 112 poteins were up-regulated. Table 2 shows the number of peptides found for the enzymes involved in the oxidation of alkane. From Table 2 it is clear that an alkane-1-monooxygenase, 8 out of 10 alcohol dehydrogenases and 2 out of the 4 aldehyde dehydrogenases are exclusively found in decane-grown cells while the remaining proeteins are up-regulated. Among the β-oxidation enzymes one out of two acyl CoA dehydrogenase, AMP-dependent synthetase and ligase, two out of three enoyl CoA hydratases and L-3 hydroxyacyl dehydrogenase were found exclusively in decane-grown cells. The β-ketothiolase was also found to be up-regulated.

Table 2: Differential expression of proteins involved in initial oxidation of *n*-alkanes and subsequent β-oxidation

Enzymes (gene #)	Acetate + Oxygen	Acetate + Chlorate	Decane + Oxygen	Decane + Chlorate
Oxidation of alkane				
Alkane-1-monooxygenase (136900)	0	0	14	17
Alcohol dehydrogenase (137184)	0	0	31	25
Short chain alcohol dehydrogenase (125520)	0	0	6	12
Alcohol dehydrogenase (93630)	0	0	0	9
Short chain alcohol dehydrogenase (155080)	0	0	0	5
Alcohol dehydrogenase Zn containing (113655)	0	0	5	0
Alcohol dehydrogenase (107190)	0	0	6	0
Alcohol dehydrogenase Zn containing (86361)	0	0	11	0
Alcohol dehydrogenase quinoprotein (84317)	2	0	28	0
Alcohol dehydrogenase Zn containing (88444)	0	5	8	15
Alcohol dehydrogenase (134297)	3	0	10	0
Aldehyde dehydrogenase (28668)	0	0	0	7
Aldehyde dehydrogenase (133138)	0	0	14	0
Aldehyde dehydrogenase (33979)	1	0	9	0
Aldehyde dehydrogenase (99095)	4	0	21	0
β-oxidation				
Acyl CoA synthetase (122243)	2	4	26	17
Acyl CoA dehydrogenase (17036)	0	0	5	0
Acyl CoA dehydrogenase like 125344	0	0	25	24
AMP-dependent synthetase and ligase (125436)	0	0	8	34
Enoyl CoA hydratase (101528)	0	0	0	5
Enoyl CoA hydratase (90735)	0	1	0	17
Enoyl CoA hydratase (2008)	0	0	5	0
Multifunctional fatty acid oxidation complex subunit alpha (95559)	11	14	110	130
L-3 hydroxyacyl dehydrogenase (102464)	0	0	0	5
β-Ketothiolase (95560)	4	6	32	25
β-Ketothiolase (103700)	1	0	8	0

Apart from these proteins which are directly involved in the degradation of alkanes some other proteins were also regulated. Transport proteins i.e. 5 porins and 6 TonB-dependent outer membrane receptors were found to be up-regulated in alkane and oxygen grown culture. An organic solvent tolerant protein was also found in decane and chlorate grown cells. A sensor histidine kinase/ response regulator was also found to be up-regulated. Trehalose phosphate synthase, trehalose synthase were up-regulated in alkane-grown cells. Other enzymes like malic enzyme, phosphoenolpyruvate carboxylase and glutamate dehydrogenase, glycosyl hydrolase and α-amylase were also up-regulated.

Differential expression of proteins involved in electron acceptor utilization and enzyme activities

A total of 65 proteins were up-regulated during growth on acetate and chlorate compared with acetate and oxygen. Sixteen of these proteins were exclusively expressed in chlorate-grown cells. Similarly a total of 56 proteins were up-regulated in cells grown with *n*-decane and chlorate in comparison with *n*-decane and oxygen and among these 7 were only present when grown with chlorate. All the proteins which were up-regulated are presented in Table 3.

Table 3: Number of peptides detected for each protein and experimentally determined activity of enzymes grown under different conditions

	Protein	**Acetate + Oxygen**	**Acetate + Chlorate**	**Decane + Oxygen**	**Decane + Chlorate**	**Acetate + Nitrate**
Number of peptides	Chlorate reductase (alpha subunit)	14	81	47	143	48
	Chlorate reductase (beta subunit)	10	25	18	40	13
	Chlorate reductase (gamma subunit)	4	15	3	11	0
	Chlorate reductase (delta subunit)	1	10	2	4	2
Activity (U/mg)	Chlorate reductase	1.7 ± 0.1	21.3 ± 0.1	0.47 ± 0.04	46.3 ± 3.2	10.6 ± 0.9
Number of peptides	Chlorite dismutase (134786)	24	97	5	111	23
	Chlorite dismutase (127405)	8	10	12	27	26
Activity (U/mg)	Chlorite dismutase	0.15 ± 0.01	6.8 ± 1.2	0.25 ± 0.0	7.4 ± 2.1	7.9 ± 1.5
Number of peptides	Nitrate reductase (catalytic subunit)	3	16	20	28	28
Activity (U/mg)	Nitrate Reductase	bdl	bdl	bdl	bdl	0.73 ± 0.10
Number of peptides	Cyt *cbb3* oxidase	1	5	0	8	0

bdl: below detection limit

Apart from the proteins listed in Table 2, an oxygen-independent coproporphyrinogen III oxidase, a molybdenum ABC transporter, a filamentation induced by cAMP protein Fic, an UDP-N-acetylmuramate-alanine ligase, ClpB protein ATP dependent chaperone were also up-regulated when grown with decane and chlorate. One ABC transporter, TonB-dependent receptor, DNA-binding stress protein, siderophore biosynthesis protein, an ubiquinol-

cytochrome-*c* reductase and an iron ABC transporter were found to be up-regulated in acetate and chlorate-grown cells.

Activities of the enzymes i.e. chlorate reductase, chlorite dismutase and nitrate reductase of cells grown under different growth conditions are also given in Table 3. Nitrate reductase activity also includes the nitrite reductase activity. Nitrite reductase activity was 0.06 $\pm$ 0.01 U/mg of protein with acetate and nitrate grown cells while it was 2.52 $\pm$ 0.42 in acetate and chlorate grown cells.

Discussion

n-alkane degradation

P. chloritidismutans AW-1^T is known to degrade *n*-alkanes via an oxygenase mediated pathway, but previously we were unable to amplify any alkane oxygenase gene using various primers targeting different classes of alkane oxygenases (Mehboob et al. 2009). By looking at Table 1 it is evident that an alkane-1-monooxygenase is exclusively expressed when the bacterium is grown with *n*-alkanes. The sequence of alkane-1-monooxygenase obtained was 76% identical with the putative alkane-1-monooxygenase of *P. mendocina* ymp and 39% identical with the alkane-1- monoxygenase of *P. aeruginosa* PAO1. Only one alkane-1-monooxygenase was found irrespective of growth with oxygen or chlorate confirming our previous conclusion that oxygen released during chlorite dismutation is used by the alkane oxygenase to form an alcohol (Mehboob et al. 2009). We were unable to find rubredoxin encoded by genome, in the proteome probably due to its small size. We did not found any other oxygenase or cytochrome P450, which may be involved in alkane degradation as found by Sabirova et al. (2006).

We were unable to find an alcohol oxygenase in the proteome as we postulated before (Mehboob et al. 2009). Instead we found 10 different alcohol dehydrogenases to be either exclusively expressed or up-regulated during growth on *n*-alkanes. Two of these alcohol dehydrogenase are expressed both during growth on alkanes with either oxygen or chlorate, showing the similarity between the second step. But some differences were also found. Two of these alcohol deydrogenases are only expressed when cells are grown with alkanes and chlorate, while 3 of them are only expressed during growth with alkanes and oxygen. In contrast to the study on *Alcanivorax borkumensis* (Sabirova et al. 2006) and *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007) we found a variety of isozymes for alcohol dehydrogenase and aldehyde dehydrogenase expressed under different conditions showing the adaptability of *P. chloritidismutans*. Similarly, four aldehyde dehydrogenases were also differentially expressed. One was alkane and chlorate specific while three others were specific for growth on alkanes and oxygen.

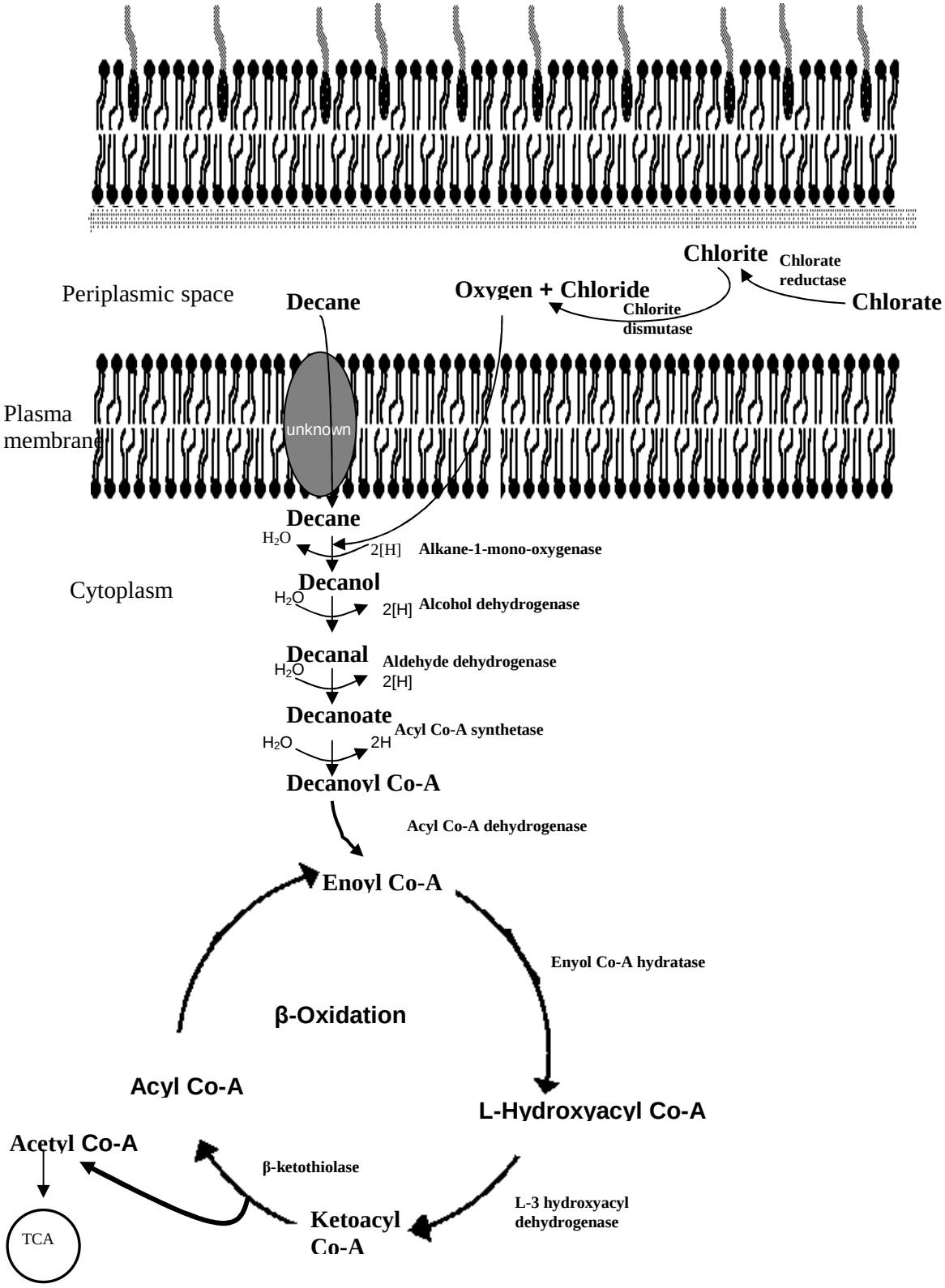

Fig 3: Degradation pathway of n-decane coupled with chlorate. All the enzymes shown in the pathway were either induced or up-regulated during growth on *n*-decane.

Apart form these proteins which are involved in direct alkane degradation some other proteins involved in alkane degradation are also up-regulated. Like Sabirova et al. (2006), we found some transport proteins, like large porin proteins which allow the passive diffusion of specific proteins are up-regulated. Similarly, TonB-dependent outer membrane proteins which can take up poorly soluble compounds with high affinity via energy-dependent process into the periplasmic space are also up-regulated. An up-regulation of some stress related proteins was also found. An organic solvent tolerant protein was also found in decane and chlorate grown cells, which is apparently helping the bacterium to cope with the harmful effect of *n*-alkanes. Due to the stress faced due to alkanes another sensor histidine kinase/ response regulator which enables bacteria to sense and adapt to a stress or growth condition was also found to be up-regulated during growth on alkanes. Instead of polyhydroxyalkanoate production (Sabirova et al. 2006) we found up-regulation of enzymes involved in trehalose synthesis. Trehalose is not only a storage molecule but also a stress response factor for environmental stimuli and helps to retain cellular integrity by presumably preventing the denaturation of protein (Jain & Roy 2008). An up-regulation of the glyoxylate pathway is suggested by up-regulation of malate synthase. This has also been observed by the (Sabirova et al. 2006; Feng et al. 2007). Due to unknown reasons enzymes involved in carbohydrate metabolism like glycosyl hydrolase and α-amylase were also up-regulated. This was not observed by others. Like Sabirova et al. (2006) we also observed an up-regulation of pilus assembly protein. However, unlike Sabirova et al. (2006) we did not find any up-regulation of fatty acid and phospholipid synthesis.

Electron acceptor utilization

The sequences of all the subunits of chlorate reductase were matching the N-terminal sequences found before (Wolterink et al. 2003). The arrangement of the chlorate reductase genes was found to be similar to *Ideonella dechloratans*, i.e. *clrABDC* (Danielsson et al. 2003). A transposase was found downstream of the chlorate reductase genes, which points towards the lateral transfer of the gene. A NCBI blast analysis (Altschul 1990) shows that molybdopterin containing alpha subunit of chlorate reductase (*ClrA*) is 70% identical to the alpha subunit of dimethylsulfide dehydrogenase (DMSO) of *Citreicella* sp. SE45 and 44% identical with the *ClrA* of *Ideonella dechloratans*. Similarly, the Fe-S cluster containing the beta subunit of chlorate reductase (*ClrB*) of *P. chloritidismutans* is 82% identical with the beta subunit of nitrate reductase of *Sagittula stellata* E-37 and 59% identical to *ClrB* of *Ideonella dechloratans.* The gamma subunit of chlorate reductase (*Clrc*) has 54% identity with the hypothetical protein of *Sagittula stellata* E-37 and only 34% identity with the *Clrc* of *Ideonella dechloratans.* The delta subunit of chlorate reductase *(ClrD)* has 46% identity with protein *DdhD* of *Citreicella* sp. SE45 and 40% with

ClrD Ideonella dechloratans. The *ClrD* is not part of the mature enzyme, but is a chaperone required for the assembly of the enzyme (*ClrABD*) (Danielsson et al. 2003; Bender et al. 2005).
A signal peptide was detected in the *ClrA* and *ClrC,* but not in the *ClrB* and *ClrD.* An alignment of the alpha subunit of chlorate reductase of *P. chloritidismutans* with the other related enzymes shows a twin-arginine motif with a consensus (S/T)-R-R-x-F-L-K motif (Fig. 4) which is involved in export of the assembled enzyme across the cell membrane via Tat pathway (Berks 1996, Danielsson 2003). This is in contrast to the findings of Wolterink et al. (2003) where based on the maximum activity the cytoplasmic localization of chlorate reductase of *P. chloritidismutans* was proposed. We also found an 8.6 kDa periplasmic cytochrome in the genome which has the motif (KLVGPxxKDVAAK) found in the 6 kDa *c* cytochrome. This cytochrome is able to donate electron for chlorate reduction (Backlund et al. 2009). Due to the presence of a conserved twin arginine motif for Tat pathway, presence of signal peptide in alpha, gamma and cytochrome we suggest that the chlorate reductase of *P. chloritidismutans* is periplasmic. Based upon the these findings about localization of chlorate reductase, differential expression of subunits of chlorate reductase, chlorite dismutase, ubiquinol-cytochrome-*c*-reductase, cytochrome *cbb3* oxidase and presence of an 8.6 kDa cytochrome in genome, we propose the respiratory pathway as presented in Fig. 5.
Table 3 shows that the enzymes involved in chlorate reduction i.e. chlorate reductase and chlorite dismutase are constitutive in nature and a basal level of protein and their activity is always present. An up-regulation of the all the subunits of chlorate reductase and chlorite dismutase, and increase in activity was observed during growth with chlorate. A down-regulation of all the subunits of chlorate reductase and chlorite dismutase and hence a decrease in activity was observed during growth with oxygen.
Though transcriptional analysis has already shown that chlorite dismutase is constitutively expressed at a basal level and is negatively regulated by oxygen (Bender et al. 2002), the (per)chlorate reductase was found to be transcribed only in the anaerobic conditions with (per)chlorate (Bender et al. 2005). Our results are in marked contrast to the findings of Bender et al. (2005), but are in agreement with the previous findings that although the chlorate reductase of *P. chloritidismutans* is oxygen sensitive (Wolterink et al. 2003), the bacterium can simultaneously reduce chlorate and oxygen when oxygen is added to a chlorate-reducing culture (Wolterink et al. 2002). Similarly, the chlorate reductase of *Pseudomonas* sp. PDA is reported to be constitutive in nature (Steinberg et al. 2005). An up-regulation of proteins and higher activity were observed when grown with decane and chlorate as compared with acetate and chlorate.

Only the catalytic subunit of nitrate reductase was found in all conditions within proteome data. However, nitrate reductase activity was only observed during growth on nitrate. The nitrite reductase activity was present in both nitrate-grown and chlorate-grown cells. The nitrite reductase activity was even higher in chlorate-grown cells than on nitrate-grown cells. Detection of catalytic subunit of periplasmic nitrate reductase and activity shows that it is not a fortuitous activity of chlorate reductase, but that there are two separate systems for chlorate and nitrate reduction.

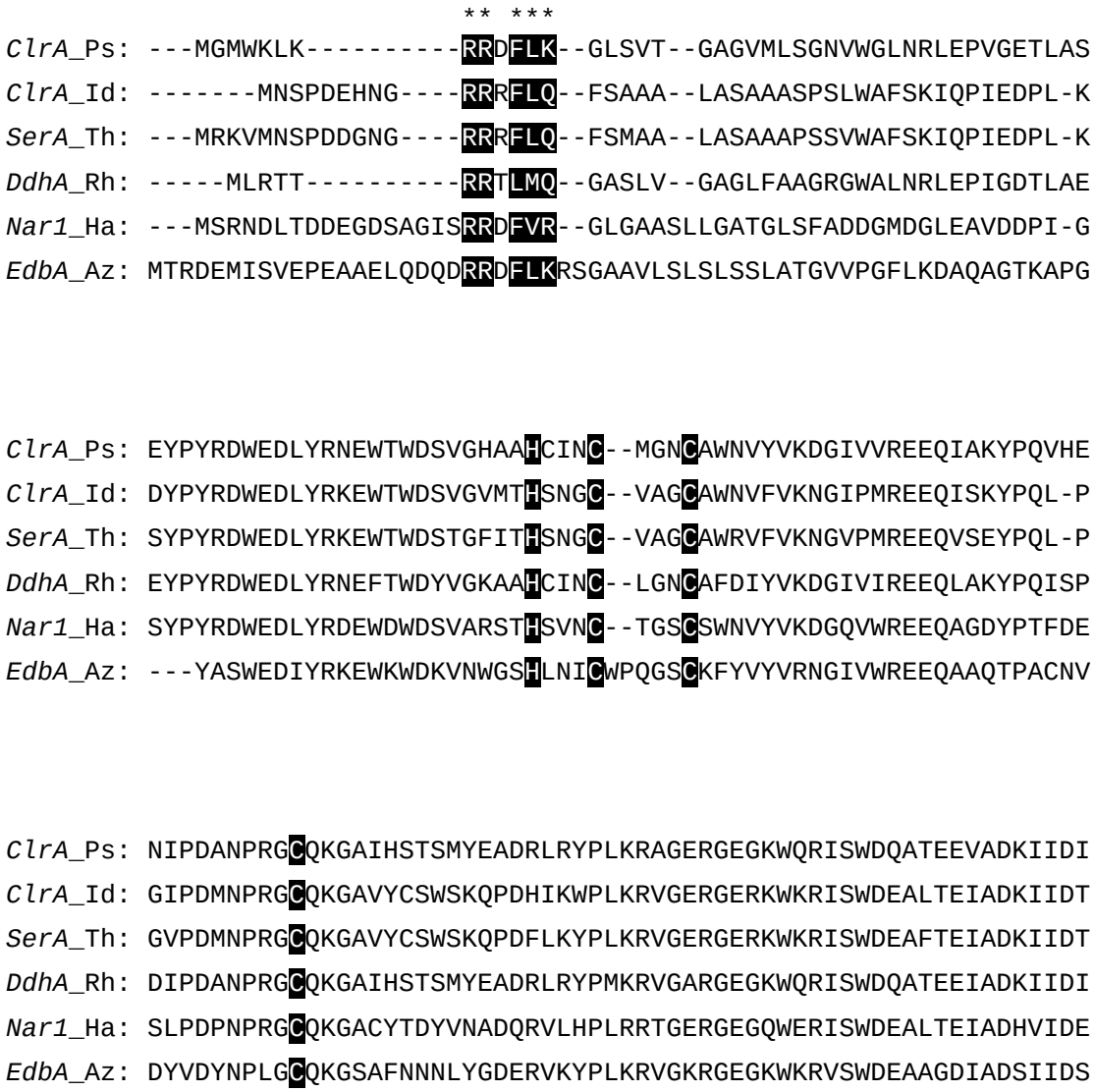

Fig 4: Alignment of alpha subunit of chlorate reductase (*ClrA*) of *Pseudomonas chloritidismutans*, *ClrA* of *Ideonella dechloratans* (AJ566363), selenate reductase (*SerA*) of *Thauera selenatis* (AJ007744), dimethylsulfide dehydrogenase (*DdhA*) of *Rhodovulum sulfidophilum* (AF453479), nitrate reductase (*Nar1*) of *Haloarcula Marismortui* (AJ277440) and ethylbenzene dehydrogenase (*EbdA*) of *Azoarcus* sp. EB1 (AF337952). Asteriks and shaded residues represent the twin arginine motif and other conserved residues of twin-arginine motif. The characteristic conserved N-terminal motif of type II DMSO reductases are also highlidhted. Corresponding gene names and accession numbers are shown in parenthesis. Only first 180 amino acids are shown.

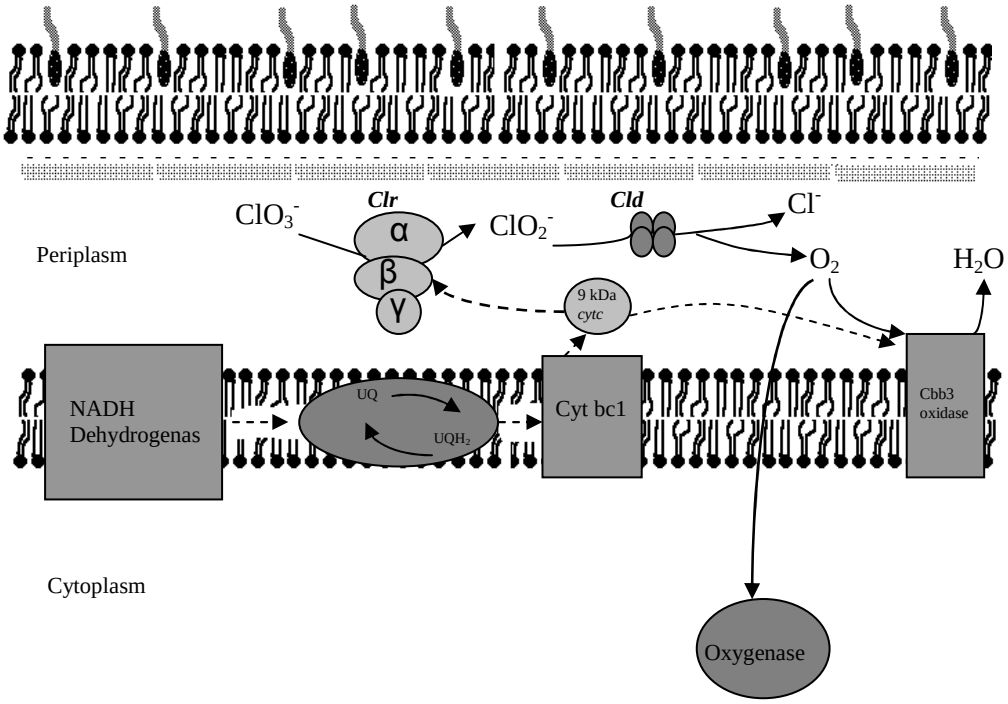

Fig. 5: Proposed respiratory pathway in *Pseudomonas chloritidismutans* illustrated with the help of proteogenomics data. The dotted lines show the electron transfers.

We found two different chlorite dismutase like sequences inside the proteome. The sequence of one chlorite dismutase (134786) was 100% identical to the previously known sequence found by Cladera et al. (2006), while the second protein (127405) has highest similarity (34% identical) with chlorite dismutase of *Dechloromonas aromatica* RCB and is only 30% identical with the already know one. The peptide count shows that the first chlorite dismutase (134786) is up-regulated when growth with chlorate, but the other one has a constitutive expression under anaerobic conditions.

Apart from these enzymes a very interesting finding was the up-regulation of cytochrome *cbb3* oxidase. The *cbb3*- type oxidases are unique heme copper oxidases, which have 6-8 fold lower K_m value for oxygen than *aa3*-type cytochrome *c* oxidases (Preisig et al. 1996). Their high affinity for oxygen allows the bacteria to colonize oxygen limited environments (Pitcher & Watmough 2004). The up-regulation of cytochrome *cbb3* type oxidase during chlorate grown condition shows the adaptation of chlorate reducing bacteria to low oxygen concentration. Though oxygen is formed during chlorate reduction, an accumulation of oxygen is never observed. Oxygen formation is observed only when chlorite is added to cell.

Other proteins related with these functions were also up-regulated. Since chlorite dismutase is a heme-containing enzyme an increase in the formation of heme is essential. This is accompanied by an up-regulation of oxygen-independent coproporphyrinogen III oxidase which is involved in heme formation. Similarly since the catalytic unit of chlorate reductase contains molybdenum and iron, a molybdenum ABC transporter is up-regulated to compensate the increased demand of molybdenum. Similarly, a siderophore biosynthesis protein, and an iron ABC transporter were up-regulated. An ABC transporter and a TonB-dependent receptor were also up-regulated on growth with acetate and chlorate. The function of these in *P. chloritidismutans* is not clear.

Some stress related proteins like a DNA stress binding protein were also up-regulated when grown on chlorate. An UDP-N-acetylmuramate-alanine ligase protein involved in cell wall synthesis is also up-regulated during growth on decane and chlorate, likely to strengthen the cell. Another stress protein i.e. ClpB protein, which is an ATP dependent chaperone is also up-regulated when grown on decane and chlorate. Clp stress proteins are expressed in reponse to high temperature, oxidative stress, high salt or ethanol concentration and iron limitation (Ekaza et al. 2001). In our case, it could be oxidative stress or iron limitation or decanol concentration.

A cAMP protein Fic is also up-regulated. It is involved in the post translational regulation of protein functions.

This is the first report about the proteogenomics of a bacterium which is able to degrade *n*-alkanes in the absence of external oxygen, while generating oxygen via chlorite dismutation. It is also the first report of the proteome of a chlorate reducing bacterium. The study confirms the conclusion of previous findings that an oxygenase mediated pathway is employed by the *P. chloritidismutans* during growth on *n*-alkanes and chlorate. It further shows that there are two separate pathways for growth on chlorate and nitrate. Further it is demonstrated that the chlorate reductase and chlorite dismutase are up-regulated when grown with chlorate and down-regulation when grown with oxygen. The finding of the presence of two different chlorite dismutases inside the genome is remarkable.

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Chapter 7

General Discussion

Industrialization of the world has led to the pollution. Pollutants are of concern due to their persistence and toxicity. Pollutants show a varying degree of persistence in oxic and anoxic environments. Most of the pollutants like aliphatic and aromatic hydrocarbons are quite easily degraded in the oxic environment. Aerobic degradation pathways are known since long time and are generally well characterized (Söhngen 1913, Berthe-Corti & Fetzner 2002, Head et al. 2006, Vaillancourt et al. 2006, Fuchs 2008). In anoxic environments only the compounds which are substituted with strong electron withdrawing groups, like highly chlorinated compounds are easily degraded (Field et al. 1995). The degradation products of these compounds are often found accumulated in the anoxic environments (Fennel et al. 2001, Coleman et al. 2002, van Doesburg et al. 2005). In anoxic environments the degradation is generally slow and incomplete and the mechanism of degradation is not completely understood (Wentzel et al. 2007, Fuchs 2008). Introduction of oxygen in pure form or in the form of hydrogen peroxide or some oxygen releasing compounds have been suggested as alternatives but each of them has its own limitation with respect to solubility, toxicity or dispersal. (Per)chlorate reduction has been suggested as an alternative (Coates et al. 1999, Tan et al. 2006, Weelink et al. 2008). Though (per)chlorate reduction has been studied in detail (Xu et al. 2003, Coates & Achenbach 2004) the bio-remediation potential of (per)chlorate-reducing bacteria is not fully explored. In this thesis we tried to explore and highlight the tremendous hidden unexplored potential of (per)chlorate-reducing bacteria for the remediation of organic pollutants.

In the first chapter a brief comparison of aerobic and anaerobic degradation and limitations of anaerobic degradation were discussed. Then, the role that (per)chlorate-reducing bacteria can play to overcome the limitation of anaerobic degradation in anoxic zones was identified. The (per)chlorate reduction was reviewed with a particular emphasis on the bio-remediation potential. Using the bioinformatics tools some putative (per)chlorate reducers were also identified.

The second chapter of this thesis was an effort to combine the oxidation of organic chlorinated compounds with low number of substituted chlorines (TCE, DCEs, DCAs), with the reduction of an inorganic chlorinated compound (chlorate). Unfortunately, we were not successful in enriching such kind of microorganism. The reasons of failure include the nature of the samples, their long term storage and low aerobic degradation potential. However, it is suggested that for such a study fresh samples should be taken from a suboxic zone having already an exposure of organic chlorinated compounds so that the target organisms that possess oxygenases with high affinity for oxygen are present. Moreover, a change in enrichment strategy is suggested i.e. to

first enrich the chlorate reducer and afterwards further enrichment of chlorinated compound-oxidizing bacteria should be done or vice versa.

The third chapter deals with the degradation of *n*-alkanes with chlorate as electron acceptor by *Pseudomonas chloritidismutans* AW-1^T. It was known that chlorate-reducing bacteria can degrade aromatics like BTEX compounds. Here for the very first time we show that chlorate-reducing bacteria have the ability to degrade aliphatic compounds like alkanes too. The rates of degradation of alkanes were similar on chlorate and on oxygen showing the similarity of the biochemical mechanism and the high efficiency of degradation with chlorate. This bacterium also grew with aerobic intermediates of the presumed pathway, like decanol and decanoate. The activity of the enzymes chlorate reductase and chlorite dismutase was demonstrated in decane- and chlorate-grown cells free extract. An alkane oxygenase activity could be only demonstrated with decane and chlorate grown whole cells. However, with the custom designed and available primers we were unable to amplify any alkane oxygenase genes. Since the bacterium was able to grow on alkanes with oxygen and chlorate but not with nitrate so we suggested an oxygenase dependent pathway for alkane degradation. This study opened the way for the possibility of application of chlorate as oxygen source for remediation of alkane polluted soils.

The fourth chapter is about the purification and characterization of chlorite dismutase from *Pseudomonas chloritidismutans*. Chlorite dismutase is the key enzyme in the chlorate reduction pathway as this is the enzyme which produces oxygen. In fact it is the only enzyme beside photosystem II which forms oxygen-oxygen double bond. By using ^{18}O labelled water, we excluded water as the oxygen source showing that both oxygen atoms in dioxygen are derived form the chlorite molecule. The purification of chlorite dismutase was done by one step hydroxyapatite chromatography. The heme-containing tetrameric enzyme showed most of the typical features of other purified chlorite dismutases. High concentrations of chlorite resulted in the disappearance of the Soret peak and loss of activity, showing the toxic effect of chlorite for the enzyme. In the chlorite dismutase assays, an optimum amount of enzyme was assured. Unlike the chlorite dismutase of *Dechloromonas aromatica* RCB the chlorite dismutase of *P. chloritidismutans* was not inhibited by upto 1.5 M chloride. One remarkable finding was the drastic effect of salts on the activity of the enzyme. The activity was increased by kosmotropic salts and decreased by chaotropic salts. This could have a practical consequence as the enzyme might not work optimally if a high concentration of a chaotropic salt is in the surrounding environment.

Chapter 5 describes the degradation of benzoate and catechol coupled with chlorate reduction by *P. chloritidismutans.* The degradation of benzoate and catechol with chlorate, but not with

nitrate, suggests an oxygenase-mediated mechanism of degradation. Catechol was detected as intermediate of benzoate degradation. Cell free extracts of bacteria grown on benzoate and chlorate showed chlorate reductase, chlorite dismutase, benzoate 1,2-dioxygenase and catechol 1,2-dioxygenase activity. A proteogenomics approach was applied to determine the presence and regulation of oxygenases. The bacterium was sequenced via 454 life science technology and a whole proteome analysis was done with acetate-grown and benzoate-grown cells. Proteins involved in benzoate degradation i.e. three subunits of benzoate 1,2-dioxygenase, dihydrodihydroxybenzoate dehydrogenase, benzoate specific porin and benzoate MFS transporter were exclusively present in benzoate grown cells. Similarly, proteins involved in the catechol degradation i.e. catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, 3-oxoadipate: 2 subunits of succinyl-CoA transferse and β-ketoadipyl CoA thiolse were also only present in benzoate grown cells. The presence of the benzoate operon in the genome and up-regulation of most of its protein during growth on benzoate and oxygen is an indication that that this bacterium utilizes the oxygen produced by chlorate reduction to degrade benzoate and catechol via an oxygenase dependent pathway when oxygen is replaced by chlorate. *P. chloritidismutans*, which was found to grow with alkanes, is also able to grow with simple aromatics and their intermediates. This makes this bacterium attractive to be used for bio-remediation purposes.

Chapter 6 describes the proteogenomics *P. chloritidismutans.* The bacterium was grown at 5 different conditions i.e. with acetate and decane as electron donors and chlorate, oxygen and nitrate as electron acceptors. A six frame translation was used for analysis of proteomics data. The differential expression of the proteome was analyzed. By comparing the alkane-grown cells (both with oxygen and with chlorate) with the acetate-grown cells (with oxygen and with chlorate) we were able to identify an alkane-1-monoxygenase responsible for growth with alkanes. The exclusive presence of alkane-1-monoxygenase and the up-regulation of chlorate reductase and chlorite dismutase show that oxygen released during chlorate reduction is used by the alkane oxygenase, resulting in a hydroxylation of the alkane to an alcohol. We also found a variety of alcohol dehydrogenases and aldehyde dehydrogenases either induced or up-regulated when grown on alkanes with oxygen or chlorate as electron acceptors. There was one alkane-1-monoxygenase present during growth on alkanes with either oxygen or chlorate, but differences were found in the expression of alcohol and aldehyde dehydrogenases, some of which were expressed only at one of the growth conditions.

By comparing the differential expression of proteins of cells grown with oxygen (both with alkanes and with acetate) with chlorate-grown cells (with alkanes and with acetate) and also

with nitrate-grown cells (with acetate) we were able to see the regulation of enzymes involved in chlorate reduction and nitrate reduction. The results of the proteogenomics analysis were compared and verified by the enzyme activity measurements. The analysis show that a basal level of chlorate reductase and chlorite dismutase is always expressed, which is up-regulated when grown with chlorate and down-regulated when grown with oxygen. There is a separate nitrate reductase in *P. chloritidismutans* which is induced when grown with nitrate. However, nitrite reductase showed a constitutive activity. We also found up-regulation of cytochrome *cbb3* oxidase during growth on chlorate as compared with oxygen or nitrate. Cytochrome *cbb3* oxidase has a very high affinity for oxygen. This shows an adaptation of the bacterium to low oxygen concentrations created during chlorate reduction. A bioinformatics analysis of chlorate reductase showed the similarity of all of its subunits with the subunits of chlorate reductase of *Ideonella dechloratans* (Danielsson et al. 2003). A transposon sequence was found downstream the chlorate reductase genes indicating its possible horizontal transfer. A twin arginine motif which may be involved in the transport the folded protein through Tat pathway (Berks 1996) was identified. A signal peptide was detected in the alpha and gamma subunit. Moreover, a periplasmic cytochrome *c* containing the conserved motif of a cytochrome *c* of *Ideonella* which is able to donate electrons to chlorate reductase was found. These findings suggest a periplasmic nature of chlorate reductase of *P. chloritidismutans* as against the previously suggested cytoplasmic localization (Wolterink et al. 2003). Based upon the proteomics data we suggest a respiratory pathway for the bacterium. Overall all the proteogenomics data indicate the high flexibility and high versatility of the bacterium to thrive in different environments. It is also concluded that 454 sequencing followed by differential proteomic analysis is the quickest method to identify the unknown genes involved in a certain metabolic function and to explore the overall pollutant degradation potential of the bacteria.

(Per)chlorate reduction and chlorite dismutation in a broader context

Apart from providing oxygen for degradation of persistent pollutants other physiological functions of chlorite dismutatase might be possible. The weak activity of chlorite dismutase enzyme for chlorite but higher activity for hydrogen peroxide in *Thermus thermophilus* HB8 led to the conclusion that the enzyme might be involved in the detoxification of hydrogen peroxide produced within the cell (Ebihara et al. 2005). However, based on the conserved residues in the active site de Geus et al. (2009) showed that this protein is functionally unrelated with other chlorite dismutases.

A functional interaction of the chlorite dismutase and antibiotic biosynthesis monooxygenase in *Haloferax volcanii* Pit A has been proposed. Since fusion of these two domains only occurs in

halophilic archaea it may play a role in an adaptation to live in hypersaline environments where the bacterium is growing in oxygen limited conditions (Bab-Dinitz et al. 2006).

Of the organisms listed in Table 6 of the introduction we can deduce that putative (per)chlorate-reducing bacteria are wide spread. Presently, most of the known (per)chlorate reducers can be placed in the phylum proteobacteria, but most of the bacteria listed in Table 6 of the introduction belong to Actinobacteria or Firmicutes. Many pathogenic strains seem to contain the chlorite dismutase genes. So it might help the pathogens to survive at oxygen limited environments. The gene neighbourhood of chlorite dismutase genes shows that often genes coding for enzymes involved in heme formation (protoporphyrinogen oxidase and uroporphyrinogen decarboxylase) are in close vicinity of the chlorite dismutase gene. It is not clear whether these enzymes are involved in the formation of heme of the chlorite dismutase or that the chlorite dismutase activity has a function in heme formation in the absence of external oxygen.

From an environmental point of view *Mycobacterium* KMS, which is a known aerobic pyrene degrading organism (Miller et al. 2004) and *Geobacillus thermodenitrificans strain NG80-2,* a known aerobic long chain alkane degrader, are interesting bacteria. One unlisted bacterium is *P. aeruginosa* which also contains a putative chlorite dismutase and has nitrate reductase, while it is a known alkane degrader.

Keeping in view the diversity and ubiquity of (per)chlorate reducers, their functions in nature, their ability to adopt to the niches with oxygen limitation and with the recent discovery of perchlorate on Mars (Hecht et al. 2009) it might be worth to speculate about the possibility of life on Mars.

Research needs

A solution for the biological remediation of hydrocarbons-contaminated soils where oxygen is the main limiting factor is the introduction of oxygen in a highly soluble form. (Per)chlorate, which acts both as electron acceptor and as oxygen source, is an appealing compound. It has a high solubility, a redox potential comparable to oxygen, and there are numerous (per)chlorate-reducing bacteria in nature.

We are at the beginning of deciphering and understanding the diversity, relevance and application of per(chlorate)-reducing bacteria that can degrade hydrocarbons and other compounds that are rather recalcitrant under anoxic conditions. Thus, in this respect studying the ecology of biodegradation deserves continued investigation efforts.

Only a few hydrocarbon-degrading chlorate-reducing bacteria have been enriched and isolated up to now. Considering the enormous potential of (per)chlorate-reducing bacteria, there is a

need to enrich and isolate (per)chlorate-reducing bacteria over a broader range of hydrocarbons and a wider range of environmental conditions (pH, temperature, salt concentration). In addition, most of the known (per)chlorate reducers have been isolated on simple substrates like fatty acids. So further screening of these bacteria for hydrocarbon degradation is suggested. The bacteria listed in Table 6 of the introduction which have putative chlorite dismutase and putative nitrate reductase genes should be screened for their (per)chlorate-reducing ability. Similarly metagenome analysis may shed light on the occurrence and distribution of genes linked to the oxygenase-dependent breakdown of recalcitrant compounds and to (per)chlorate reduction. This may also provide insight into the biodegradation potential of polluted soils.

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Summary

Aliphatic and aromatic hydrocarbons are two groups of compounds that are widespread pollutants. The aerobic microbial degradation of aliphatic and aromatic hydrocarbons proceeds in general fast and has been widely studied, while the biodegradation in anoxic environments is often incomplete, proceeds at lower rates and is less characterized. The different techniques for the introduction of oxygen in anoxic zones are economically not attractive and have severe limitations. A promising solution for this could be to make use of the reduction of chlorate. Chlorate reduction is a unique process which yields molecular oxygen upon microbial reduction. This can be of practical significance, since the oxygen released can be incorporated inside the anaerobically recalcitrant compounds by oxygenases to form hydroxylated derivatives which can be further degraded easily either aerobically or anaerobically.

We have found that *Pseudomonas chloritidismutans* AW-1^T, which is a known chlorate-reducing bacterium, can combine the oxidation of *n*-alkanes and the reduction of chlorate. The bacterium was able to grow on *n*-alkanes with oxygen and chlorate, but not with nitrate. The bacterium was able to grow with the intermediates of the aerobic pathway. The specific growth rates were almost equal on chlorate and oxygen. Hence we suggest that oxygen released during chlorate reduction is used via an alkane oxygenase to degrade *n*-alkanes. Chlorite dismutase is the key enzyme which splits chlorite into chloride and oxygen. We have isolated the chlorite dismutase for *Pseudomonas chloritidismutans* AW-1^T. By using ^{18}O labelled water, we demonstrated that both oxygen atoms in dioxygen are derived form the chlorite molecule and not from the water. High concentrations of chlorite resulted in the disappearance of the Soret peak and loss of activity, showing the toxic effect of chlorite for the enzyme. The activity of the enzyme was drastically increased by kosmotropic salts and decreased by chaotropic salts.

Pseudomonas chloritidismutans AW-1^T was also able to grow on benzoate with oxygen and chlorate but not with nitrate, demonstrating the use of the oxygenase mediated pathway. Catechol has been detected as an intermediate, further confirming this hypothesis. Cell free extracts of bacteria grown on benzoate and chlorate showed chlorate reductase, chlorite dismutase, benzoate-1,2-dioxygenase and catechol-1,2 dioxygenase activity. By differential proteomic analysis we found that the proteins involved in benzoate degradation were exclusively present in benzoate grown cells. Similarly, proteins involved in the catechol degradation were also only present in benzoate grown cells.

Pseudomonas chloritidismutans AW-1^T was sequenced via 454 life science technology and a whole proteome analysis was done under 5 different growth conditions i.e. with acetate and decane as electron donors and chlorate, oxygen and nitrate as electron acceptors. By comparing the alkane-grown cells (both with oxygen and with chlorate) with the acetate-grown cells (with oxygen and with chlorate), we were able to identify an alkane-1-monoxygenase responsible for growth with alkanes. The exclusive presence of alkane-1-monoxygenase and the up-regulation of chlorate reductase and chlorite dismutase show that oxygen released during chlorate reduction is used by the alkane oxygenase, resulting in a hydroxylation of the alkane to an alcohol. We also found other enzymes involved in the pathway i.e. alcohol dehydrogenases, aldehyde dehydrogenases and enzymes of the beta-oxidation either induced or up-regulated when grown on alkanes.

By comparing the enzyme activities with the differential expression of proteins of cells grown with oxygen, chlorate and nitrate we found that a basal level of chlorate reductase and chlorite dismutase is always expressed, but these enzymes are up-regulated when grown with chlorate and down-regulated when grown with oxygen. Apart from a chlorate reductase there is a separate nitrate reductase in *P. chloritidismutans*. We also found up-regulation of cytochrome *cbb3* oxidase during growth on chlorate as compared with oxygen or nitrate. This shows an adaptation of the bacterium to low oxygen concentrations created during chlorate reduction. A bioinformatics analysis of chlorate reductase showed the similarity of all of its subunits with the subunits of chlorate reductase of *Ideonella dechloratans*. Due to the presence of a twin arginine motif, the presence of a signal peptide on a gamma subunit and the detection of a periplasmic cytochrome *c* responsible for donating electrons to chlorate reductase, we suggest a periplasmic localization of the chlorate reductase. Our findings suggest that oxygen released during chlorate reduction can be used to degrade the anaerobically recalcitrant compounds and chlorate reduction has a very high potential for bioremediation of anoxic soils.

Samenvatting

Alifatische en aromatische koolwaterstoffen komen wijdverspreid als verontreinigingen voor. De aërobe microbiële afbraak van alifatische en aromatische koolwaterstoffen verloopt in algemeen snel, terwijl de biologische afbraak in anaërobe milieus vaak langzaam en onvolledig is. Van de anaërobe afbraak is minder bekend dan van de aërobe afbraak. Er bestaan verschillende technieken om zuurstof in anaërobe bodems in te brengen teneinde microbiële afbraak te stimuleren. Deze technieken zijn echter economisch niet aantrekkelijk en hebben veelal ook technische beperkingen. Een veelbelovende oplossing voor dit probleem is het gebruik van chloraat. De microbiële omzetting van chloraat is een uniek proces omdat er moleculaire zuurstof vrij gemaakt wordt. Dit kan de afbraak van koolwaterstoffen versnellen, aangezien de vrijgekomen zuurstof via oxygenases ingebouwd kan worden in organische moleculen. De gevormde gehydroxyleerde verbindingen zijn dan verder makkelijk afbreekbaar, zowel aëroob als anaëroob. Wij hebben ontdekt dat *Pseudomonas chloritidismutans* stam AW-1, een chloraat-reducerende bacterie die in ons laboratorium geïsoleerd is, de oxidatie van n-alkanen kan koppelen aan de reductie van chloraat. De bacterie kan op *n*-alkanen met zuurstof en chloraat, maar niet met nitraat, groeien en blijkt dit met de intermediairen van de bekende aërobe afbraakroute van n-alkanen te kunnen. De gemeten specifieke groeisnelheden met chloraat en zuurstof waren ongeveer gelijk. Op grond daarvan concluderen wij dat zuurstof die tijdens chloraatreductie vrijkomt via een alkaan oxygenase wordt gebruikt om n-alkanen af te breken. Chorietdismutase is het enzym dat chloriet omzet in chloride en zuurstof. Wij hebben het chlorietdismutase van *P. chloritidismutans* stam AW-1 geïsoleerd en gekarakteriseerd. Door ^{18}O water te gebruiken konden we aantonen dat beide zuurstofatomen van het chloriet molecuul omgezet worden naar zuurstof en dat niet één van de zuurstofatomen uit water afkomstig is, zoals eerder aangenomen werd. Hoge concentraties chloriet resulteerden in de verdwijning van de Soret piek en het verlies van activiteit van het chlorietdismutase. De activiteit van het enzym werd aanzienlijk verhoogd met kosmotropische zouten en werd verlaagd met chaotropische zouten. *P. chloritidismutans* stam AW-1 bleek ook op benzoaat met zuurstof en chloraat te kunnen groeien, maar niet met nitraat. Dit doet vermoeden dat ook benzoaat middels oxygenases wordt afgebroken. De detectie van catechol als intermediair bevestigt deze hypothese. In celvrije extracten van bacteriën die op benzoaat en chloraat gekweekt waren kon een activiteit van chloraatreductase, chlorietdismutase, benzoaat-1,2-dioxygenase en catechol-1,2-dioxygenase gemeten worden. Door gebruik te maken van differentiële proteoomanalyse kon aangetoond worden dat enzymen die voor benzoaat- en catecholafbraak nodig zijn, alleen in benzoaat-

gekweekte cellen aanwezig waren en niet in acetaatgekweekte cellen Op dezelfde manier werd het alkaanmetabolisme van *P. chloritidismutans* stam AW-1 nader onderzocht. Cellen werden onder vijf verschillende condities gekweekt, namelijk met acetaat en decaan als elektronendonor en met chloraat, zuurstof en nitraat als elektronenacceptor. Door alkaangekweekte cellen (zowel met zuurstof als met chloraat) met acetaatgekweekte cellen (met zuurstof en met chloraat) te vergelijken, konden wij een alkaan-1-monoxygenase identificeren als enzym dat betrokken is bij groei met alkanen. De aanwezigheid van alkaan-1-monoxygenase en van chloraatreductase en chlorietdismutase tonen aan dat de zuurstof die tijdens chloraatreductie vrijkomt, door alkaan-1-oxygenase wordt gebruikt. Andere enzymen die betrokken zijn bij de afbraak van alkanen, zoals alcoholdehydrogenases, aldehydedehydrogenases en enzymen die verantwoordelijk zijn voor bèta-oxidatie zijn eveneens gevonden De bevindingen van de differentiële proteoomanalyse konden bevestigd worden middels enzymactiviteitsmetingen. We vonden tevens dat behalve chloraatreductase er ook een apart nitraatreductase in *P. chloritidismutans* aanwezig is, en dat het cytochroom cbb3 oxydase belangrijk is bij groei met chloraat. Dit cytochroom cbb3 oxidase is karakteristiek voor groei bij lage zuurstofspanning. Dit duidt erop dat de bacterie tijdens chloraatreductie onder condities van lage zuurstofconcentraties groeit. Uit bioinformatica-analyse bleek dat de oriëntatie van genen, die betrokken zijn bij chloraatreductie, gelijkenis vertoont met die van *Ideonella dechloratans*. De aanwezigheid van een twin-arginine motief in de gamma-subeenheid van chloraatreductase en de detectie van een periplasmatisch cytochroom c duidt op een periplasmatische lokalisatie van chloraatreductase. De bevindingen in dit proefschrift tonen aan dat zuurstof die tijdens chloraatreductie vrijkomt bij bioremediatie, gebruikt kan worden om verbindingen die onder anaërobe condities recalcitrant zijn versneld af te breken zijn door chloraat toe te dienen.

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Curriculum Vitae

Farrakh Mehboob was born in Pakistan on 17th February, 1977. He did his Bachelors in Botany, Zoology, Chemistry from University of the Punjab. After completing his Masters Degree in Biological Sciences with specialization in Biochemistry/ Molecular Biology from Quaid-i-Azam University Islamabad, he joined Pakistan Agricultural Research Council as scientific officer. The major focus of his research was pesticide residues in food chain and ecosystem. In 2005, he won the overseas Ph.D. scholarship from the Higher Education Commission (HEC) of Pakistan for completing a Doctoral Research in The Netherlands. Before starting his Ph.D., he did 6 months training on anaerobic microbial physiology techniques. He started his Ph.D. at the Laboratory of Microbiology, Wageningen University and Research Centre in 2005. His research was focused on the anaerobic degradation of organic pollutants using chlorate as electron acceptor. This thesis is the result of the work carried out during his Ph.D.

List of Publications

Mehboob F*, Wolterink AFWM*, Vermeulen AJ, Jiang B, Hagedoorn P-L, Stams AJM, Kengen SWM (2009) Purification and characterization of a chlorite dismutase from *Pseudomonas chloritidismutans*. *FEMS Microbiol Lett* 293:115-121
*Both authors contributed equally

Mehboob F, Howard J, Schraa G, Stams AJM (2009) Growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes with chlorate as electron acceptor. *Appl Microbiol Biotechnol* 83:739-747

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- **Teaching and supervising Thesis students**
- **Phylogeny and ARB/SILVA**

Research and Management Skills:

- **Scientific exchange visit to Laboratory of Microbial Ecology and General Microbiology, University of Konstanz and Max-Planck-Institute for Terrestrial Microbiology Marburg, Germany**
- **Writing PhD proposal**
- **Seminars, Colloquia and Workgroup Meetings at Microbiology**
- **PhD Trip to East Coast USA**

Oral Presentations:

- **Oxidation of alkanes coupled to chlorate reduction by *Pseudomonas chloritidismutans* AW-1^T , The Third International Meeting on Environmental Biotechnology and Engineering (3IMEBE), 24 September 2008, Palma de Mallorca, Spain**
- **Growth of *Pseudomonas chloritidismutans* AW-1^T on *n*-alkanes with chlorate as electron acceptor, Innovative Techniques for a Sustainable Environment, 19 February 2009, Wageningen, The Netherlands**
- **Martian solution for planet earth pollution, Series of Minisymposia, 16 April 2009 & 20 April 2009, Woods Hole Oceanographic Institution (WHOI), MA and New Brunswick, New Jersey, USA**

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