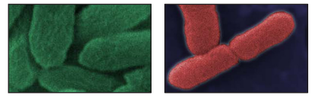
Physiology and biochemistry of aromatic hydrocarbon-degrading bacteria that use chlorate and/or nitrate as electron acceptor

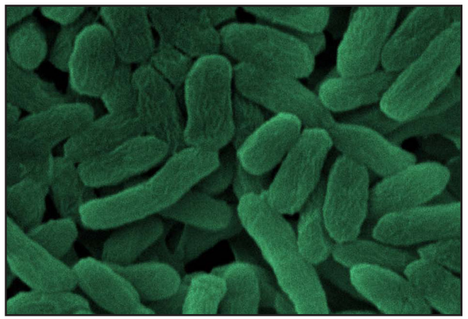
Margreet J. Oosterkamp

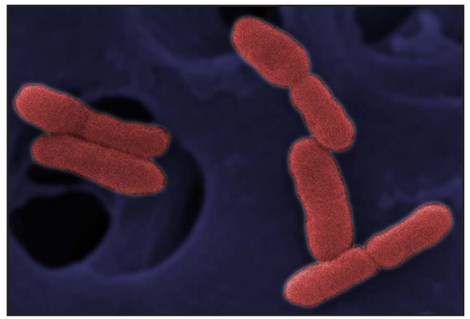
Invitation

for the public defense
of my thesis



Physiology and biochemistry of aromatic hydrocarbon-degrading bacteria that use chlorate and/or nitrate as electron acceptor





The public defense of my thesis will take place in the Aula of Wageningen University (Generall Faulkesweg 1, Wageningen) on December 18 2013 at 4:00 pm.

This defense is followed by a reception in Café Carré (Vijzelstraat 2, Wageningen).

Margreet J. Oosterkamp

Paranimphs
Ton van Gelder
(t.vangelder@wur.nl)
Aura Widjaja
(a.widjaja@wur.nl)

Marjet Oosterkamp
(911 W Springfield Ave Apt 19,
Urbana, IL 61801, USA;
marjet.oosterkamp@gmail.com)

Physiology and biochemistry of aromatic hydrocarbon-degrading bacteria that use chlorate and/or nitrate as electron acceptor

Margreet J. Oosterkamp

Thesis committee

Thesis supervisor

Prof. dr. ir. A. J. M. Stams
Personal Chair at the Laboratory of Microbiology
Wageningen University

Thesis co-supervisors

Dr. C. M. Plugge
Assistant Professor at the Laboratory of Microbiology
Wageningen University

Dr. P. J. Schaap
Assistant Professor at the Laboratory of Systems and Synthetic Biology
Wageningen University

Other members

Prof. dr. L. Dijkhuizen, University of Groningen

Prof. dr. H. J. Laanbroek, University of Utrecht

Prof. dr. ir. D. Springael, Catholic University of Leuven

Prof. dr. S. C. de Vries, Wageningen University

This project was conducted under the auspices of the graduate school SENSE (Socio-Economic and Natural Sciences of the Environment)

Physiology and biochemistry of aromatic hydrocarbon-degrading bacteria that use chlorate and/or nitrate as electron acceptor

Margreet J. Oosterkamp

Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff
the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public on Wednesday December 18 2013
at 4:00 p.m. in the Aula of Wageningen University

Margreet J. Oosterkamp
Physiology and biochemistry of aromatic hydrocarbon-degrading bacteria
that use chlorate and/or nitrate as electron acceptor
191 pages.

Thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in English and Dutch

ISBN 978-94-6173-777-9

Dedicated to my parents

'Je kunt het altijd proberen (You can always try)'

Preface

In situ bioremediation by using naturally occurring or introduced microorganisms is a very attractive option for remediation of anoxic polluted soil sites. Often the appropriate conditions for microorganisms need to be created and the introduction of electron acceptors is required. Oxygen can be introduced by aeration, however, this is difficult to achieve and very costly. We focused on the use of the alternative electron acceptors nitrate and chlorate. Nitrate and chlorate are more soluble than oxygen and easy to introduce in soil via the groundwater. Some microorganisms are known to degrade soil pollutants with nitrate and/or chlorate.

Nitrate-reduction is used as a method for bioremediation of anaerobic polluted soil sites. Degradation of aromatic hydrocarbons is known to involve anaerobic pathways. Interestingly, an alternative nitrate reduction pathway in which oxygen is produced was found. This light-independent *in situ* oxygen production offers innovative possibilities for bioremediation of anaerobic soils polluted with aromatic hydrocarbons, for example. Biochemical routes for the degradation of such hydrocarbons require mono- and dioxygenases. These enzymes cannot function under anaerobic conditions. Because of the production of oxygen from an alternative nitrate reduction pathway, oxygenases could function. Similarly, oxygen is produced when chlorate is the electron acceptor. Laboratory experiments and additional tests in the field showed that chlorate-enhanced anaerobic benzene degradation with chlorate is possible. A stable enrichment that degraded benzene with chlorate at comparable high rates as reported for aerobic benzene-degrading cultures and at 10 to 100 times higher rates than reported for anaerobic benzene-degrading cultures was obtained. One of the bacteria from this enrichment, a novel *Alicycliphilus* strain, has been obtained in pure culture. This bacterium degrades benzene and some other aromatic hydrocarbons with chlorate as electron acceptor, but not with nitrate as electron acceptor. The general aim of this project was to get insight into the physiological and biochemical properties of this bacterium and of other aromatic hydrocarbon degrading bacteria that use nitrate and/or chlorate as electron acceptor.

Contents

Chapter 1

General introduction

Author and affiliations
Margreet J. Oosterkamp[1(a)]
1 Wageningen University, Agrotechnology and food sciences group, Laboratory of microbiology, Dreijenplein 10, 6703HB, Wageningen, The Netherlands
(a) Present address: Department of Animal Sciences, Energy Biosciences Institute, Institute of Genomic Biology, University of Illinois at Urbana-Champaign, 1207 W Gregory Drive, Urbana, Illinois 61801, USA.

Keywords
Perchlorate-reducing bacteria, chlorate-reducing bacteria, nitrate-reducing bacteria, chlorate reductase, nitrate reductase, chlorite dismutase

Manuscript status
Part of this thesis chapter has been published (Oosterkamp, Mehboob *et al.* 2011)

Abstract

Bacteria that grow with aromatic and aliphatic hydrocarbons and use perchlorate, chlorate and/or nitrate as electron acceptor can be applied for bioremediation of soils polluted with aromatic and aliphatic hydrocarbons. Perchlorate, chlorate and nitrate are more soluble than oxygen and can reach anaerobic soil sites more readily than oxygen. Besides the beneficial effect of the addition of an electron acceptor, (per)chlorate-reducing bacteria possess chlorite dismutase that forms oxygen. This oxygen can be used to degrade aromatic and aliphatic hydrocarbons using aerobic pathways. Aerobic degradation of these compounds is a faster process than anaerobic degradation. (Per)chlorate- and nitrate-reducing microorganisms are present in various ecosystems and interact with each other. The nitrate reduction pathway has been studied in more detail than the perchlorate and chlorate reduction pathways. The nitrate reductase of the NarGHI-type, that converts nitrate to nitrite, can also convert chlorate to chlorite. Furthermore, the subunits of NarGHI-type nitrate reductase and of chlorate reductase are structurally very similar. To get more insight in the metabolism of aromatic and aliphatic hydrocarbon degrading bacteria that can use nitrate and/or chlorate as electron acceptor, further studies are needed. In this introduction chapter the research described in this thesis is defined. The background information summarized above is provided to increase understanding of the research described in this thesis and of the need to do this research.

Introduction

Compared to cost-intensive chemical and physical methods for the clean-up of soil sites polluted with aromatic and aliphatic hydrocarbons, methods using naturally occurring or introduced microorganisms are an attractive option. Recent advances in genomics and proteomics allow more detailed research on such microorganisms (Zhao and Poh 2008). Optimal conditions for pollutant-degrading microorganisms often need to be created. For aromatic and aliphatic hydrocarbon-polluted anaerobic soils the introduction of oxygen via aeration may be used. However, aeration of anaerobic soils is difficult to achieve and very costly. An effective alternative is the use of chlorate or nitrate as terminal electron acceptors. Unlike oxygen, chlorate and nitrate are highly soluble (Table 1). Chlorate and nitrate are easy to introduce in soil via the groundwater. Biological chlorate reduction is a unique type of anaerobic respiration, because in the conversion of the intermediate chlorite, molecular oxygen is produced (Ginkel, Rikken *et al.* 1996). Biochemical pathways in which mono- and dioxygenases initiate degradation of aromatic and aliphatic hydrocarbons cannot be employed under anaerobic conditions, because these pathways require oxygen. However, these pathways can be employed with chlorate as electron acceptor, as oxygen is produced *in situ*. A similar pathway is used with perchlorate as electron acceptor. Furthermore, oxygen production from nitric oxide was postulated as an alternative mechanism of respiratory nitrate reduction (Zedelius, Rabus *et al.* 2011). This allows the use of aerobic degradation pathways with nitrate as electron acceptor as well. In this chapter we provide background information about microorganisms that can use perchlorate, chlorate and/or nitrate as electron acceptors. Detailed information about the enzymes involved in perchlorate, chlorate and nitrate reduction is summarized and discussed. Furthermore, we give more information about aerobic and anaerobic microbial degradation of aromatic and aliphatic hydrocarbons such as benzene, toluene and *n*-alkanes. In the outline of this thesis, we will describe our studies. These studies were performed to get a better understanding of the metabolism of bacteria that degrade aromatic and aliphatic hydrocarbons with chlorate and/or nitrate as electron acceptor.

Table 1. Solubility of oxygen, sodium chlorate and nitrate in water (NIST-MML 2007). The solubility in water (in grams per 100 grams of water) was determined in a temperature range from 0 to 100°C and with 1 atm pressure. The solubility of oxygen was determined with oxygen from air at 1 atm pressure.

Substance	Formula	0°C	10°C	20°C	30°C	40°C	60°C	80°C	90°C	100°C
Oxygen at 21 kPa	O_2	0.000456	0.000353	0.000284	0.000238	0.000203				
Sodium chlorate	$NaClO_3$	7.48	8.23	9.01	9.86	10.8	12.9	15.7	17.3	19.2
Sodium nitrate	$NaNO_3$	8.6	9.5	10.3	11.2	12.0	14.4	17.4		21.2

Origin, toxicity and fate of perchlorate and chlorate in the environment

Perchlorate ($ClO4^-$) and chlorate ($ClO3^-$), are used as solid rocket fuel, in road flares, fireworks, matches, blasting agents, explosives and lubricating oils (Urbansky 1998; Gullick, Lechvallier et al. 2001; Aziz and Hatzinger 2008). Besides from anthropogenic origin, perchlorate and chlorate may also occur in the environment by natural production (Dasgupta, Martinelango et al. 2005; Kang, Jackson et al. 2008; Kounaves, Stroble et al.). Natural deposits of large amounts of perchlorate and chlorate can be found in the hyper arid region of the Atacama desert of Chile (Orris, Harvey et al. 2003). Furthermore, NASA's Phoenix lander detected perchlorate, indicating the natural occurence of perchlorate on Mars (Hecht, Kounaves et al. 2009).

Chlorite is more reactive than chlorate and perchlorate (Taylor, Whitte et al. 1940). Perchlorate can bind to the sodium-iodide symporter and inhibits the uptake of iodide by the thyroid gland in humans. Long-term reduction of iodide uptake in an adult can eventually

cause too low hormone production by the tyroid (Wolff 1998). In the 1950's and 1960's perchlorate was used to treat too high hormone production by the tyroid (Mattie, Strawson et al. 2006). In the 1960's treatment using perchlorate was limited since bone marrow diseases were reported (Soldin, Braverman et al. 2001). Both chlorate and chlorite have been reported to cause red blood cell disease in laboratory rats (Couri, Abdel-Rahman *et al.* 1982). To most of the freshwater and marine species of the aquatic organisms chlorate is nontoxic, but to certain macro brown algal species chlorate was highly toxic (Wijk and Hutchinson 1995). Chlorate is applied in agriculture as a herbicide and defoliant, but is also damaging sensitive plant species (Logan 1998). Chlorate and chlorite have been found to induce chromosomal damage in plants (Feretti, Zerbini et al. 2008).

Due to their high solubility (Table 1), perchlorate and chlorate salts are readily transported and are detected in surface water and groundwater on Earth (Urbansky 1998; Xu, Song et al. 2003). Removal of perchlorate and chlorate from drinking water is difficult and costly using typical physical-chemical water treatment technologies, such as ion exchange and carbon absorption(Logan 1998; Urbansky 1998). Bacteria are able to use perchlorate and chlorate as electron acceptors for growth (Rikken, Kroon et al. 1996; Wolterink, Jonker et al. 2002), which allows the use of such bacteria for perchlorate and chlorate removal. Characteristically, molecular oxygen is evolved upon reduction of perchlorate and chlorate (Rikken, Kroon et al. 1996; Mehboob, Junca et al. 2009).

Perchlorate- and chlorate-reducing microorganisms

Penicillium, Aspergillus and *Fusarium* species grown on hay-surface were found to use chlorate as electron acceptor (Aslander 1928). This is generally credited to be the first report of chlorate-reducing microorganisms. Microbial chlorate reduction was patented as a means of anaerobic wastewater treatment (Korenkov, Romanenko et al. 1976). (Per)chlorate-reducing microorganisms, such as *Alicycliphilus denitrificans* strain BC, *Pseudomonas chloritidismutans* strain AW-1^{T}and *Dechloromonas aromatica* strain RCB might be implemented in bioremediation of soil pollutants, such as benzene, toluene or *n*-alkanes (Chakraborty, O'Connor *et al.* 2005; Weelink, Tan *et al.* 2008; Mehboob, Junca *et al.* 2009).

In wetland close to the effluent of a cellulose plant, a great variety of chlorate-reducing microorganisms were detected (Schwarz, Urrutia *et al.* 2012). Dominant species were related to *Dechlorospirillum* sp., *Alicycliphilus denitrificans*, *Dechloromonas agitata*, *Dechloromonas* sp. LT1, and *Ideonella dechloratans*. Native microbial communities in vadose soil were found to have potential for perchlorate reduction (Nozawa-Inoue, Scow *et al.* 2005). Dominant perchlorate-reducing microorganisms in the vadose soil belong to the *Dechlorospirillum, Dechloromonas, Dechlorosoma* and *Azospirillum* genera. In fact, perchlorate- and chlorate-reducing capacity is present in many ecosystems using various energy substrates (Ginkel, Plugge *et al.* 1995).

Bacteria can grow using perchlorate and chlorate as electron acceptors when they are able to reduce perchlorate and/or chlorate, produce chloride and oxygen from the toxic intermediate chlorite and couple perchlorate and/or chlorate reduction to electron-transport phosphorylation. The first reported isolated dissimilatory perchlorate-reducing microorganism is *Vibrio dechloraticans* strain Cuznesove B-1168 (Korenkov, Romanenko et al. 1976). More than 100 perchlorate- and chlorate-reducing microorganisms have been isolated and this number continues to increase (Table 2). They were isolated from various environments including pristine and contaminated soil and sediment (Coates, Bruce et al. 1999). Isolated (per)chlorate-reducing microorganisms are spread among Clostridia and Alpha-, Beta-, Gamma-, Epsilonproteobacteria and even Archaeoglobi. The majority is Gram negative, facultative anaerobic, mesophilic and has a pH optimum around neutral (Table 2). Furthermore, the majority belongs to the Betaproteobacteria and within this family (per)chlorate-reducing strains of the *Dechloromonas* and *Azospira* genera are predominantly known. The archaeon *Archaeoglobus fulgidus* has an atypical (per)chlorate reduction pathway including an interplay of abiotic and biotic redox reactions involving sulphur compounds (Liebensteiner, Pinkse *et al.* 2013).

Table 2. Selected cultivated and isolated (per)chlorate-reducing microorganisms with references.

Strain	Class	Gram stain	Electron acceptors	Optimal temperature	Optimal pH	Application	Reference
Vibrio dechloraticans Cuznesove B-1168	γ-Proteobacteria	Negative	ClO_4^-, ClO_3^-, CO_2, NO_3^-	-	-	Anaerobic wastewater treatment	(Korenkov, Romanenko et al. 1976)
Acinetobacter thermotoleranticus	γ-Proteobacteria	Negative	ClO_3^-, SO_4^{2-}	Mesophilic (tolerates up to 47°C)	-	Sewage treatment	(Stepanyuk, Smirnova et al. 1992)
Ideonella dechloratans	β-Proteobacteria	Negative	ClO_3^-, O_2, NO_3^-	-	-	-	(Malmqvist, Welander et al. 1994)
Azospira oryzae strain GR-1	β-Proteobacteria	Negative	ClO_4^-, ClO_3^-, O_2, NO_3^-, Mn^{4+}	Mesophilic	Neutral	Domestic wastewater treatment	(Rikken, Kroon et al. 1996)
Wolinella succinogenes strain HAP-1	ε-Proteobacteria	Negative	ClO_4^-, ClO_3^-, NO_3^-	Mesophilic	Neutral-basic	Sewage treatment	(Wallace, Ward et al. 1996)
Azospira sp. strain Perc1ace	β-Proteobacteria	Negative	ClO_4^-, NO_3^-	-	-	Perchlorate-contaminated groundwater treatment	(Herman and Frankenberger 1999)
Dechloromonas agitata strain CKB^T	β-Proteobacteria	Negative	ClO_4^-, ClO_3^-, O_2	Mesophilic	Neutral	Environmental (per)chlorate contamination	(Coates, Michaelidou et al. 1999; Achenbach, Michaelidou et al. 2001)
Dechloromonas sp. strain JM	β-Proteobacteria	Negative	ClO_4^-, ClO_3^-, NO_3^-, O_2, H_2	-	Neutral	Drinking water treatment	(Miller and Logan 2000)
Dechloromonas aromatica strain RCB	β-Proteobacteria	Negative	ClO_4^-, ClO_3^-, NO_3^-, O_2	Mesophilic	-	Benzene contaminated environments	(Coates, Chakraborty et al. 2001)
Dechloromonas aromatica strain JJ	β-Proteobacteria	Negative	NO_3^-, O_2	Mesophilic	-	Benzene contaminated environments	(Coates, Chakraborty et al. 2001)
Pseudomonas chloritidismutans strain AW1^T	γ-Proteobacteria	Negative	ClO_3^-, O_2	Mesophilic	Neutral-basic	-	(Wolterink, Jonker et al. 2002)
Dechloromonas hortensis strain MA-1^T	β-Proteobacteria	Negative	ClO_4^-, ClO_3^-, NO_3^-, O_2	Mesophilic	Neutral	Bioremediation	(Wolterink, Kim et al. 2005)
Pseudomonas chloritidismutans strain ASK1	γ-Proteobacteria	Negative	ClO_3^-, O_2	Mesophilic	Neutral	Bioremediation	(Wolterink, Kim et al. 2005)
Azospirillum lipoferum strain DSM1691^T	α-Proteobacteria	Negative	ClO_4^-	Mesophilic	-	-	(Peng, Wang et al. 2006)
Moorella perchloratireducens	Clostridia	Positive	ClO_4^-, ClO_3^-, NO_3^-, $S_2O_3^{2-}$, neutralized Fe(III) complexes, AQDS	Thermophilic	Neutral	-	(Balk, van Gelder et al. 2008)
Alicycliphilus denitrificans strain BC	β-Proteobacteria	Negative	ClO_3^-, O_2, NO_3^-	Mesophilic	Neutral	Bioremediation	(Weelink, Tan et al. 2008)
Sporomusa ovata strain An4	Clostridia	Negative	ClO_4^-, ClO_3^-, NO_3^-, CO_2	Mesophilic	Neutral	-	(Balk, Mehboob et al. 2010)
Moorella humiferrea strain 64-FGQ^T	Clostridia	Positive	ClO_4^-, NO_3^-, $S_2O_3^{2-}$, humic acid, AQDS	Thermophilic	Neutral	-	Nepomnyashchaya, Slobodkina et al. 2011)
Archaeoglobus fulgidus	Archaeoglobi	Negative	ClO_4^-, ClO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, SO_3^{2-}	Thermophilic	Neutral	-	(Stetter, Lauerer et al. 1987; Liebensteiner, Pinkse et al. 2013)

Nitrate-reducing microorganisms

In nitrate respiration, substrate oxidation is coupled to nitrate reduction to generate free energy, protonmotive force for the synthesis of ATP, solute uptake and motility. This process of denitrification, which follows the pathway: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, cannot be assigned characteristic of particular taxonomic clades of microorganisms (Zumft 1997; Jones, Stres et al. 2008). There are also microorganisms that generate ammonium from nitrate reduction, via the pathway $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$, which is assumed to occur when the amount of nitrate is limited compared to organic carbon (Cole and Brown 1980).

Previous studies have shown that nitrate-reducing microorganisms can be used for degradation of aromatic and aliphatic hydrocarbons (Heider, Spormann *et al.* 1998; Spormann and Widdel 2000; Weelink, van Eekert *et al.* 2010; Vogt, Kleinsteuber *et al.* 2011). Benzene is generally considered more recalcitrant than the other BTEX compounds, toluene, ethylbenzene and xylene isomers (Vogt, Kleinsteuber et al. 2011). In a BTEX degrading bacterial consortium study, toluene was degraded with higher degradation rates than benzene and xylene isomers (Dou, Liu et al. 2008). Most of the isolated anaerobic BTEX degrading microorganisms are denitrifying (Weelink, van Eekert *et al.* 2010). These bacteria are *Magnetospirillum* (of the Alpha-proteobacteria) *Azoarcus, Thauera, Dechloromonas* and *Georgfuchsia* (of the Beta-proteobacteria) species. Most of the isolated bacteria are toluene degraders that are also reported to degrade one or more of the other BTEX. *D. aromatica* strain RCB is the only microorganism that was reported to degrade all the BTEX hydrocarbons with nitrate (Chakraborty, O'Connor et al. 2005).

Principle of metabolism

In (per)chlorate-reducing microorganisms, perchlorate and chlorate function as electron acceptor or oxidant by taking-up electrons that are released from the oxidation of an electron donor or reductant. Metabolism with any electron donor and electron acceptor combination is based on this principle. Standard reduction potentials ($E^{0'}$ at pH 7.0 and 25°C) of redox (reductant/oxidant) couples are listed (Table 3). For electron transport, acceptors must have a more positive E^0 compared to donors. The difference in reduction potential between the electron acceptor and donor ($\Delta E^{0'}$) is a measure for the free energy change (the Gibbs free energy change, $\Delta G^{0'}$, at pH 7.0 and 25°C) via the equation $\Delta G^{0'} = -n \times F \times \Delta E^{0'}$ (in which n is the number of electrons transferred and F is the Faraday constant, 96.5 kJ M^{-1} V^{-1}).

Nitrate has a lower reduction potential than chlorate and perchlorate, and chlorate and perchlorate even have a higher reduction potential than oxygen (Table 3). Given these values, nitrate, perchlorate and chlorate are good electron acceptors. Furthermore, perchlorate and chlorate might even be stronger electron acceptors than nitrate and oxygen.

Table 3. Selected microbiologically important reduction potentials (Thauer, Jungermann *et al.* 1977).

Redox pair	$E^{0'}$ (Volts)
SO_4^{2-}/HSO_3^-	-0.52
$2H^+/H_2$	-0.41
$S_2O_3^{2-}/HS^- + HSO_3^-$	-0.27
S^0/HS^-	-0.40
SO_4^{2-}/HS^-	-0.22
$HSO_3^-/S_3O_6^{2-}$	-0.17
HSO_3^-/HS^-	-0.12
$Fe(OH)_3 + HCO_3^-/FeCO_3$	0.20
$S_3O_6^{2-}/S_2O_3^{2-} + HSO_3^-$	0.23
NO_2^-/NO	0.35
$NO_3^-/NO2^-$	0.43
Fe^{3+}/Fe^{2+}	0.77
Mn^{4+}/Mn^{2+}	0.80
O_2/H_2O	0.82
ClO_3^-/Cl^-	1.03
NO/N_2O	1.18
ClO_4^-/Cl^-	1.29
N_2O/N_2	1.36

Biochemistry of perchlorate and chlorate reduction

Perchlorate and chlorate are reduced by perchlorate reductase and chlorate reductase. Microorganisms that reduce perchlorate, can also use perchlorate reductase to reduce chlorate to chlorite. Chlorite dismutase converts chlorite to chloride and dioxygen (Figure 1).

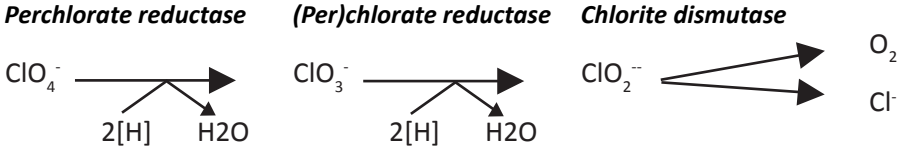


Figure 1. The perchlorate-reduction pathway by (per)chlorate-reductase and chlorite dismutase. Perchlorate reductase reduces both perchlorate and chlorate.

Perchlorate and chlorate reductases belong to the type II DMSO (dimethyl sulfoxide) family of enzymes (Thorell, Stenklo et al. 2003; Bender, Shang et al. 2005). Type II DMSO reductases have a common molybdenum cofactor known as bis molybdopterin guanine dinucleotide (MGD) (Moura, Brondino et al. 2004). Other type II DMSO reductases are nitrate and selenate reductase and ethyl benzene dehydrogenase. All perchlorate and chlorate reductases known thus far are periplasmic (Table 4). The α (60-100 kDa) and β (35-63 kDa) subunits form a membrane-associated complex, while the γ subunit (27-56 kDa) is separate from this complex and not membrane-anchored (Bender, Shang et al. 2005), which might explain why in some cases only the αβ complex was described (Table 4). Like in the other known (per)chlorate reductases, the γ subunit of chlorate reductase of *Ideonella dechloratans* contains heme *b* (Karlsson and Nilsson 2005). In *Azospira oryzae* GR-1 enzyme activity for perchlorate and chlorate was determined, this showed that the maximum reaction rate and the affinity for chlorate was higher than for perchlorate (Table 4), which would ensure reduction of chlorate to chlorite by the perchlorate reductase in presence of both perchlorate and chlorate. In the chlorate-accumulating strain *Azospira* sp. strain HCAP-C the maximum reaction rate and affinity for perchlorate was higher than for chlorate (Dudley, Salamone et al. 2008).

Chlorite dismutase is a key enzyme in the perchlorate and chlorate reduction pathway. It catalyzes the detoxification of the intermediate chlorite. This detoxification reaction is essential for (per)chlorate-reducing microorganisms. The enzyme is involved in the reduction of chlorite to chloride while producing molecular oxygen (Hagedoorn, De Geus et al. 2002). Labeling studies confirmed that both atoms of the dioxygen that is formed, originate from chlorite (Lee, Streit et al. 2008; Mehboob, Wolterink et al. 2009). It is one of the few oxygen-generating enzymes in nature besides photo system II and an uncharacterized enzyme from an anaerobic methane-degrading microorganism (Streit and DuBois 2008; Ettwig, Butler *et al.* 2010). Chlorite dismutase is a heme-containing homotetrameric enzyme, which is present in the periplasm (Ginkel, Rikken *et al.* 1996; Stenklo, Danielsson Thorell *et al.* 2001; Streit and DuBois 2008; Mehboob, Wolterink *et al.* 2009).

Chlorite dismutases (and chlorite dismutase-like enzymes) can be divided into two 'lineages' (Mlynek, Sjöblom et al. 2011). The lineage-one chlorite dismutases comprise the functionally validated (for chlorite transformation) enzymes. A separate phylogenetic lineage of chlorite dismutase-like enzymes form the lineage-two chlorite dismutases. Since, except for the *Nitrobacter* species, the lineage-two chlorite dismutases were not reported to be involved in (per)chlorate reduction, most of these enzymes have a yet-unknown function (Mlynek, Sjöblom et al. 2011). Structurally, the chlorite dismutase of *Nitrobacter winogradskyi* is different from all other known chlorite dismutase structures (Table 5). Whereas other chlorite dismutases are homotetramers or homopentamers, the chlorite dismutase of *N. winogradskyi* is a homodimer

(Mlynek, Sjöblom et al. 2011). A chlorite dismutase-like protein from *Thermus thermophilus* was reported to have weaker chlorite dismutase activity than the functionally validated chlorite dismutases (Mlynek, Sjöblom et al. 2011). The kinetic parameters of this protein from *T. thermophilus* are different from functionally validated chlorite dismutases and the dismutase of *N. winogradskyi* (Table 5). Perchlorate and chlorate reductases and chlorite dismutase have been discussed in more detail recently (Nilsson, Rova *et al.* 2012).

Perchlorate and chlorate reduction versus nitrate reduction

The membrane-bound nitrate reductase Nar catalyses chlorate reduction, while the periplasmic nitrate reductase Nap poorly reduces chlorate (Bell, Richardson et al. 1990; Zumft 1997; Bedzyk, Wang et al. 1999; Moreno-Vivián, Cabello et al. 1999; Simon, Sänger et al. 2004). Recently, a novel respiratory membrane-bound nitrate reductase that is chlorate-sensitive was identified in *Sinorhizobium meliloti* strain 2011 (Ferroni, Rivas et al. 2011). Early research in spinach and *Chlorella vulgaris* showed that nitrate reductase had a higher affinity for nitrate than for chlorate (Solomonson and Vennesland 1972; Nakagawa and Yamashita 1986) and in *Aspergillus oryzae* and *Escherichi coli* the enzyme functions in presence of chlorate (Goksøyr 1951; Goksøyr 1952). A membrane-bound nitrate reductase in the bacterium *Thiospaera pantotropha* showed higher catalytic activity for chlorate than for nitrate and membrane-bound nitrate reductases from soil and sediment bacteria had different rates of catalytic activity with chlorate and nitrate (Bell, Richardson et al. 1990; Carter, Hsiao et al. 1995). Growth conditions influenced enzyme activity in the perchlorate-reducing *Dechlorosoma sp*. KJ. In nitrate-grown cells perchlorate reductase activity was lower, while in chlorate-grown cells perchlorate reductase activity was higher than nitrate reductase activity (Xu, Trimble et al. 2004). Furthermore, in the chlorate-reducing and denitrifying *I. dechloratans*, chlorate-grown cells were found to lose the capability of reducing nitrate and similar results were obtained in a study of chlorate reduction in environmental and enrichment cultures (Malmqvist, Welander *et al.* 1994; Ginkel, Plugge *et al.* 1995).

Comparison of subunits of perchlorate and chlorate reductases to nitrate reductases

The common property of all studied perchlorate, chlorate and nitrate reductases is the presence of the molybdenum factor (Mo) and co-factor (Mo-co) in the active center or α-subunit of the enzymes (Frunzke, Hoffmüller et al. 1991; Frunzke, Heiss et al. 1993). The size of the α-subunit of perchlorate and chlorate reductases (PcrA and ClrA) varies from 60 to 100 kDa, while the α-subunit of Nar-type nitrate reductase (NarG) is larger and varies from 104 to 150 kDa (Morozkina and Zvyagilskaya 2007). The β-subunit of perchlorate and chlorate reductases (PcrB and ClrB) and of Nar nitrate reductases (NarH), contain Fe-S clusters which are involved in electron transfer to the catalytic subunit. The size of PcrB and ClrB ranges from 35 to 63 kDa which is similar to the 43 to 63 kDa size of NarH (Morozkina and Zvyagilskaya 2007).The γ-subunit of respiratory nitrate reductase (NarI), contains two b-type hemes and the γ-subunit of perchlorate and chlorate reductases (PcrC and ClrC) is the cytochrome b moiety of the enzymes (Karlsson and Nilsson 2005). The size of PcrC and ClrC ranges from 27 to 56 kDa and is larger than the 19 to 28 kDa size of NarI (Morozkina and Zvyagilskaya 2007). Furthermore, NarI is a membrane spanning protein and PcrC and ClrC are not (Bender, Shang et al. 2005). Similar to the δ-subunit of nitrate reductase (NarJ) which is necessary for Nar assembly, the δ-subunit of perchlorate and chlorate reductases (PcrD and ClrD) is a chaperone involved in the assembly of the αβ-complex (Thorell, Stenklo et al. 2003; Bender, Shang et al. 2005; Morozkina and Zvyagilskaya 2007). NarJ of *Escherichia coli* is a system-specific chaperone that stays in the cytosol after assembly of the αβγ-complex in the membrane (Liu and DeMoss 1997) and is involved in the acquisition of the molybdenum cofactor (Rothery, Magalon et al. 1998).

Table 4. Characteristics of (per)chlorate reductases. [a]Only chlorate and nitrate were tested. [b]Location was indicated to be cytoplasmic, but periplasmic based on the sequence of the enzyme encoding gene. [c]Purified enzyme was not checked for chlorate or nitrate reductase activity.

Microorganism	*Proteus mirablis*	*Azospira oryzae* strain GR-1	*Pseudomonas chloritidismutans* strain AW-1^T	*Ideonella dechloratans*	*Azospira sp.* strain Perc1ace	*Pseudomonas sp.* strain PDA	*Azospira sp.* strain KJ
Location	membrane	periplasmic	periplasmic[b]	periplasmic	periplasmic	periplasmic	periplasmic
Electron acceptor	chlorate[a]	perchlorate, chlorate, nitrate, iodate & bromate	chlorate, bromate	chlorate, bromate, iodate, nitrate & selenate	perchloratec	chlorate	chlorate, perchlorate
Size of subunit (kDa)	75, 63, 56	95, 40	97, 38, 34	94, 35.5, 27	75, 35	60, 48, 27	100, 40
Composition native enzyme	heterotrimer	trimer of heterodimers	heterotrimer	heterotrimer	heterodimer	heterotrimer	heterodimer
	α_1-β_1-γ_1	α_3-β_3	α_1-β_1-γ_1	α_1-β_1-γ_1	α_1-β_1	α_1-β_1-γ_1	α_1-β_1
Vmax (U/mg)	-	13.2 for ClO_3^- 3.8 for ClO_4^-	51 for ClO_3^-, 26 for BrO_3^-	-	4.8 for ClO_4^-	-	-
Km (µM)	-	< 5 for ClO_3^- 27 for ClO_4^-	159 for ClO_3^-, 50 for BrO_3^-	850 for ClO_3^-, 909 for BrO_3^-, 178 for IO_3^-, 136 for NO_3^-, 59 for SeO_3^-	34.5 for ClO_4^-	-	-
Reference	(Oltmann, Reijnders et al. 1976)	(Kengen, Rikken et al. 1999)	(Wolterink, Schiltz et al. 2003; Mehboob 2010)	(Thorell, Stenklo et al. 2003)	(Okeke and Frankenberger 2003)	(Steinberg, Trimble et al. 2005)	(Steinberg, Trimble et al. 2005)

Table 5. Characteristics of chlorite dismutases.

Microorganism	*Azospira oryzae* strain GR-1	*Ideonella dechloratans*	*Dechloromonas aromatica* strain RCB	*Pseudomonas chloritidismutans* strain AW-1^T	*Candidatus Nitrospira defluvii*	*Nitrobacter winogradskyi*	*Thermus thermophilus* strain HB8
Size of subunit (kDa)	32	25	27	31	30	43	26
Composition native enzyme	homopentamer/ homohexamer (De Geus, Thomassen et al. 2009)	homotetramer	homopentamer (Goblirsch, Streit et al. 2009)	homotetramer	homopentamer	homodimer	homopentamer
Vmax (U/mg)	2.2 x 10^3	4.3 x 10^3	4.7 x 10^3	440	1.9 x 10^3	no data	1.6
Km (µM)	170	260	220	80	15.8 x 10^3/58	90	13 x 10^3
Reference	(Ginkel, Rikken et al. 1996)	(Stenklo, Danielsson Thorell et al. 2001)	(Streit and DuBois 2008)	(Mehboob, Wolterink et al. 2009)	(Maixner, Wagner et al. 2008; Kostan, Sjöblom et al. 2010)	(Mlynek, Sjöblom et al. 2011)	(Ebihara, Okamoto et al. 2005; Maixner, Wagner et al. 2008)

Unlike the cytoplasmic Nar, perchlorate and chlorate reductases are periplasmic in nature. Therefore, their catalytic subunit has the motif of the twin-arginine translocation (Tat) pathway (Berks 1996; Thorell, Stenklo et al. 2003; Bender, Shang et al. 2005). The αβ-complex is assembled in the cytoplasm with the help of the δ-subunit and then hitchhiked to the periplasm via the Tat pathway (Thorell, Stenklo et al. 2003). This is similar to the mechanism of Nap (Richardson, Berks et al. 2001). However, unlike the γ-subunit of Nap (NapC), PcrC and ClrC are not membrane spanning. As a result, a mediator is required for electron transfer to the (per)chlorate reductase. In *I. dechloratans* this mediator is a 6 kDa periplasmic *c* cytochrome-containing peptide (Bäcklund, Bohlin et al. 2009; Bohlin, Bäcklund et al. 2010; Bäcklund and Nilsson 2011). Analogues of this periplasmic c cytochrome have also been found in the genomes of the chlorate-reducing *D. aromatica* and *A. denitrificans* (Bäcklund, Bohlin et al. 2009; Oosterkamp, Veuskens et al. 2011). A periplasmic 'Archaeal' Nar also uses the Tat pathway to translocate the αβ-complex to the periplasmic side of the cell membrane. However, this enzyme is different from the cytoplasmic Nar, Pcr and Clr. NarGH is attached to a membrane-associated cytochrome *b* protein. Furthermore, a membrane-associated NarB subunit contains Fe-S clusters and transfers electrons to the cytochrome *b* protein. NarB is attached to the membrane-spanning NarC, which contains cytochrome *b* (Martinez-Espinosa, Dridge *et al.* 2007).

Comparison of catalytic subunits of perchlorate, chlorate and nitrate reductase

Multiple sequence alignment of the catalytic subunits of perchlorate, chlorate and nitrate reductases shows the presence of an aspartate residue that is highly conserved in these enzymes (Figure 2). This residue acts as a ligand that binds with Mo in Nar (Bertero, Rothery et al. 2003). The catalytic subunit of Nap is not closely related to these enzymes. It contains molybdenum, but this is co-ordinated by a cysteine and not by an aspartate ligand and is catalytically different (Jormakka, Richardson *et al.* 2004).

```
ClrA_Id: TAYTRMTKLLGAISPDATSMTGDLYTGIQTVRVPASTVSTFDDWFTSDLILMWHKN
PcrA_KJ: SAGHRFAHYIGAHAHTFCDWYGDHPTGQTQTCGVQGDTCETADWFNSKYIILWGSN
PcrA_PC: SAGHRFAHYIGAHTHTFFDWYGDHPTGQTQTCGVQGDTAETADWFNSRYIILWGSN
PcrA_Da: SAGHRFAHYIGAHTHTFFDWYSDHPTGQTQTCGVQGDSAECSDWFNSKYIILWGAN
NarG_Ec: ASGARYLSLIGGTCLSFYDWYCDLPPASPQTWGEQTDVPESADWYNSSYIIAWGSN
NarG_Pf: AAGSRYLSLIGGVCLSFYDWYCDLPPASPMVWGEQTDVPESADWYNSNYIIAWGSN
NarG_Bs: ASGSRFMSLIGGPMLSFYDWYADLPPASPQIWGDQTDVPESSDWYNSGYIITWGSN
```

Figure 2. Multiple sequence alignment of the α subunits of chlorate reductase (ClrA) of *Ideonella dechloratans* (Id), perchlorate reductase (PcrA) of *Dechlorosoma* KJ (KJ), *Dechlorosoma* PCC (PC), *Dechloromonas agitata* (Da), and of nitrate reductase (NarG) of *Escherichia coli* (Ec) *Pseudomonas fluorescens* (Pf), *Bacillus subtilis* (Bs). Protein sequences were obtained from the GenBank database (Benson, Karsch-Mizrachi et al. 2008). GenBank accession numbers are as follows: ClrA Id P60068, PcrA KJ ACB69917, PcrA PC ABS59782, PcrA Da AAO49008, NarG Ec CAQ32609, NarG Pf AAG34373, and NarG Bs P42175. The conserved aspartate residue which co-ordinates with Mo is shaded.

Phylogenetic analysis shows that the perchlorate reductases of *Dechloromonas agitata* and two *Dechlorosoma* strains form a separate monophyletic group from the chlorate reductase of *I. dechloratans* and the catalytic subunit of the perchlorate reductases are more closely related to catalytic subunits of nitrate reductases than of chlorate reductases, which is similar to a previous study (Bender, Shang et al. 2005). The putative NarG of *Sagittula stellata* is highly similar to the ClrA of *I. dechloratans* (Figure 3). Furthermore, *S. stellata* cannot reduce nitrate (Gonzalez, Mayer et al. 1997).

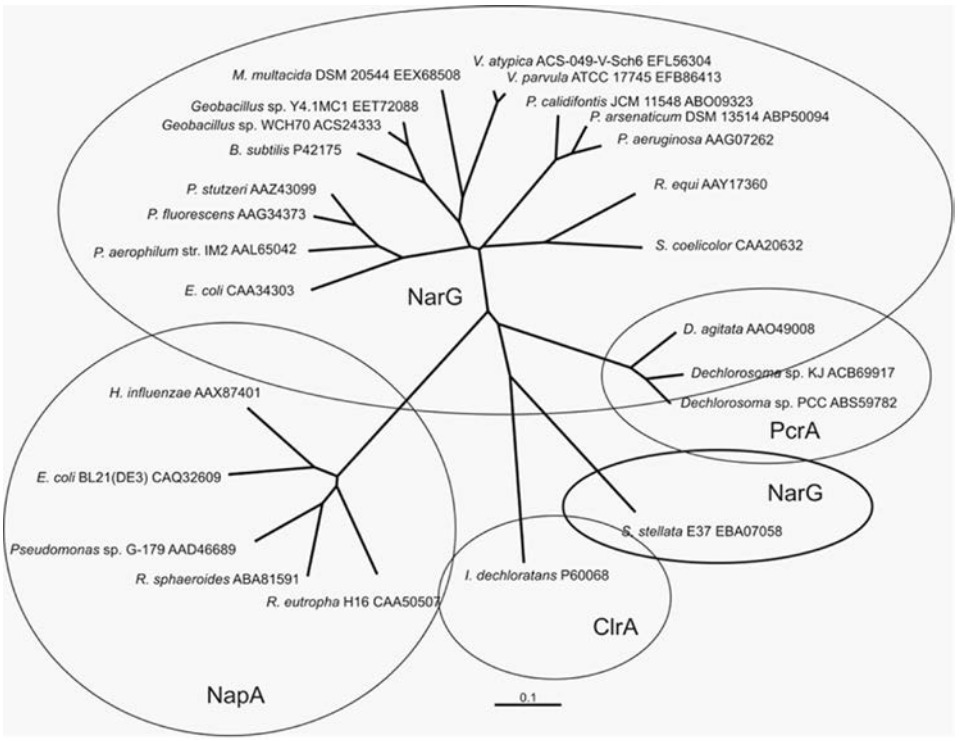


Figure 3. Unrooted neighbor-joining tree indicating the evolutionary distances of NarG, NapA, PcrA, and ClrA, the α subunits of respiratory nitrate reductase, perchlorate reductase, and chlorate reductase, respectively. Protein sequences were obtained from the GenBank database (Benson, Karsch-Mizrachi et al. 2008). GenBank accession numbers are indicated. The tree was assembled using ClustalX 1.83 (Thompson, Gibson et al. 1997), visualized using TreeView 1.6.6 software (Page, 2001), and any make-up was performed using CorelDraw version 12.0 (Corel Corporation, 2003). This unrooted tree was bootstrapped with the number of bootstrap trials set to 1000.

Aerobic degradation of hydrocarbons

Microbial perchlorate and chlorate reduction yields oxygen in the conversion of chlorite by chlorite dismutase (Ginkel, Rikken *et al.* 1996), also in nitrate and nitrite reduction oxygen production has been observed (Ettwig, Butler *et al.* 2010; Zedelius, Rabus *et al.* 2011; Ettwig, Speth *et al.* 2012). This allows microorganisms to employ aerobic degradation of pollutants, such as aromatic and aliphatic hydrocarbons, in essentially anaerobic environments. In aerobic degradation of these hydrocarbons, oxygen functions as electron acceptor and oxygen molecules are also incorporated in the hydrocarbons. Such incorporation of oxygen molecules is catalyzed by oxygenases. There are two types of oxygenases, mono- and dioxygenases. An important group of monooxygenases, the soluble di-iron monooxygenases, have a limited phylogenetic distribution in the bacteria domain (Leahy, Batchelor et al. 2003). Dioxygenases are distributed widely in nature and are involved in both anabolic and catabolic processes (Eltis and Bolin 1996).

Petroleum hydrocarbons form perhaps the most complex mixture of organic compounds on Earth and are a non-homogeneous mixture of aromatic hydrocarbons as well as of *n*-alkanes, or saturated hydrocarbons, resins (pyridines, quinolines, carbazoles, sulfoxides

and amides) and asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) (Leahy and Colwell 1990; Head, Jones et al. 2006). Due to their hydrophobicity, *n*-alkanes can absorb aromatic compounds, as a result degradation of *n*-alkanes may increase the bioavailability of fuel aromatics (Widdel and Rabus 2001).

The majority of oxygenases involved in aerobic degradation of *n*-alkanes, are mono-oxygenases. These are mainly the rubredoxin-dependent *alkB* and cytochrome P450-type mono-oxygenases (Beilen, Funhoff *et al.* 2006; Beilen and Funhoff 2007). Alcohols formed by the incorporation of oxygen molecules in *n*-alkanes, are further degraded via the beta-oxidation pathway (Wentzel, Ellingsen et al. 2007).

Compared to other aromatic and saturated hydrocarbons, BTEX are relatively water-soluble and therefore these contaminants can spread via the groundwater (Coates, Chakraborty et al. 2002; Chakraborty and Coates 2004). The initial step in aerobic degradation of aromatic hydrocarbons is ring dihydroxylation, the incorporation of oxygen in the aromatic ring, which is mediated by monooxygenases or ring-hydroxylating dioxygenases. The phenol hydroxylases and the four-component alkene/aromatic monooxygenases are soluble, multicomponent enzymes that could be involved in degradation of hydrocarbons. The other two groups of monooxygenases are the methane monooxygenases and the alkene monooxygenases (Leahy, Batchelor et al. 2003). Ring-hydroxylating dioxygenases, or Rieske nonheme iron oxygenases, are soluble, multi-component iron-sulfur enzymes. These enzymes are composed of a terminal oxygenase component (NAD(P)H-reductase) and electron transport proteins (ferredoxin) (Butler and Mason 1996; Gibson and Parales 2000). Ring-cleavage dioxygenases are involved in fission of the aromatic ring. Depending on the cleavage, there are two pathways of aromatic hydrocarbon degradation. These are initiated by *ortho*-cleavage or by *meta*-cleavage. In *ortho*- or intradiol-cleavage, the aromatic ring is cleaved between two hydroxyl-groups. This pathway leads eventually to the formation of acetyl-CoA and succinate, which can enter the tricarboxylic acid cycle. In the *meta*- or extradiol-cleavage pathway, the ring is cleaved adjacent to a hydroxyl-group, which leads eventually to the production of pyruvate and acetaldehyde, both intermediates of the tricarboxylic acid cycle (Harwood and Parales 1996; Vaillancourt, Bolin *et al.* 2006). In general, intradiol dioxygenases contain a nonheme Fe(III) cofactor and extradiol dioxygenases have nonheme Fe(II) or Mn(II) as a cofactor (Vaillancourt, Bolin et al. 2006).

Anaerobic degradation of hydrocarbons

Aromatic and aliphatic hydrocarbons often persist in the anaerobic zones of the environment, microorganisms can degrade such compounds anaerobically with degradation rates that are much slower than aerobic degradation rates (Heider, Spormann *et al.* 1998; Ehrenreich, Behrends *et al.* 2000; Spormann and Widdel 2000; Wentzel, Ellingsen *et al.* 2007; Weelink, van Eekert *et al.* 2010; Vogt, Kleinsteuber *et al.* 2011). Degradation of *n*-alkanes was proposed to occur via carboxylation (So, Phelps et al. 2003), and via fumarate addition (Rabus, Wilkes et al. 2001; Callaghan, Wawrik et al. 2008; Grundmann, Behrends et al. 2008).

In anaerobic benzene degradation, activation of the aromatic ring is difficult due to the stability of benzene. Possible initial activation can proceed by hydroxylation, carboxylation and methylation (Vogt, Kleinsteuber *et al.* 2011). Facultative anaerobes can use a 'hybrid pathway' in which benzene is converted to benzoate and benzoate is further degraded via CoA-thioesters (Zaar, Eisenreich et al. 2001; Gescher, Ismail et al. 2006). Fumarate addition is used to degrade toluene (Leutwein and Heider 1999; Heider 2007; Fuchs 2008). In this pathway toluene is degraded to benzylsuccinate, which is mediated by the enzyme benzylsuccinate synthase. Benzylsuccinate is oxidized to benzoyl-CoA, which is degraded via reductive ring cleavage to carbon dioxide. Other pathways for toluene conversion have also been described, these are direct methyl-group hydroxylation to benzyl alcohol or hydroxylation to cresol (Frazer, Coschigano et al. 1995). The degradation pathway of xylene isomers is proposed to be initiated with fumarate addition (Beller and Spormann 1997; Krieger, Beller et al. 1999; Widdel and Rabus 2001).

Ethylbenzene degradation was also found to include fumarate addition (Kniemeyer, Fischer et al. 2003). Additionally, an ethylbenzene degradation pathway was described in which the enzyme ethylbenzene dehydrogenase catalyzes an oxygen-independent oxidation of the methyl-group of ethylbenzene, which eventually leads to conversion to benzoyl-CoA and ultimately to carbon dioxide (Ball, Johnson et al. 1996).

Outline of the thesis

Nitrate and chlorate-reducing microorganisms are present in many ecosystems. This is not surprising, since nitrate and chlorate are naturally occurring compounds. Recent evidence of a mechanism of nitrate reduction which has similarities with the mechanism of perchlorate and chlorate reduction has been presented. The proposed pathway involves the yet hypothetical "nitric oxide dismutase", which like chlorite dismutase releases molecular oxygen (Ettwig, Butler *et al.* 2010; Zedelius, Rabus *et al.* 2011). Such oxygen-releasing reduction pathways can be used to develop methods for soil bioremediation. The main aim of the research presented in this thesis was to further explore the chlorate and nitrate reduction pathways in bacteria that degrade aromatic and aliphatic hydrocarbons with chlorate or nitrate as electron acceptor. This information can help to determine the potential of bacterial chlorate and nitrate reduction as mechanisms for biodegradation of aromatic and aliphatic hydrocarbon-polluted anaerobic soils.

There are physiological differences between the denitrifying bacteria *A. denitrificans* strains K601^T and BC. Strain BC uses chlorate as electron acceptor and strain K601^T does not. Furthermore, strain K601^T grows using the cyclic hydrocarbon cyclohexanol as a substrate and strain BC does not (Weelink, Tan *et al.* 2008). The genome sequences (Oosterkamp, Veuskens *et al.* 2011) and physiology of strains BC and K601^T gave insight into the biodegradation capacity and biochemical pathways of the strains (Chapter 2). A detailed physiological study with strain BC using acetate as electron donor and nitrate, chlorate or oxygen as electron acceptor was performed. Insight was obtained in the biochemical pathways involved in metabolism of the different electron acceptors and in the metabolic flexibility of the strain with respect to the electron acceptors (Chapter 3). The gene coding for NarG, the alpha subunit of respiratory nitrate reductase can contain a transposon in chlorate-adapted strain BC cells. This is proposed to be a mechanism for NarG regulation on gene level (Chapter 4). Acetone can occur as environmental pollutant. Strain BC is known to be able to degrade acetone with the enzyme acetone carboxylase. Proteomics revealed more about nitrate-depended acetone degradation in strain BC (Chapter 5).

Pseudomonas stutzeri strain BN that degrades benzene using nitrate as electron acceptor was isolated from a chemostat. Furthermore, its genome sequence gave insight into the biochemical pathways of the strain and phylogenetical analyses and physiological experiments characterized the strain in more detail (Chapter 6).

The genome sequence of *Georgfuchsia toluolica* strain G5G6^T, that degrades toluene with nitrate as electron acceptor (Weelink, Van Doesburg *et al.* 2009), was used to characterize the strain and the biochemical pathways of the strain in more detail. Furthermore, the metagenome of the polluted aquifer from which the strain was isolated gave insight in the natural occurrence of the strain (Chapter 7).

Pseudomonas chloritidismutans strain AW-1^T degrades *n*-alkanes with chlorate as electron acceptor (Mehboob, Junca *et al.* 2009). Genome sequencing and proteomics gave more insight in the biochemical pathways involved in *n*-decane degradation with chlorate (Chapter 8).

The findings of the research described have been summarized and placed in a broader perspective in the general discussion of this thesis. In addition, future studies are suggested (Chapter 9).

Chapter 2

Genome analysis and physiological comparison of *Alicycliphilus denitrificans* strains BC and K601^{T}

Authors and affiliations
Margreet J. Oosterkamp[1(a)]*, Teun Veuskens[1]*, Flávia Talarico Saia [1(b)], Sander A.B. Weelink[1(c)], Lynne A. Goodwin[2], Hajnalka E. Daligault[2], David C. Bruce[2], John C. Detter[2], Roxanne Tapia[2], Cliff S. Han[2], Miriam L. Land[3], Loren J. Hauser[3], Alette A.M. Langenhoff[4(d)], Jan Gerritse[4], Willem J.H. van Berkel[5], Dietmar H. Pieper[6], Howard Junca[7], Hauke Smidt[1], Gosse Schraa[1], Mark Davids[8], Peter J. Schaap[8], Caroline M. Plugge[1] and Alfons J.M. Stams[1,9#]

1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
2 Los Alamos National Laboratory, Bioscience Division B-6, Genome Science, Joint Genome Institute, Los Alamos, New Mexico 87545, USA.
3 BioEnergy Science Center and Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA.
4 Deltares, Princetonlaan 6, 3584 CB Utrecht, The Netherlands.
5 Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA, Wageningen, The Netherlands.
6 Microbial Interactions and Processes Research Group, HZI – Helmholz Centre for Infection Research, Inhoffenstrasse 7, D-38124 Braunschweig, Germany.
7 Research Group Microbial Ecology: Metabolism, Genomics and Evolution of Communities of Environmental Microorganisms, CorpoGen, Carrera 5 # 66A-34, Bogotá, Colombia.
8 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
9 Centre of Biological Engineering, Campus de Gualtar, University of Minho, 4710-057, Braga, Portugal.
(a) Present address: Department of Animal Sciences, Energy Biosciences Institute, Institute of Genomic Biology, University of Illinois at Urbana-Champaign, 1207 W Gregory Drive, Urbana, Illinois 61801, USA.
(b) Present address: Laboratory of Biological Process, Department of Hydraulic and Sanitation, São Carlos School of Engineering, University of São Paulo, Av. Trabalhador São-Carlense, 400, São Carlos 13566-590, SP, Brazil.
(c) Present address: Tauw B.V., Handelskade 11, P.O. Box 133, 7400 AC Deventer, The Netherlands.
(d) Present address: Sub-Department of Environmental Technology, Wageningen University, P.O. Box 17, 6700 AA The Netherlands.
* These authors contributed equally
# Correspondent: e-mail: fons.stams@wur.nl; tel.: (+31) 317 483101; fax: +(31) 317 483829

Keywords
Alicycliphilus denitrificans, genome, anaerobic, chlorate, nitrate, benzene, aromatic compound, oxygenase, aliphatic compound

Manuscript status
Published article (Oosterkamp, Veuskens *et al.* 2013)

Abstract

The genomes of the Betaproteobacteria *Alicycliphilus denitrificans* strains BC and K601^T have been sequenced to get insight into the physiology of the two strains. Strain BC degrades benzene with chlorate as electron acceptor. The cyclohexanol-degrading denitrifying strain K601^T is not able to use chlorate as electron acceptor, while strain BC cannot degrade cyclohexanol. The 16S rRNA sequences of strains BC and K601^T are identical and the fatty acid methyl ester patterns of the strains are similar. Basic Local Alignment Search Tool (BLAST) analysis of predicted open reading frames of both strains showed most hits with *Acidovorax* sp. JS42, a bacterium that degrades nitro-aromatics. The genomes include strain-specific plasmids (pAlide201 in strain K601^T and pAlide01 and pAlide02 in strain BC). Key genes of chlorate reduction in strain BC were located on a 120 kb megaplasmid (pAlide01), which was absent in strain K601^T. Genes involved in cyclohexanol degradation were only found in strain K601^T. Benzene and toluene are degraded via oxygenase-mediated pathways in both strains. Genes involved in the *meta*-cleavage pathway of catechol are present in the genomes of both strains. Strain BC also contains all genes of the *ortho*-cleavage pathway. The large number of mono- and dioxygenase genes in the genomes suggests that the two strains have a broader substrate range than known thus far.

Introduction

Microbial (per)chlorate-reducing bacteria are able to produce oxygen as intermediate during anaerobic respiration with perchlorate and chlorate (Coates, Bruce *et al.* 1999; Coates and Achenbach 2004; Weelink, Tan *et al.* 2008; Weelink, van Eekert *et al.* 2010). This process can create possible advantages in *in-situ* bioremediation of anaerobic environments where pollutants like aromatic hydrocarbons persist that are more prone to aerobic degradation (Coates, Bruce et al. 1999; Logan and Wu 2002). *Alicycliphilus denitrificans* strain BC is able to couple benzene and toluene degradation to chlorate reduction (Weelink, Tan et al. 2008). Strain BC and *A. denitrificans* strain K601^T are members of the Comamonadaceae family of the Betaproteobacteria (Weelink, Tan et al. 2008). *A. denitrificans* strain K601^T was isolated with cyclohexanol and nitrate as substrates (Mechichi, Stackebrandt et al. 2003). Contrary to strain BC, strain K601^T lacks the chlorate-reducing capability. Strain BC, on the other hand, cannot degrade cyclohexanol (Weelink, Tan et al. 2008; Jin, Straathof et al. 2011).

Benzene degradation coupled to chlorate reduction was proposed to be an aerobic process, in which oxygen is derived from the conversion of chlorate is used in oxygenase-dependent pathways (Ginkel, Rikken *et al.* 1996; Rikken, Kroon *et al.* 1996; Logan 1998; Weelink, Tan *et al.* 2008). Aerobic degradation of benzene and other aromatic hydrocarbons is well-studied (Cao, Nagarajan et al. 2009; Morikawa 2010; Di Gennaro, Bargna et al. 2011). In aerobic microorganisms, benzene degradation may be initiated by Rieske non-heme iron oxygenases, which catalyze a one-step incorporation of dioxygen into their substrates (Gibson and Parales 2000; Suenaga, Koyama et al. 2009) or by two successive monooxygenations. These monooxygenations are catalyzed by distinct multicomponent toluene/benzene monooxygenases, which produce intermediate phenols, and successively by multicomponent phenol monooxygenases, forming catechols (Pérez-Pantoja, Gonzáles *et al.* 2010). The end product of catechol degradation is acetyl-CoA that can enter the citric acid cycle.

Degradation of aliphatic hydrocarbons, such as cyclohexanol and cyclohexanone can occur via aerobic and anaerobic pathways (Norris and Trudgill 1971; Trudgill 1984; Dangel, Tschech et al. 1988; Dangel, Tschech et al. 1989). Aerobic degradation of cyclohexanol is mediated by monooxygenases that cleave the aromatic ring. *A. denitrificans* strain K601^T degrades cyclohexanol under anaerobic conditions (Mechichi, Stackebrandt et al. 2003). Likely, in the anaerobic cyclohexanol degradation pathway cyclohexanol is oxidized to 2-cyclohexenone via cyclohexanone. The enzyme that mediates conversion of 2-cyclohexenone is a bifunctional oxidoreductase that catalyzes both the Michael addition of water to 2-cyclohexenone and the subsequent oxidation of the resulting 3-hydroxycyclohexanone to 1,3-cyclohexanedione (Jin, Straathof et al. 2011).

We aimed to obtain insight in the physiological properties of *A. denitrificans* strains BC and K601^T and in the pathways involved in degradation of aromatic and alicyclic compounds with different electron acceptors. Therefore, we studied the genome sequences of *A. denitrificans* strain BC and strain K601^T and performed more detailed physiological comparisons of these strains.

Materials and Methods

Cultivation and DNA isolation

A. denitrificans strain BC (DSM 18852, JCM 14587) was isolated and described previously (Weelink, Tan et al. 2008). *A. denitrificans* strain K601^T (DSM 14773, CIP 107495) was purchased from the DSMZ, (Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH, Braunschweig, Germany). The strains were cultivated in AW1-sulfate medium as described previously (Weelink, Tan et al. 2007). For DNA isolation cells were grown in 1.2 L-bottles containing 500 mL medium with acetate (10 mM) and nitrate (10 mM). Cultures were incubated at 30^{o}C without agitation. Cells were harvested by centrifugation and genomic DNA was isolated

following the protocol for bacterial genomic DNA isolation using CTAB of DOE JGI (U.S. Department Of Energy, Joint Genome Institute, CA, USA). DNA concentration was measured using Nanodrop (Thermo scientific) and DNA integrity and quality were determined by loading the genomic DNA on a 1% agarose gel with size and concentration markers according to the instructions of DOE JGI.

Growth experiments

To determine the substrate spectrum of the *A. denitrificans* strains, different electron donors were tested in duplicate batches with nitrate (10 mM), oxygen (5% in headspace) or chlorate (10 mM) as electron acceptor. Late log-phase cells of strain BC grown on acetate (10 mM) and nitrate (10 mM) were used as inoculum (5%) for all batches except for batches with aromatic compounds as substrate. In these batches late log-phase cells grown on either benzene (repeated feeds of 0.5 mM) or acetate (10 mM) and chlorate (10 mM) were used as inoculum (5%-10%). Physiological properties of strain K601^T were described before (Mechichi, Stackebrandt et al. 2003), but additional substrate tests were performed. Late log-phase cells of strain K601^T grown on acetate (10 mM) and either oxygen (5% in headspace) or nitrate (10 mM) were used as inoculum in these tests. Growth was monitored by visual observation of turbidity and the decrease in electron acceptor and donor concentration. Analytical procedures were as described previously (Weelink, Tan et al. 2008).

Genome sequencing, assembly and annotation

High molecular weight genomic DNA of *A. denitrificans* strains BC and K601^T was provided to the DOE JGI. For cloning and a combination of Illumina GAii and 454 shotgun sequencing (Bennett 2004; Margulies, Egholm et al. 2005), a combination of small and large insert libraries were prepared. For strain BC the Illumina GAii shotgun library generated 32,476,780 reads comprising 1,169 Mb and for strain K601^T this generated 28,774,946 reads comprising 2,186 Mb. A 454 Titanium standard library generated 198,756 reads for strain BC and 637,992 reads for strain K601^T. For strain BC a paired end 454 library generated 83,659 reads comprising 191 Mb and for strain K601^T this generated 314,193 reads comprising 281.7 Mb of 454 paired end data. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov/. The initial draft assembly of strain BC contained 120 contigs in 3 scaffolds and the draft assembly of strain K601^T contained 175 contigs in 2 scaffolds.

The 454 Titanium standard data and the 454 paired end data were assembled together with Newbler, version 2.3. The Newbler consensus sequences were computationally shredded into 2 kb overlapping fake reads (shreds). Illumina sequencing data were assembled with VELVET, version 0.7.63 (Zerbino and Birney 2008), and the consensus sequences were computationally shredded into 1.5 kb shreds. We integrated the 454 Newbler consensus shreds, the Illumina VELVET consensus shreds and the read pairs in the 454 paired end library using parallel phrap, version SPS - 4.24 (High Performance Software, LLC). The software Consed was used in the following finishing process (Ewing and Green 1998; Ewing, Hillier et al. 1998; Gordon, Abajian et al. 1998). Illumina data were used to correct potential base errors and increase consensus quality using the software Polisher developed at JGI (www.jgi.doe.gov/software). Possible mis-assemblies were corrected using gapResolution (www.jgi.doe.gov/software), Dupfinisher (Han and Chain 2006), or sequencing cloned bridging PCR fragments with subcloning. Gaps between contigs were closed by editing in Consed, by PCR and by Bubble PCR primer walks (Cheng, unpublished). To close gaps and to raise the quality of the finished sequence, a total of 511 additional reactions were necessary for strain BC, for strain K601^T a total of 415 additional reactions were necessary. The total size of the genome of strain BC is 4,835,713 bp and the genome size of strain K601^T is 5,070,751 bp.

The final assembly is based on 191 Mb and 227 Mb of 454 draft data for strains BC and K601^T, respectively. This provides an average 40x coverage for the genome of strain BC

and an average 45x coverage of the genome of strain K601^T. Additionally, the final genomes are based on 650 Mb and 2,099 Mb of Illumina draft data for strains BC and K601^T, respectively, which provides an average 135x coverage of the genome of strain BC and an average 416.3x coverage of the genome of strain K601^T.

Genes were identified using Prodigal (Hyatt, Chen et al. 2010) as part of the Oak Ridge National Laboratory genome annotation pipeline, followed by a round of manual curation using the JGI GenePRIMP pipeline (Pati, Ivanova et al. 2010). The predicted CDSs were translated and used to search the National Center for Biotechnology Information (NCBI) nonredundant database, UniProt, TIGRFam, Pfam, PRIAM, KEGG, COG, and InterPro databases. These data sources were combined to assert a product description for each predicted protein. Non-coding genes and miscellaneous features were predicted using tRNAscan-SE (Lowe and Eddy 1997), RNAMMer (Lagesen, Hallin et al. 2007), Rfam (Griffiths-Jones, Bateman et al. 2003), TMHMM (Krogh, Larsson et al. 2001), and signalP (Bendtsen, Nielsen et al. 2004).

The complete final assemblies were released on September 3 2010 (strain BC) and on January 7 2011 (strain K601^T). The genomes were implemented in GenBank. For strain BC the accession numbers are CP002449 (chromosome), CP002450 (megaplasmid), CP002451 (plasmid). For strain K601^T the accession numbers are CP002657 (chromosome) and CP002658 (plasmid).

Bidirectional BLAST analysis

The genomes of *A. denitrificans* strains BC and K601^T were compared using bidirectional BLAST analysis. The FTP server of NCBI (http://www.ncbi.nlm.nih.gov/Ftp) was used to download the protein sequence files obtained from the genome sequences of strains BC and K601^T. Bidirectional best hits were obtained by BLAST using a similarity threshold of 50% and a sequence length mismatch of 80 to 120% (Altschul, Madden et al. 1997).

Comparative DNA and cellular property analysis of strains BC and K601^T

The G+C content, DNA-DNA hybridization and cellular fatty acids analysis were performed by the DSMZ (Braunschweig, Germany). For cellular fatty acid analysis, strains BC and K601^T (DSM 14773^T) were grown under identical conditions, i.e. acetate (10 mM) and nitrate (10 mM) in 2 L AW-1-sulfate medium at 30°C (Weelink, Tan et al. 2007). ANI and TETRA of strain BC against strain K601^T and against *Acidovorax* sp. JS42, were determined using the software program JSpecies (http://www.imedea.uib.es/jspecies/). ANIb, ANIm and TETRA are calculated as described by Richter and Rosselló-Móra (Richter and Rosselló-Móra 2009).

Results and Discussion

Comparison of strains BC and K601^T

The genomes of *A. denitrificans* strains BC and K601^T have been annotated (Oosterkamp, Veuskens et al. 2011). Based on 16S rRNA gene sequence analysis *A. denitrificans* clusters in the family Comamonadaceae of the Betaproteobacteria (Weelink, Tan et al. 2008). Strains K601^T and BC showed 99.7% 16S rRNA gene similarity (Weelink, Tan et al. 2008). This study reveals that the three 16S rRNA gene copies present in each genome are identical for both strain BC and K601^T, but have different gene location and orientation in each of the strains (Table S1), indicating that the genomes have a different topology. However, the general characteristics of the genomes of strains BC and K601^T are similar (Table 1). Furthermore, the strain-specific fatty acid methyl ester patterns of strains BC and K601^T are similar (Table S2).

Table 1. General features of the genomes of *A. denitrificans* strains BC and K601^T.

		Strain BC	Strain K601^T
Genome size		4,835,713 bp	5,070,751 bp
G+C content		67.9 %	67.8 %
DNA scaffolds		3	2
Chromosome	Size	4,637,013 bp	4,995,263 bp
	Coding DNA	91%	90%
	G+C content	68%	67%
Plasmid	Size	78,982 bp	75,488 bp
	Coding DNA	84%	87%
	G+C content	64%	62%
Megaplasmid	Size	119,718 bp	-
	Coding DNA	78%	-
	G+C content	58%	-
Total gene number		4709	4899
Protein coding genes		4542	4696
Pseudogenes		101	136
rRNA genes			
5S rRNA		3	3
16S rRNA		3	3
23S rRNA		3	3
tRNA genes		53	54

The G+C content of *A. denitrificans* strains K601^T and BC is 66.0 and 67.6 mol%, respectively, as determined by conventional methods (Mechichi, Stackebrandt et al. 2003; Weelink, Tan et al. 2008). These values are comparable to those determined from the genomic DNA sequences of strains BC and K601^T, which gave values of 67.9% and 67.8%, respectively. Experimental DNA-DNA hybridization of strain BC against K601^T showed 74.5 $\pm$ 3.5% similarity. Based on the genome size difference this value seems low, but the genome of strain K601^T is about 7% larger than the genome of strain BC. Genome size differences may affect DNA-DNA hybridization values. However, the experimental error of DNA-DNA hybridization is too high for the genome size difference to have an effect. For species circumscription, a cut-off point of 70% DNA-DNA hybridization similarity is generally used. This cut-off point corresponds to 95% average nucleotide identity of genes present in both strains tested. Based on this cut-off for average nucleotide identity, a tetranucleotide frequency correlation coefficient of >0.99 may support species delineation (Goris, Konstantinidis *et al.* 2007; Richter and Rosselló-Móra 2009). Values for average nucleotide identity (ANI) and the tetranucleotide frequency correlation coefficient (TETRA) can be determined using the software programme JSpecies (Richter and Rosselló-Móra 2009). According to JSpecies, the ANIb (BLAST calculation of ANI) value of strain BC compared to K601^T is 98.71% and the ANIm (MUMmer calculation of ANI) value is 99.60%, both well above the threshold of 95% for circumscribing species. The TETRA value was 0.9995, which is above the boundary of 0.99. In summary, ANIb, ANIm and TETRA values also indicate that both strains belong to the same species. As a comparison, we determined the values when comparing strain BC and another member of the Comamonadaceae family, *Acidovorax* sp. JS42, to which strain BC is closely related. In this case the ANIb value was 84.11%, the ANIm value 87.04% and the TETRA value 0.9756, confirming that these strains are different species, while there was 97% 16S rRNA similarity between the strains.

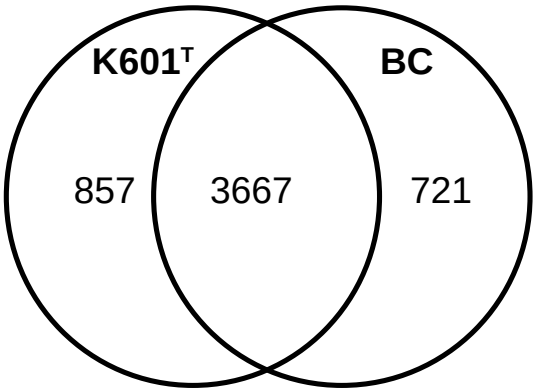


Figure 1. Bidirectional BLAST analysis of the genomes of *A. denitrificans* strains K601^T and BC. The amount of protein sequences present only in strains K601^T (left) and BC (right) and in both strains (center) is shown in the VENN diagram. 172 protein sequences of strain K601^T and 154 of strain BC could not be assigned (for instance duplicates of sequences).

Bidirectional BLAST analysis showed that strain K601^T contains 857 proteins that are not present in strain BC and that strain BC has 721 proteins not present in strain K601^T (Fig. 1). An overview of the main metabolic pathways deduced from the genomes of the *A. denitrificans* strains is depicted in Fig. 2, and specific pathways for strain BC or K601^T are indicated. Lists of genes involved in these pathways are given from Table S3 to S5.

Chlorate, nitrate and oxygen respiration pathways

In contrast to strain K601^T, strain BC contains a megaplasmid harboring the genes involved in respiratory chlorate reduction (Alide01) (Oosterkamp, Veuskens et al. 2011). To date, it is unknown if the megaplasmid can be transferred to other strains, e.g. to strain K601^T and if this plasmid allows other strains to grow by respiratory chlorate reduction.

Alide_4611-4614 encode subunits of a DMSO reductase family type II enzyme, or more specifically these genes encode chlorate reductase. Chlorate reductase is composed of four subunits encoded by the *clrABCD* genes (Alide_4611-4614). The chlorite dismutase gene (*cld*, Alide_4615) is transcribed in opposite direction (forward) compared to the genes encoding chlorate reductase (reverse). The gene cluster for chlorate reduction of strain BC is highly similar to the cluster of *Ideonella dechloratans* (Fig. 3), though it is not known whether the genes are plasmid-encoded in this bacterium (Thorell, Karlsson et al. 2002; Thorell, Stenklo et al. 2003). In *Dechloromonas aromatica* strain RCB genes encoding perchlorate reductase and chlorite dismutase are clustered and located on the chromosome (Daro_2580-2584, GenBank accession number of genome CP000089). These genes are clustered in *D. agitata* as well (Bender, Shang et al. 2005). Recently, it was found that the genes encoding (per)chlorate reductase and chlorite dismutase in these *Dechloromonas* strains and two other (per)chlorate-reducing bacteria are located on a genomic island in the chromosomes (Melnyk, Engelbrektson et al. 2011).

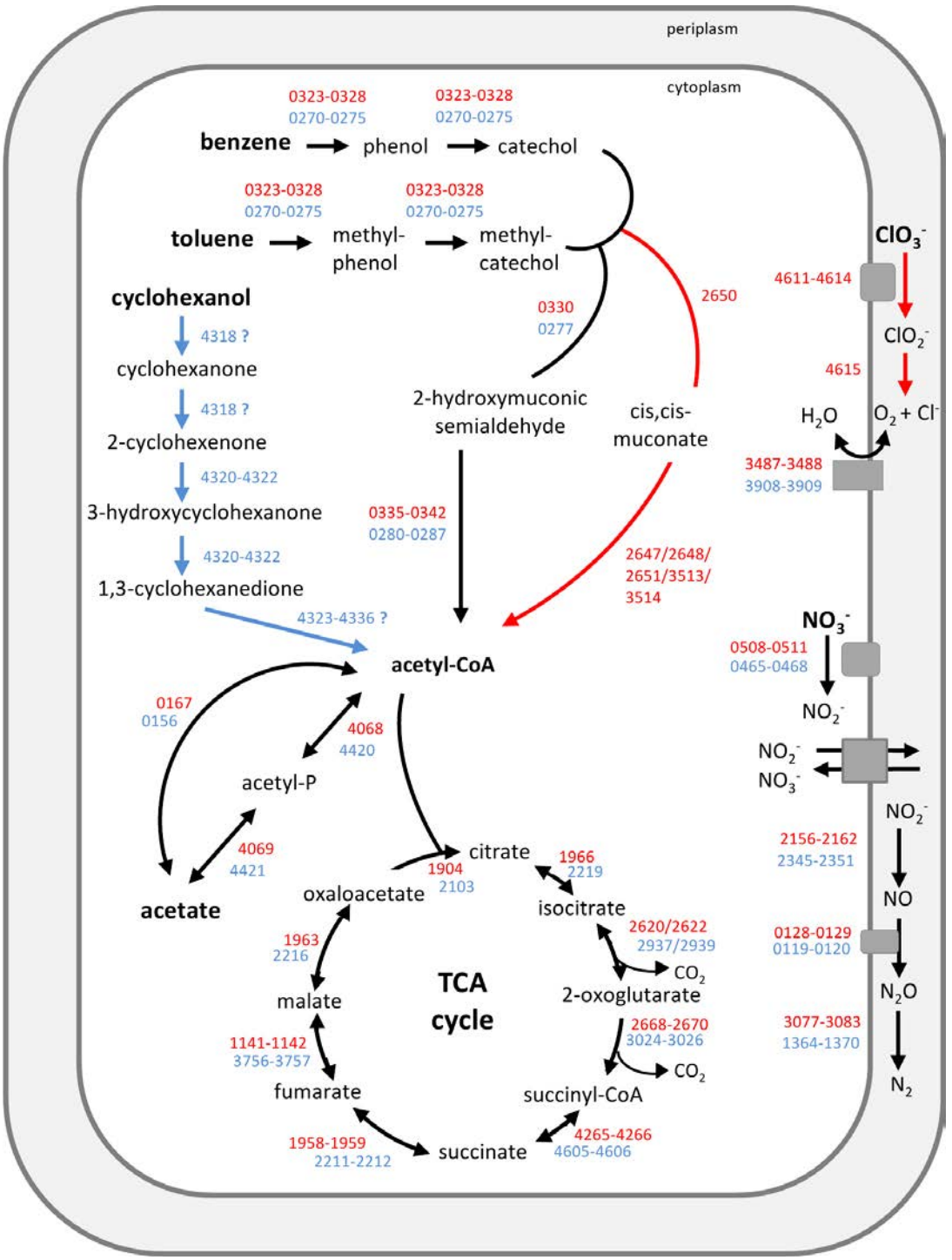


Figure 2. Main metabolic pathways of *A. denitrificans*. Pathways are indicated using arrows. Black arrows indicate pathways of both strain BC and K601^T, red arrows indicate pathways of strain BC, and blue arrows pathways of strain K601^T. Red gene numbers indicate genes of strain BC (geneID is Alide_red gene number) and blue gene numbers genes of strain K601^T (geneID is Alide2_blue gene number).

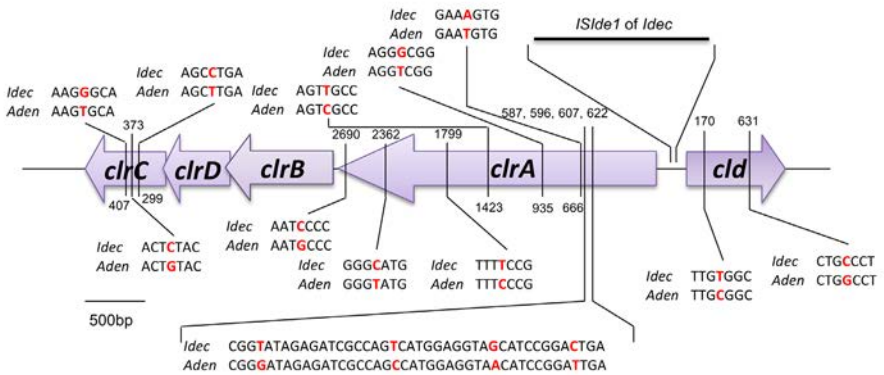


Figure 3. Gene cluster for chlorate reduction in *A. denitrificans* strain BC (*Aden*) compared to *I. dechloratans* (*Idec*). The gene cluster for chlorate reduction comprises of chlorite dismutase (*cld*), chlorate reductase subunit A, B, C and D (*clrA, clrB, clrC, clrD*), and in *I. dechloratans* it also includes an insertion sequence (*ISIde1*). The numbers represent the location of nucleotide differences (in red) of strain BC compared to *I. dechloratans* counted from the first nucleotide of each gene. The scale bar represents 500 bp. Sequences for the chlorate reduction gene cluster of *I. dechloratans* were obtained from the EMBL nucleotide sequence database (accession numbers AJ296077 and AJ566363).

Three types of nitrate reductases are known, the dissimilatory membrane-associated Nar, the dissimilatory periplasmic Nap and the assimilatory cytoplasmic Nas. Nas is exclusively involved in assimilatory nitrate reduction to ammonium (Richardson, Berks et al. 2001; Stolz and Basu 2002). Nar and Nap differ with respect to chlorate reduction; Nar can catalyze chlorate reduction, but Nap cannot or only poorly reduce chlorate (Bell, Richardson et al. 1990; Bedzyk, Wang et al. 1999; Rusmana and Nedwell 2004; Oosterkamp, Mehboob et al. 2011). Strains BC and K601^T have a functional Nar (the gene product of Alide_0508-0511 in BC and of Alide2_0465-0468 in K601^T), but no functional Nap or Nas.

Putative nitrite reductase, nitric oxide reductase and nitrous oxide reductase encoding genes (*nir* genes: Alide_2156-2162 in BC and Alide2_2345-2351 in K601^T, *nor* genes: Alide_0128 in BC and Alide2_0119 in K601^T, and *nos* genes: Alide_3077-3083 in BC and Alide2_1364-1370 in K601^T) indicate that nitrate is reduced to N_2, which is in accordance with physiological tests (Mechichi, Stackebrandt et al. 2003; Weelink, Tan et al. 2008).

A. denitrificans strains BC and K601^T are facultative anaerobes (Mechichi, Stackebrandt et al. 2003; Weelink, Tan et al. 2008). In the presence of oxygen, cytochrome oxidases catalyze the reduction of oxygen to water, resulting in proton translocation and generating ATP by electron transport phosphorylation (Brunori, Giuffrè et al. 2005; Brzezinski and Gennis 2008). Several cytochrome oxidases are known (Thony-Meyer 1997). The genomes of strains BC and K601^T encode cytochrome oxidases that can be used under aerobic and micro-aerophilic (high-oxygen affinity) conditions. Cytochrome *c* oxidase genes are present (Alide_2815, 2816, 3608, 3609, 3487, 3488 in strain BC and Alide2_1643, 1644, 3908, 3909, 3953, 3954 in strain K601^T). Furthermore, the genomes contain genes encoding cytochrome *o* ubiquinol oxidase (Alide_1992-1995 in strain BC and Alide2_2246-2249 in strain K601^T) that are employed at

high oxygen concentration. At low oxygen concentration, high-affinity cytochrome oxidases are used. The genomes of strains BC and K601^T contain cytochrome *bd* ubiquinol oxidase coding genes (Alide_2141 and 2142 in strain BC and Alide2_2330 and 2331 in strain K601^T) and cytochrome *c* oxidase cbb_3-type coding genes (Alide_3325-3328 in strain BC and Alide2_1119-1122 in strain K601^T). An overview of genes involved in respiration in *A. denitrificans* strains BC and K601^T is shown in Table S4.

Degradation pathways of aromatic and alicyclic compounds

Strains K601^T and BC are able to degrade benzene and toluene with oxygen, but not with nitrate as electron acceptor (Table 2), indicating that oxygenases are involved in the initial degradation steps of these aromatic compounds. In previous research, two oxygenase-coding genes involved in the initial successive oxidation reactions (*BC-BMOa*) and the subsequent cleavage of catechol (*BC-C23O*) were identified in the *Alicycliphilus* strains (Weelink, Tan et al. 2008). Monooxygenases that catalyze the conversion of benzene or toluene to phenol or methylphenol (benzene/toluene monooxygenases) and of phenols to catechols (phenol monooxygenases), belong to an evolutionary related family of soluble diiron monooxygenases (Leahy, Batchelor et al. 2003). Based on their alpha subunits, which are assumed to be the site of substrate hydroxylation, phenol as well as benzene/toluene monooxygenases can be differentiated within this family (Leahy, Batchelor et al. 2003), (Pérez-Pantoja, Donoso et al. 2012). Genome analysis confirmed the presence of a multicomponent phenol monooxygenase (Alide_0323–0328 in BC; Alide2_0270–0275 in K601^T) (Weelink, Tan et al. 2008). The absence of other benzene/toluene mono- and/or dioxygenases suggests that the phenol monooxygenase is responsible for both the hydroxylation of benzene (and/or toluene) to (methyl-)phenol and the subsequent hydroxylation of (methyl-)phenol to (methyl-)catechol. Hydroxylation of the benzene ring catalyzed by phenol monooxygenases has been reported (Pérez-Pantoja, Gonzáles *et al.* 2010). Phenol monooxygenase of *Pseudomonas stutzeri* strain OX1 transforms benzene and toluene to catechol and 3-methylcatechol (via phenol and 2-methylphenol), respectively (Cafaro, Izzo et al. 2004). Moreover, toluene-2-monooxygenase of *Burkholderia cepacia* strain G4 oxidizes toluene to 3-methylcatechol (Newman and Wackett 1995). Although often lacking among phenol hydroxylase clusters (Leahy, Batchelor et al. 2003), both strain BC and K601^T contain a gene coding for a ferredoxin (Alide_0329 in BC; Alide2_0276 in K601^T) clustered with the phenol monooxygenase encoding genes. Furthermore, the phenol monooxygenase gene clusters contain genes encoding sigma54 specific transcriptional regulators (Alide_0322 and 0334 in BC; Alide2_0269 and 0279 in K601^T) (Fig. 4). The protein products of Alide_0322 and Alide2_0269 have homology to regulatory proteins comprised in previously described phenol monooxygenase gene clusters, such as DmpR of *Pseudomonas sp.* strain CF600 (45% homology on protein level) that regulates transcription based on direct interaction with aromatic compounds (Shingler and Moore 1994). A similar multicomponent phenol monooxygenase cluster is present in the close relative *Acidovorax sp.* strain JS42 (Ajs_0206-0210, which has 72% similarity on protein level) (Kivisaar 2009). *D. aromatica* strain RCB and *Comamonas* sp. strain E6 contain similar monooxygenase clusters with 76% and 86% identity on protein level, respectively (Salinero, Keller et al. 2009), and *D. aromatica* strain RCB also contains a benzene/toluene monooxygenase gene cluster.

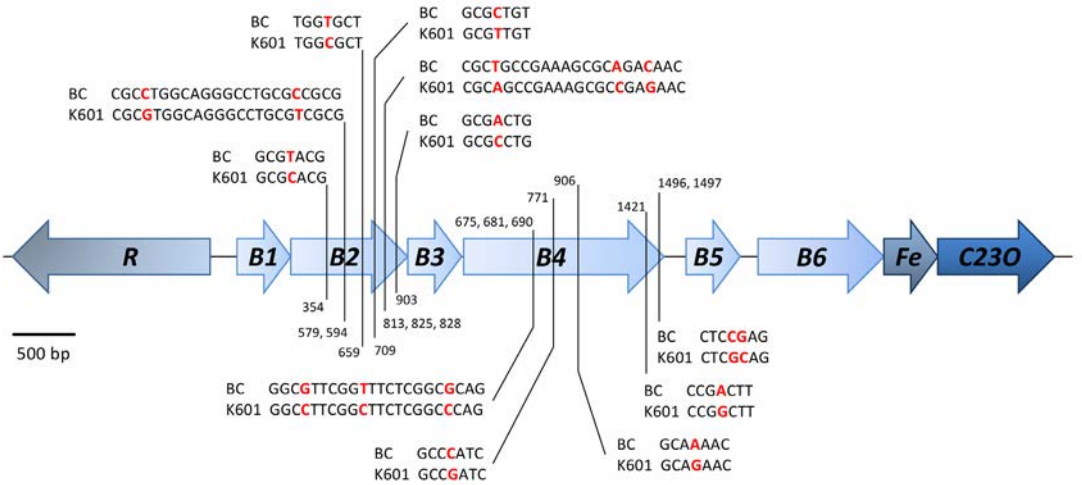


Figure 4. Organization of the multicomponent benzene/phenol monooxygenase cluster (B1-B6) and catechol dioxygenases (C23O) of *A. denitrificans* strains BC and K601^T. In this gene cluster a gene coding for a transcriptional regulator (R) and a gene coding for a ferredoxin (Fe) were also found. Both strains BC and K601^T have highly similar gene clusters (99%) with differences only in subunit B2 and B4. The numbers represent the location of the nucleotide differences (in red) of strain BC compared to K601^T counted from the first nucleotide of each gene. The scale bar represents 500 bp.

Benzene and toluene degradation leads to the formation of (methyl)catechol. There are two routes of aerobic catechol degradation, the *meta*- and the *ortho*-cleavage pathway. All genes involved in the *meta*-cleavage pathway of (methyl)catechol degradation are present in the genomes of strains BC and K601^T. We confirmed the presence of genes encoding a catechol 2,3-dioxygenase in strain BC as reported previously (Weelink, Tan et al. 2008) and found homologous genes in strain K601^T (Alide_0330 in BC; Alide2_0277 in K601^T). This catechol 2,3-dioxygenase catalyzes the extradiol cleavage of catechol to 2-hydroxymuconic semialdehyde. Among the 16 sequenced strains of Comamonadaceae only in five strains catechol 2,3-dioxygenase genes are present (Pérez-Pantoja, Donoso et al. 2012). *Acidovorax sp*. JS42 contains a catechol 2,3-dioxygenase homologous to that of strains BC and K601^T with 92% similarity on protein level (Ajs_0214) (Kivisaar 2009). Further degradation of 2-hydroxymuconic semialdehyde can proceed via the hydrolytic or the oxalocrotonate branch of the *meta*-cleavage pathway (Harayama, Mermod et al. 1987). Genes encoding enzymes involved in both of these branches are present in the genomes of strains BC and K601^T (Fig. 2). The hydrolytic branch is used when toluene is converted via 3-methylcatechol and involves degradation of 2-hydroxymuconic semialdehyde to 2-oxopent-4-enoate. The enzyme catalyzing this conversion is a 2-hydroxymuconic semialdehyde hydrolase (Alide_0336 in BC; Alide2_0281 in K601^T). Methyl-catechol and catechol are converted to 2-oxopent-4-enoate using the oxalocrotonate branch of the *meta*-cleavage pathway, which proceeds via a dehydrogenase, tautomerase and decarboxylase (Alide_0335+0340+0342 in BC; Alide2_0280+0285+0287 in K601^T). Finally, 2-oxopent-4-enoate is converted to acetyl-CoA that can enter the citric acid cycle and the genes encoding the enzymes involved in this conversion are Alide_337-339 in strain BC and Alide2_282-284 in strain K601^T.

Strain BC, but not K601^T, also contains all genes essential for *ortho*-cleavage of catechol. In this pathway, catechol is converted by catechol 1,2-dioxygenase (encoded by Alide_2650) to *cis,cis*-muconate, which via muconolactone, 3-oxoadipate-enol-lactone, 3-oxoadipate and 3-oxoadipyl-coA, is converted to acetyl-coA (Alide_2647+2648+2651+3513+3514 of strain BC) (Harwood and Parales 1996). Strain K601^T lacks genes coding for catechol 1,2-dioxygenase, muconate cycloisomerase and 3-oxoadipate-enol-lactonase, rendering the *ortho*-cleavage pathway incomplete.

Although anaerobic benzene degradation was described for some pure bacterial cultures, information about the degradation pathways is incomplete (Musat and Widdel 2008; Abu Laban, Selesi *et al.* 2009; Weelink, van Eekert *et al.* 2010; Vogt, Kleinsteuber *et al.* 2011). *D. aromatica* strain RCB is capable of anaerobic degradation of all BTEX compounds with nitrate as electron acceptor, but *A. denitrificans* strain BC and K601^T cannot degrade these compounds with nitrate while acetate can be degraded with nitrate as electron acceptor (Fig. 5). This is confirmed by the absence of genes that code for known key enzymes for anaerobic aromatic compound degradation in the genomes of strain BC and K601^T, such as

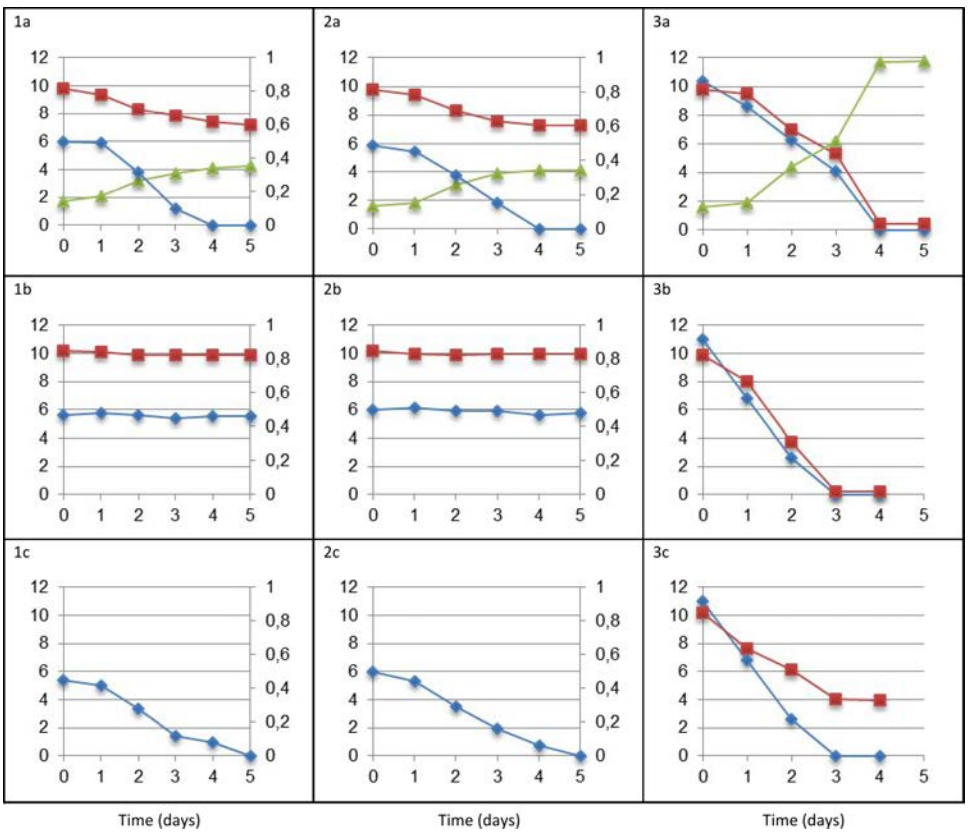


Figure 5. Degradation of benzene (1), toluene (2) and acetate (3) with chlorate (a), nitrate (b) or oxygen (c) as electron acceptor by *A. denitrificans* strain BC. Benzene, toluene and acetate degradation is indicated with diamonds. Benzene and toluene concentrations are outlined on a secondary y-axis while acetate and electron acceptor concentrations are indicated on the primary y-axis. Chlorate, nitrate and oxygen consumption is depicted with squares. Chloride production when chlorate is used as electron acceptor is indicated with triangles and no electron acceptor consumption is shown when no significant difference could be observed because of presence of the electron acceptor in abundance.

benzylsuccinate synthase or ethylbenzene dehydrogenase. Remarkably, these key enzymes are also not present in *D. aromatica* strain RCB (Salinero, Keller et al. 2009). Strain RCB is able to couple benzene degradation to nitrate reduction, but the occurrence of a strict anaerobic pathway is not proven, and it has been suggested that strain RCB might activate benzene with oxygen produced from the reduction of nitrate or uses hydroxyl free radicals (Chakraborty and Coates 2005; Weelink, van Eekert *et al.* 2010). Oxygen production by nitrite reduction was found in the anaerobic methane degrading *Candidatus Methylomirabilis oxyfera* (Ettwig, Butler *et al.* 2010; Wu, Ettwig *et al.* 2011) and was reported as a possible mechanism for the initial alkane activation in strain HdN1 (Zedelius, Rabus et al. 2011). A similar mechanism was proposed for benzene and toluene degradation using chlorate as electron acceptor in strain BC (Weelink, Tan et al. 2008). Physiological tests showed that these compounds are degraded in 3 to 5 days in presence of chlorate or oxygen (Fig. 5). Both strains BC and K601^T are unable to aerobically utilize ethylbenzene, xylenes and benzoate (Table 2). Accordingly, enzymes involved in the initial steps of degradation of these compounds are not present in the genomes.

Cyclohexanol degradation by strain K601^T follows a proposed pathway via the intermediates cyclohexanone, 2-cyclohexenone, 3-hydoxycyclohexanone to 1,3-cyclohexanedione and subsequent cleavage of the ring (Jin, Straathof et al. 2011). Genes encoding a three subunit molybdoenzyme (Alide2_4320 – Alide2_4322) are present in the genome of strain K601^T, but are absent in strain BC. This gene cluster, homologous to carbon monoxide dehydrogenase (CO-DH) and xanthine dehydrogenase (Xdh), is identified as a bifunctional hydratase/alcohol dehydrogenase (MhyADH), belonging to the molybdopterin binding oxidoreductase family with the double function of hydration of cyclohexenone and oxidation of its product 3-hydroxycyclohexanone to 1,3-cyclohexanedione (Jin, Straathof et al. 2011). An ORF located close to the MhyADH shows a sequence (Alide2_4318) with similarity to a flavin-containing domain of fumarate reductase/succinate dehydrogenase and 3-ketosteroid dehydrogenase (KSTD). Based on the reactions catalyzed by those types of enzymes, this sequence might be coding for a cyclohexanone dehydrogenase (Jin, Straathof et al. 2011). Depending on the substrate specificity of the coding enzyme, the conversion of cyclohexanol to cyclohexanone could also be catalyzed by this enzyme. Since members of this group of enzymes are known to catalyze the oxidative hydroxylation of a wide range of aldehydes and aromatic heterocyclic compounds (Andrade, Brondino et al. 2000) the substrate range of strain K601^T might be broader than known so far. More genes closely located to the MhyADH cluster are identified as CO-DH genes (Alide2_4319; Alide2_4326) and are possibly also involved in cyclohexanol degradation.

A cyclopentanol dehydrogenase (Alide2_4312) and, furthermore, more mono- and dioxygenase genes were found in the genomes than the ones we already described, such as an extradiol ring cleavage dioxygenase (Alide_2035 in BC; Alide2_2289 in K601^T), a cytochrome P450 (Alide_3136 in BC; Alide2_1311) and several 2-nitropropane dioxygenases (Alide_0303, Alide_0687, Alide_2358, Alide_3754, Alide_3890, Alide_4340 in strain BC; Alide2_0250, Alide2_1856, Alide2_2552, Alide2_4108, Alide2_4236, Alide2_4670 in K601^T). This indicates that a broader spectrum of xenobiotic compounds might be degraded by the *Alicycliphilus* strains. A list of genes involved in degradation of aromatic and alicyclic compounds in strains BC and K601^T can be found in Table S5.

Other physiological characteristics

Strains BC and K601^T were physiologically characterized previously (Mechichi, Stackebrandt et al. 2003; Weelink, Tan et al. 2008). Additional physiological tests were performed based on the genome sequences. Genome analysis showed that there are no known sugar transporter genes in strains BC and K601^T. We did not observe growth of strains BC and K601^T with glucose and fructose (Table 2), though previously strain K601^T was described to use these sugars (Mechichi, Stackebrandt et al. 2003; Weelink, Tan et al. 2008). Comparative genome analysis showed that all genes of the tricarboxylic acid cycle are present in strains BC and K601^T (Table S3). The two bacteria use carboxylic acids like acetate, lactate,

succinate or fumarate as substrates for growth (Table 2). Strains BC and K601^T can use the amino acids glutamate and alanine as growth substrates and genes encoding glutamate dehydrogenase (Alide_0201+1063 in BC; Alide2_0190+4027 in K601^T) and beta-alanine-pyruvate transaminase (Alide_4363 in BC; Alide2_4693 in K601^T) were found in the genome.

Table 2. Overview of substrate range of *A. denitrificans* strains BC and K601^T.

		Strain BC			Strain K601^T	
Electron donor	**Concentration**	**NO_3^-**	**O_2**	**ClO_3^-**	**NO_3^-**	**O_2**
Acetate	10 mM	+	+	+	+[a]	+[a]
Lactate	10 mM	+	+	+	+[a]	+[a]
Pyruvate	10 mM	+	+	+	+[a]	+[a]
Succinate	10 mM	+	+	+	+[a]	+[a]
Propionate	10 mM	+	+	+	+[a]	+[a]
Butyrate	10 mM	+	+	+	+[a]	+[a]
Malate	10 mM	+	+	+	+[a]	+[a]
Citrate	10 mM	+	+	+	+[a]	+[a]
Fumarate	10 mM	+	+	+	+[a]	+[a]
Glucose	10 mM	-	-	-	-[a]	-
Fructose	10 mM	-	-	-	-[a]	-
Xylose	10 mM	-	n.d.	n.d.	-[a]	n.d.
Alanine	10 mM	-	+	+	+	+
Glycine	10 mM	-	-	-	-	-
Glutamate	10 mM	+	+	+	+	+
Ethanol	10 mM	-	n.d.	n.d.	-[a]	-[a]
Methanol	10 mM	-	-	-	-[a]	-[a]
Glycerol	10 mM	-	n.d.	n.d.	n.d.	+[a]
Benzene	0.25 mM	-	+	+	-	+
Toluene	0.25 mM	-	+	+	-	+
Ethylbenzene	0.25 mM	-	-	-	-	-
o-Xylene	0.1 mM	-	-	-	-	-
m-Xylene	0.1 mM	-	-	-	-	-
p-Xylene	0.1 mM	-	-	-	-	-
Benzoate	1 mM	-	-	-	-[a]	-[a]
Phenol	1 mM	-	+	+	-[a]	+
p-Hydroxybenzoate	1 mM	-	-	-	-[a]	+[a]
o-Cresol	1 mM	-	+	+	-[a]	+[a]
m-Cresol	1 mM	-	+	+	-[a]	+[a]
p-Cresol	1 mM	-	+	+	-[a]	+[a]
Monochlorobenzene	0.05 mM	-	-	-	-	-
Catechol	1 mM	-	+	+	-	+
Cyclohexanol	1 mM	-	-	-	+a	+a

+: growth, -: no growth, n.d.: not determined, [a]: previous data (Mechichi, Stackebrandt et al. 2003).

Concluding remarks

Bacteria that degrade benzene and other aromatic hydrocarbons in the absence of oxygen have two strategies for degradation: 1) employment of alternative pathways which are oxygenase-independent (Boll, Fuchs *et al.* 2002; Carmona, Zamarro *et al.* 2009; Weelink, van Eekert *et al.* 2010; Vogt, Kleinsteuber *et al.* 2011) and 2) as described here, production of oxygen in the reduction of the electron acceptor to employ oxygenase-dependent pathways. Here we present genome information that shows how *A. denitrificans* strain BC is able to couple benzene degradation to chlorate reduction. The key genes that code for enzymes that are essential for chlorate reduction and oxygen production are located at a plasmid. *A. denitrificans* strain K601^T lacks this plasmid and thus is not able to degrade benzene with chlorate. The two strains are not able to degrade benzene with nitrate. Only a few pure cultures of anaerobic benzene-degrading bacteria have been described. *Azoarcus* strain DN11 and AN9, *Dechloromonas aromatica* strain RCB and JJ and a *Bacillus cereus* strain were reported to degrade benzene with nitrate as electron acceptor (Chakraborty and Coates 2005; Kasai, Takahata *et al.* 2006; Dou, Ding *et al.* 2010; Holmes, Risso *et al.* 2011). Recently, *Bacillus subtilis* and *Pseudomonas aeruginosa* strains were found to degrade benzene with nitrate and oxygen as electron acceptors (Mukherjee and Bordoloi 2012). Thus far, it is not clear how these bacteria degrade benzene in the absence of oxygen. One of the options is an aerobic pathway involving oxygen derived from nitrate.

As aromatic hydrocarbons often accumulate in the anaerobic zones of soil, bacteria that are able to degrade hydrocarbons in the absence of oxygen are important for *in situ* bioremediation. However, oxygen is often difficult to introduce in soil. Chlorate and nitrate addition is an alternative to stimulate the breakdown of aromatic and aliphatic hydrocarbons. *A. denitrificans* strain BC has the ability to degrade some aromatic hydrocarbons, but its substrate range is limited. However, the observation that the essential genes for chlorate reduction (chlorate reductase and chlorite dismutase) are coded on a plasmid suggests that the ability to degrade hydrocarbons with chlorate can be transferred to bacteria with a wider substrate spectrum such as e.g. *Pseudomonas putida*. An important prerequisite, however, may be that the oxygenases possess a high affinity for oxygen.

Acknowledgments

This research was supported by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053, the graduate school WIMEK (Wageningen Institute for Environment and Climate Research, which is part of SENSE Research School for Socio-Economic and Natural Sciences of the Environment, www.wimek-new.wur.nl and www.sense.nl), SKB (Dutch Centre for Soil Quality Management and Knowledge Transfer, www.skbodem.nl) and the Consolider project CSD-2007-00055. The research was incorporated in the TRIAS (TRIpartite Approaches toward Soil systems processes) program (www.nwo.nl/trias). Flávia Talarico Saia was supported by a FAPESP (the State of São Paulo Research Foundation) scholarship (2006-01997/5). The work conducted by the DOE JGI is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

Table S1. Location of the 16S and 23S rRNA genes in the genomes of *A. denitrificans* strains BC and K601^T.

		Location in strain BC			Location in strain K601^T		
Gene		1	2	3	1	2	3
16S rRNA	Start (nucl)	3143946	4003821	4282912	1500507	4372093	4663323
	Stop (nucl)	3142430	4002305	4281396	1502023	4370577	4661807
	Length (bp)	1517	1517	1517	1517	1517	1517
	Orientation	-	-	-	+	-	-
23S rRNA	Start (nucl)	3141781	4001656	4280747	1502672	4369928	4661158
	Stop (nucl)	3138905	3998780	4277871	1505548	4367051	4658282
	Length (bp)	2877	2877	2877	2877	2878	2877
	Orientation	-	-	-	+	-	-

Table S2. Major cellular fatty acids of *A. denitrificans* strains BC and K601^T. Data of strain K601^{T}are from a previous study (Mechichi, Stackebrandt et al. 2003).

Major cellular fatty acids	Strain BC (this study) (%)	Strain K601^T (this study) (%)	Strain K601^T (previous study) (%)
C10:0	0.4	0.6	-
C10:0 3OH	2.4	4.2	-a
C12:0	2.7	4.3	4
C14:0	1.7	1.4	-
C15:0	n.d.	n.d.	2
C16:1 ω7c	10.9	27.0	37
C16:0	44.0	36.2	24
C17:1 ω7c	0.3	n.d	-
cycloC17:0	27.8	9.9	2
C18:3 ω6c (6,9,12)	n.d.	0.4	-
C18:1 ω7c	8.4	13.6	21
C18:0	0.25	0.4	-
cycloC19:0 ω8c	1.25	2.1	-

- n.d.: not detectable, -: unknown and [a]: occurred in 'small amounts'.

Table S3. List of genes involved in the citric acid cycle and acetate metabolism in *A. denitrificans* strains BC and K601^T.

Enzyme name	Reaction	GeneID in BC	GeneID in K601^T
Aconitase	citrate : isocitrate	Alide_1966	Alide2_2219
Isocitrate dehydrogenase	isocitrate : 2-oxoglutarate	Alide_2620	Alide2_2937
		Alide_2622	Alide2_2939
2-oxoglutarate dehydrogenase	2-oxoglutarate : succinyl-CoA	Alide_2668	Alide2_3024
		Alide_2669	Alide2_3025
		Alide_2670	Alide2_3026
Succinyl-CoA synthetase	succinyl-CoA : succinate	Alide_4265	Alide2_4605
		Alide_4266	Alide2_4606
Succinate dehydrogenase	succinate : fumarate	Alide_1958	Alide2_2211
		Alide_1959	Alide2_2212
Fumarate hydratase	fumarate : malate	Alide_1141	Alide2_3756
		Alide_1142	Alide2_3757
Malate dehydrogenase	malate : oxaloacetate	Alide_1963	Alide2_2216
Citrate synthase	oxaloacetate : citrate	Alide_1904	Alide2_2103
Acetate kinase	acetate : acetyl-phosphate	Alide_4069	Alide2_4421
Phosphate-acetyltransferase	acetyl-phosphate : acetyl-CoA	Alide_4068	Alide2_4420
Acetyl-CoA hydrolase/transferase	acetate : acetyl-CoA	Alide_0167	Alide2_0156
Acetate/CoA ligase	acetate : acetyl-CoA	Alide_1147	Alide2_3751

Table S4. List of genes involved in anaerobic respiration in *A. denitrificans* strains BC and K601^T.

Enzyme name	Reaction	GeneID in BC	GeneID in K601^T
Chlorate reductase	Chlorate : chlorite	Alide_4611	-
		Alide_4612	-
		Alide_4613	-
		Alide_4614	-
Chlorite dismutase	Chlorite : chloride	Alide_4615	-
Nitrate reductase	Nitrate : nitrite	Alide_0508	Alide2_0465
		Alide_0509	Alide2_0466
		Alide_0510	Alide2_0467
		Alide_0511	Alide2_0468
Nitrite reductase	Nitrite : nitric oxide	Alide_2156	Alide2_2345
		Alide_2157	Alide2_2346
		Alide_2158	Alide2_2347
		Alide_2159	Alide2_2348
		Alide_2160	Alide2_2349
		Alide_2161	Alide2_2350
		Alide_2162	Alide2_2351
Nitric oxide reductase	Nitric oxide : nitrous oxide	Alide_0128	Alide2_0119
		Alide_0129	Alide2_0120
Nitrous oxide reductase	Nitrous oxide : dinitrogen gas	Alide_3077	Alide2_1364
		Alide_3078	Alide2_1365
		Alide_3079	Alide2_1366
		Alide_3080	Alide2_1367
		Alide_3081	Alide2_1368
		Alide_3082	Alide2_1369
		Alide_3083	Alide2_1370

Table S5. List of genes involved in degradation of aromatic and acyclic compounds in *A. denitrificans* strains BC and K601^T.

Enzyme name	Reaction	GeneID in BC	GeneID in K601T
Bifunctional hydratase/alcohol dehydrogenase	Cyclohexenone : 1,3-cyclohexanedione	-	Alide2_4320
		-	Alide2_4321
		-	Alide2_4322
Sigma 54 transcriptional regulator		Alide_0322	Alide2_0269
Benzene/phenol-monooxygenase	Benzene/toluene : (methyl-)phenol : (methyl-)catechol	Alide_0323	Alide2_0270
		Alide_0324	Alide2_0271
		Alide_0325	Alide2_0272
		Alide_0326	Alide2_0273
		Alide_0327	Alide2_0274
		Alide_0328	Alide2_0275
Ferredoxin		Alide_0329	Alide2_0276
Catechol 2,3-dioxygenase	(Methyl-)catechol : 2-hydroxymuconic semialdehyde	Alide_0330	Alide2_0277
Putative regulator of phenolics degradation		Alide_0334	Alide2_0279
2-Hydroxymuconic semialdehyde dehydrogenase	2-Hydroxymuconic semialdehyde : 5-hydroxyhexa-2,4-dienedioate	Alide_0335	Alide2_0280
2-Hydroxymuconic semialdehyde hydrolase	2-Methylcatechol : 2-hydroxy-6-oxo-2,4-heptadienoate	Alide_0336	Alide2_0281
2-Oxopent-4-enoate hydratase	2-Oxopentenoate : 4-hydroxy-2-oxopentanoate	Alide_0337	Alide2_0282
Acetaldehyde dehydrogenase (acetylating)	Acetaldehyde : acetyl-coA	Alide_0338	Alide2_0283
4-Hydroxy-2-oxovalerate aldolase	4-Hydroxy-2-oxovalerate : acetaldehyde	Alide_0339	Alide2_0284
4-Oxalocrotonate decarboxylase	5-Oxohex-2-enedioate : 2-oxopentanoate	Alide_0340	Alide2_0285
4-Oxalocrotonate tautomerase family enzyme	5-Hydroxyhexa-2,4-dienedioate : 5-oxohex-2-enedioate	Alide_0342	Alide2_0287
Catechol 1,2-dioxygenase	Catechol : cis,cis-muconate	Alide_2650	-
Muconate cycloisomerase	Cis,cis-muconate : muconolactone	Alide_2651	-
Muconolactone isomerase	Muconolactone : 3-oxoadipate-enol-lactone	Alide_2647	Alide2_3665
3-Oxoadipate-enol-lactonase	3-Oxoadipate-enol-lactone : 3-oxoadipate	Alide_2648	-
3-Oxoacid CoA transferase	3-Oxoadipate : 3-oxoadipyl-coA	Alide_3513	Alide2_3663
		Alide_3514	Alide2_3664
Acetyl-CoA acetyltransferase	3-Oxoadipyl-coA : acetyl-coA	Alide_0140	-
		Alide_0678	-
		Alide_1564	-
		Alide_3281	-
		Alide_3761	-

Chapter 3

Metabolic response of *Alicycliphilus denitrificans* strain BC toward electron acceptor variation

Authors and affiliations
Margreet J. Oosterkamp[1], Sjef Boeren[2], Caroline M. Plugge[1], Peter J. Schaap[3] and Alfons J.M. Stams[1]*

1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
2 Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.
3 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
(a) Current address: Animal Sciences Laboratory, University of Illinois at Urbana-Champaign, 1207 West Gregory Drive, Urbana, Illinois 61801, USA.
* Correspondent: E-mail: fons.stams@wur.nl; Tel.: (+31) 317 483101; Fax: (+31) 317 483829.

Keywords
Alicycliphilus denitrificans, bioremediation, chlorate reduction, nitrate reduction

Manuscript status
Published article (Oosterkamp, Boeren *et al.* 2013)

Abstract

Alicycliphilus denitrificans is a versatile, ubiquitous, facultative anaerobic bacterium. *A. denitrificans* strain BC can use chlorate, nitrate and oxygen as electron acceptor for growth. Cells display a prolonged lag-phase when transferred from nitrate to chlorate and vice versa. Furthermore, cells adapted to aerobic growth do not easily use nitrate or chlorate as electron acceptor. We further investigated these responses of strain BC by differential proteomics, transcript analysis and enzyme activity assays. In nitrate-adapted cells transferred to chlorate and vice versa, appropriate electron acceptor reduction pathways need to be activated. In oxygen-adapted cells, adaptation to the use of chlorate or nitrate is likely difficult due to the poorly active nitrate reduction pathway and low active chlorate reduction pathway. We deduce that the Nar-type nitrate reductase of strain BC also reduces chlorate, which may result in toxic levels of chlorite if cells are transferred to chlorate. Furthermore, the activities of nitrate reductase and nitrite reductase appear to be not balanced when oxygen-adapted cells are shifted to nitrate as electron acceptor, leading to the production of a toxic amount of nitrite. These data suggest that strain BC encounters metabolic challenges in environments with fluctuations in the availability of electron acceptors.

Introduction

Chlorate is highly soluble, chemically stable under most environmental conditions and readily transported in groundwater (Logan 1998; Urbansky 1998; Kang, Jackson et al. 2008). Chlorate is used as electron acceptor by chlorate-reducing bacteria (Korenkov, Romanenko et al. 1976; Coates, Michaelidou et al. 1999). One of the intermediates of chlorate reduction is molecular oxygen (Ginkel, Rikken et al. 1996; Rikken, Kroon et al. 1996; Wolterink, Jonker et al. 2002; Mehboob, Junca et al. 2009). The oxygen can enhance degradation of compounds that are recalcitrant in the absence of oxygen, such as BTEX. Such compounds are degraded by aerobic bacteria by means of oxygenases (Coates, Bruce et al. 1999; Weelink, Tan et al. 2008; Mehboob, Junca et al. 2009).

Reduction of chlorate involves the conversion of chlorate to chlorite and the dismutation of chlorite to chloride and oxygen. Perchlorate reductase also reduces chlorate. The properties of (per)chlorate reductases and chlorite dismutase have been described recently (Nilsson, Rova et al. 2012). Historically, the reduction of (per)chlorate was considered to be a side activity of denitrifying microorganisms (Quastel, Stephenson et al. 1925; Hackenthal 1965; De Groot and Stouthamer 1969; Coates and Achenbach 2004). Two bacterial classes of respiratory nitrate reductases exist, the membrane-bound (Nar) and periplasmic (Nap) enzyme complexes. Nar has a high activity with chlorate (Bell, Richardson et al. 1990; Rusmana and Nedwell 2004; Kučera 2006) and Nap has no or only a low activity with chlorate (Kraft, Strous et al. 2011). There are two Nar enzyme systems known, one with a cytoplasmic and another with a periplasmic orientation (Martinez-Espinosa, Dridge et al. 2007; Kraft, Strous et al. 2011).

The soil bacterium *Alicycliphilus denitrificans* strain BC degrades benzene with chlorate and oxygen, but not with nitrate as electron acceptor (Weelink, Tan et al. 2008). With acetate as substrate, strain BC can grow with nitrate, chlorate and oxygen as electron acceptor. We observed that cells adapted to growth with nitrate as electron acceptor grow poorly when grown with chlorate as electron acceptor and vice versa. Furthermore, cells adapted to aerobic growth cannot switch easily to growth with chlorate or nitrate. Here we focused on this poor metabolic flexibility of strain BC with respect to changes in the electron acceptor. We performed proteome analysis, enzyme assays and qRT-PCR with strain BC adapted to growth with acetate as electron donor and chlorate, nitrate or oxygen as electron acceptor using *A. denitrificans* strain K601^T, that cannot use chlorate, as a control strain.

Materials and methods

Microorganisms

A. denitrificans strain BC (DSM 18852) was isolated in our laboratory (Weelink, Tan et al. 2008) and *A. denitrificans* strain K601^T (DSM 14773) was obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ (Braunschweig, Germany). Strains BC and K601^T were grown in AW1-sulfate medium (pH 7.3) as described previously (Weelink, Tan et al. 2007). Cultures were incubated in the dark at 30°C without agitation. The medium was prepared with anoxic water and dispensed in the flasks under continuous flushing with nitrogen gas. Bottles were closed with butyl rubber stoppers and aluminum caps, and the head space was replaced with 20 % CO_2 and 80 % N_2 gas (170 kPa). All solutions that were added to the medium were anoxic and autoclaved at 121°C for 20 minutes. Sodium salts of acetate, chlorate and nitrate were supplied from 0.4 M stock solutions to a final concentration of 10 mM and oxygen was added from a bottle with sterilized 100 % oxygen gas. For adaptation, cells were transferred to fresh medium at least five times.

Analytical procedures

Oxygen was measured by headspace analysis using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) with a packed column (Molsieve 13x 60/80 mesh, 2 m x 2.4 mm, Varian, Middelburg, The Netherlands) and a thermal conductivity detector. Chlorate, nitrate, chloride, and nitrite were measured by suppressor mediated ion chromatography (Dionex, Breda, The Netherlands) with an IonPac AS9-SC column (Dionex) and a conductivity detector. Acetate was analyzed by liquid chromatography (spectrasystem HPLC, Thermo Fisher Scientific, Waltham, MA, USA) with a column for detection of organic acids (RT 300-6,5 Polyspher OA HY pre-packed column, Merck, Darmstadt, Germany) and an RI detector.

Enzyme assays

Nitrate, chlorate, and nitrite reductase, and chlorite dismutase activities were determined with cell free extracts of strains BC and K601^T. For this, 240-mL cultures of strain BC adapted to growth with acetate and nitrate, chlorate or oxygen and of strain K601^T grown with acetate and nitrate were used. Cell free extracts were prepared under anoxic conditions as described previously (Wolterink, Jonker et al. 2002). The reduction of oxyanions was determined by following oxidation of reduced methylviologen spectrophotometrically at 578 nm and 30°C (Kengen, Rikken et al. 1999). Chlorite dismutase activity was determined by oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH, USA) (Wolterink, Jonker et al. 2002).

Proteomics

Separation of proteins from cell free extracts

Cell free extracts of strain BC adapted to growth with acetate and chlorate, nitrate or oxygen as electron acceptor were prepared for whole-proteome analysis. The cell free extracts were prepared as described for the enzyme assays and stored in 2-mL Low Binding tubes (Eppendorf, Hamburg, Germany) at -20°C. A Bio-rad protein-assay (Bio-rad Laboratories, Hercules, CA, USA) was used according to the manufacturer's instructions to determine the protein content of cell extracts. Bovine serum albumin was used as protein standard. 25 µg of protein were loaded on a 10% SDS-polyacrylamide separation gel (pH 8.8) with a stacking gel (pH 6.8) using the mini-protean 3 cell (Bio-rad Laboratories, Hercules, CA, USA) (Figure S6). An empty lane was left between each loaded lane and the electrophoresis procedure was according to the manufacturer's instructions. Gels were stained using Coomassie Brilliant Blue R-250 as indicated in the protocol of the mini-protean 3 system. Gels were scanned and Quantity One software (Bio-rad Laboratories, Hercules, CA, USA) was used to calculate the intensity of each of the lanes (whole lanes) loaded with cell free extract. The ratio of intensity of the lanes to the lane with the highest intensity was calculated and used to prepare a gel in which these intensities were similar.

Digestion of proteins from cell free extracts

SDS-PAGE separated proteins from strain BC grown with acetate and chlorate, nitrate, or oxygen as electron acceptor, were subjected to in-gel digestion. Each of the used gel lanes was cut into four slices using a clean scalpel and on a clean microscope slide. Slices were further processed to pieces of about 1 mm^{2}and the gel pieces obtained were put in 1.5-mL Low Binding tubes (Eppendorf, Hamburg, Germany) and reduced, alkylated and trypsin digested as previously described (Rupakula, Kruse et al. 2013). The supernatant obtained was used for LC-MS/MS analysis.

Liquid chromatography with tandem mass spectrometry

Protein digests obtained from cells of *A. denitrificans* strain BC grown with acetate and chlorate, nitrate, or oxygen as electron acceptor, were analyzed on nanoLC-MS/MS (at Biqualys,

Wageningen, The Netherlands). The procedure is as described previously (Lu, Boeren et al. 2011). MS/MS spectra were analyzed with MaxQuant 1.2.2.5 using default settings for the Andromeda search engine (Cox, Neuhauser et al. 2011) except that extra variable modifications were set for de-amidation of N and Q as described before (Smaczniak, Li et al. 2012). An *A. denitrificans* strain BC database downloaded from Uniprot (http://www.uniprot.org) was used together with a contaminants database that contains sequences of common contaminants like human keratins, BSA (P02769) and Trypsin (P00760, P00761). The "label-free quantification" as well as the "match between runs" (set to 2 minutes) options were enabled. De-amidated peptides were included to be used for protein quantification and all other quantification settings were kept default.

Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus 1.3.0.4 module (available at the MaxQuant suite). Accepted were peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which one should be unique and one should be unmodified. Reversed hits were deleted from the MaxQuant result table as well as all results showing a Log LFQ value of 0 for both sample and control. The normal logarithm was taken from normalised label free quantitation protein intensities (LFQ) as obtained from MaxQuant. Zero values for one of the two Log LFQ columns were replaced by a value of 3.5 to make sensible ratio calculations possible. Relative protein quantitation of sample to control was done by subtracting the Log LFQ control from the Log LFQ sample value. Total non-normalised protein intensities corrected for the number of measurable tryptic peptides (iBAQ intensity, Schwanhausser, Busse et al. 2011)) were, after taking the normal logarithm, used for plotting on the y-axis in a protein ratio versus abundance plot.

The proteomics data have been deposited to the ProteomeXchange with identifier PXD000258.

Quantitative real-time PCR

RNA isolation and reverse transcription

Strain BC cultures grown with acetate and chlorate, nitrate, or oxygen as electron acceptor were used for RNA isolation and subsequent cDNA synthesis. Cells were harvested by centrifugation in a Sorvall centrifuge with an SLA-3000 rotor and 500-mL screw-cap tubes (Thermo Fisher Scientific, Waltham, MA, USA) at 10°C and 10k rpm for 10 minutes. Subsequently, RNA was isolated as described previously (Zoetendal, Booijink et al. 2006) and cDNA was synthesized as described before (Worm, Stams et al. 2011).

Quantitative real-time PCR amplification

Quantitative real-time PCR amplification was performed using cDNA obtained from cultures of *A. denitrificans* strain BC adapted to growth with acetate and chlorate, nitrate or oxygen as electron acceptor. The reactions were performed with universal 16S rRNA primers for bacteria and specific primers targeting the genes coding for NarG and ClrA (synthesized by Biolegio, Nijmegen, The Netherlands). Universal 16S rRNA primers for bacteria were 16S rRNA-bact-1369-F and 16S rRNA-prok-1492-R (Suzuki, Béjà et al. 2001). Specific primers for *narG* were AdeBC-narG-F (5'-GCC AGC AGT TCT ACC AGG AC-3') and AdeBC-narG-R (5'-GTT GTC GCT GTA CGT GCT GT-3') and specific primers for *clrA* were AdeBC-clrA-F (5'-GGT CCG CAT TTT CAA TGA CT-3') and AdeBC-clrA-R (5'-CCG ACG TGT ACT TGA TGT GG-3'). Primers targeting *narG* and *clrA* were validated using MIQE guidlines (Bustin, Benes et al. 2009).

Reactions were performed with biological duplicates and technical triplicates and in the Bio-rad CFX96 machine and using the corresponding software package (Bio-rad Laboratories, Hercules, CA, USA). 250 pg/µL and 2.5 pg/µL of cDNA and 5 pmole of primers was used per reaction, prepared

as described before (Worm, Stams et al. 2011). The program was as follows, 98°C for 5 minutes, 45 cycles of 95°C for 1 minute, 56 (16S rRNA)/60°C (narG, clrA) for 30 seconds and 72°C for 40 seconds. Furthermore, PCR products were analyzed using a melting curve from 65 to 90°C, transcript numbers were determined by calculating ΔΔCq with 16S rRNA as a reference and with a Log relative default Cq value of 35 in case no fluorescence was detected.

Results

Electron acceptor utilization by strain BC

Physiological tests showed that cells adapted to nitrate and transferred to chlorate had an extended lag-phase before growth resumed (Fig. 1) and, similarly, cells adapted to chlorate showed an extended growth lag-phase when transferred to nitrate (Fig. 2). When adapted cells were transferred to medium with both chlorate and nitrate as electron acceptor, nitrate-adapted cells preferably used nitrate and chlorate-adapted cells preferably used chlorate as electron acceptor (Fig. S1). Furthermore, cells adapted to oxygen could not easily resume growth with nitrate or chlorate as electron acceptor (Fig. S2). However, cells adapted to nitrate or chlorate switched to oxic conditions with ease (Fig. S3).

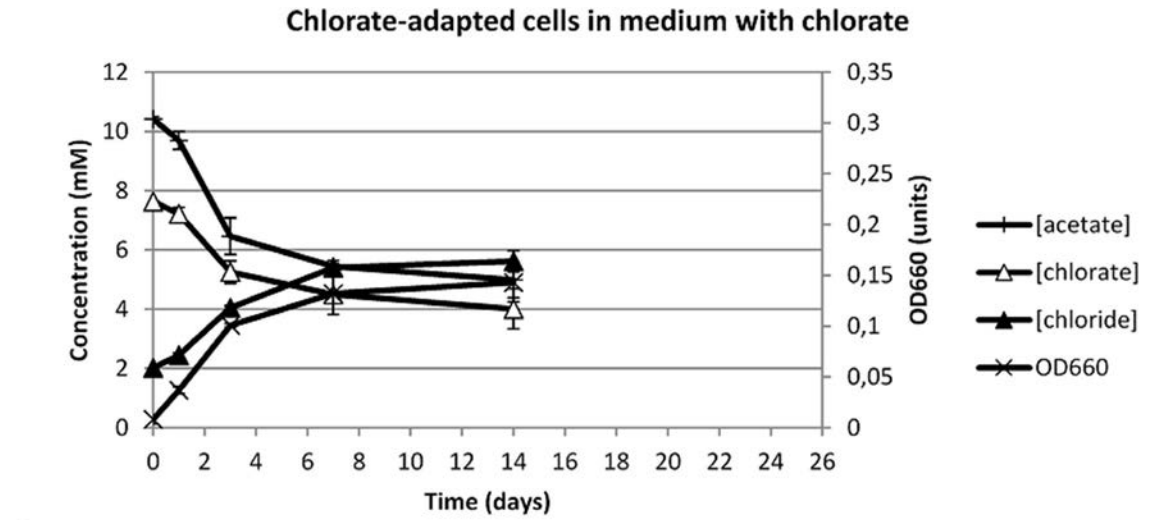
Activity of enzymes involved in chlorate and nitrate reduction

In strain BC, chlorate reductase and chlorite dismutase are encoded by *clr* and *cld* genes, respectively. The *clr* and *cld* genes are not present in the genome of strain K601^T. Furthermore, in both strains nitrate reductase and nitrite reductase are encoded by *narGHI* and *nirS* genes (Oosterkamp, Veuskens et al. 2011). Chlorate reductase, chlorite dismutase, nitrate reductase and nitrite reductase activities were determined in cell-free extracts of strain BC cells grown with acetate and chlorate, acetate and nitrate, and acetate and oxygen, and in cell-free extracts of strain K601^T cells grown with acetate and nitrate. Chlorate-adapted cells of strain BC have low Nar and Nir activities compared to nitrate adapted cells (Table 1). Cld activity was low in nitrate adapted BC cells compared to chlorate-adapted cells. Clr activity, however, was about six times higher in nitrate-adapted cells than in chlorate-adapted cells of strain BC (Table 1). Furthermore, Clr activity was also observed in nitrate-grown cells of strain K601^T which does not contain genes typically coding for Clr and Cld (Oosterkamp, Veuskens et al. 2011).

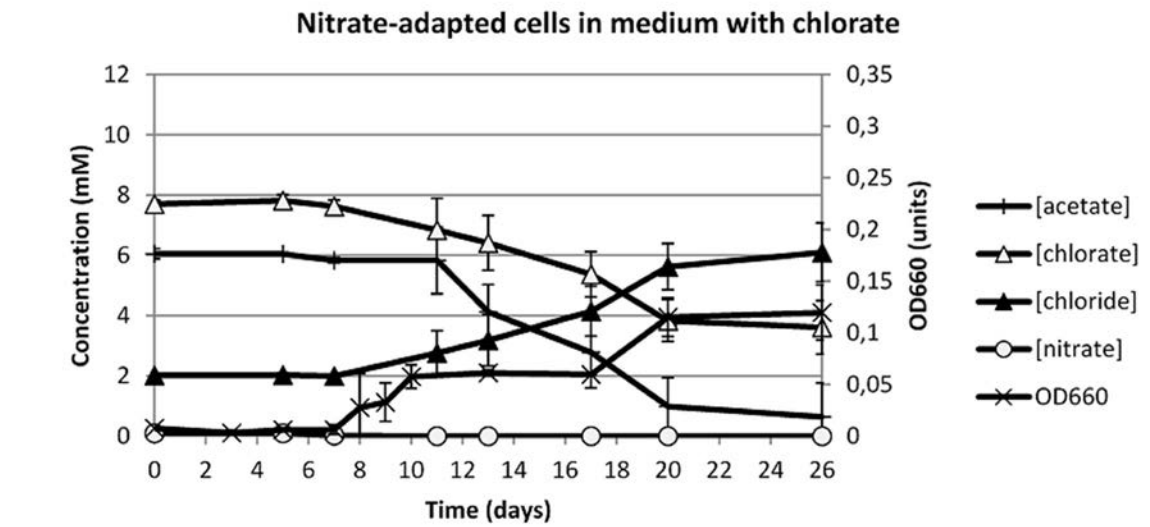
Oxygen-adapted cells of strain BC exhibited no or very low Cld activity. Clr and Nar activities in oxygen-adapted cells were higher than in chlorate-adapted cells and about two times lower than in nitrate adapted cells (Table 1). Furthermore, the specific activity of Nir in oxygen adapted cells was higher than in cells grown with nitrate or chlorate.

Whole-proteome analysis of chlorate-, nitrate- or oxygen-grown cells

Cell free extracts of strain BC cells grown with acetate and chlorate, nitrate or oxygen as electron acceptor were used for quantitative whole-proteome analyses. In this analysis, NarGHI (Alide_0508/0509/0511) is more abundant in nitrate- and oxygen-grown cells than in chlorate-grown cells (Table 1). NirS (Alide_2162), and NorB (nitric-oxide reductase, Alide_0128) were more present in nitrate- than in chlorate- or oxygen-adapted cells (Fig. 4, S4, S5). Furthermore, NosZ (nitrous oxide reductase, Alide_3077) was present in similar amounts in chlorate-, nitrate- and oxygen-grown cells. The ClrAB and Cld proteins (Alide_4613-4615) were most abundant in chlorate adapted cells (Fig. 4, S4). An overview of protein abundance of the key enzymes involved is shown (Fig. 3) and raw proteomics data can be found in Table S1.

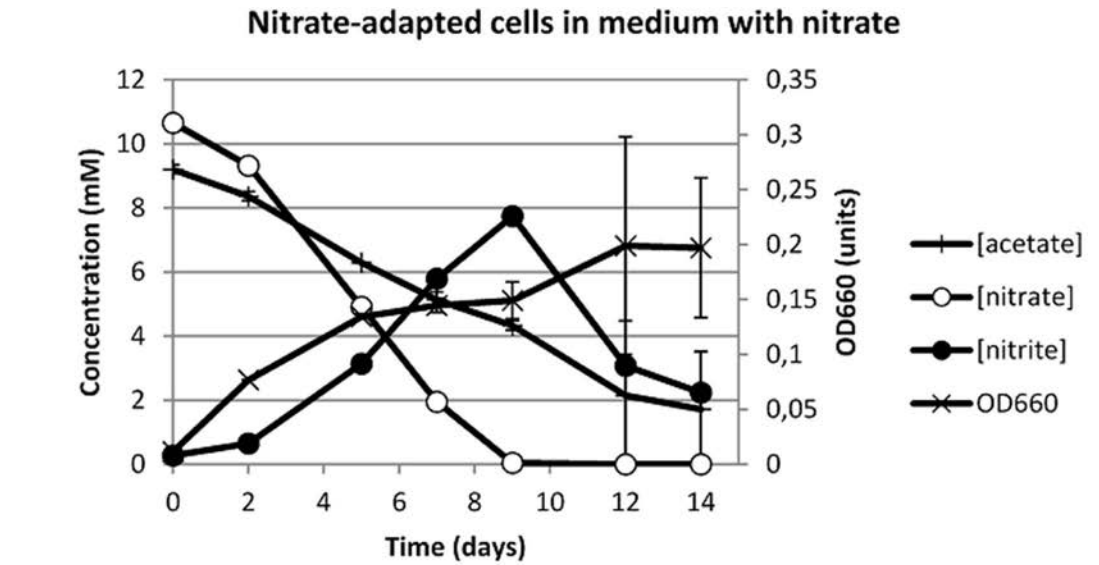


a)

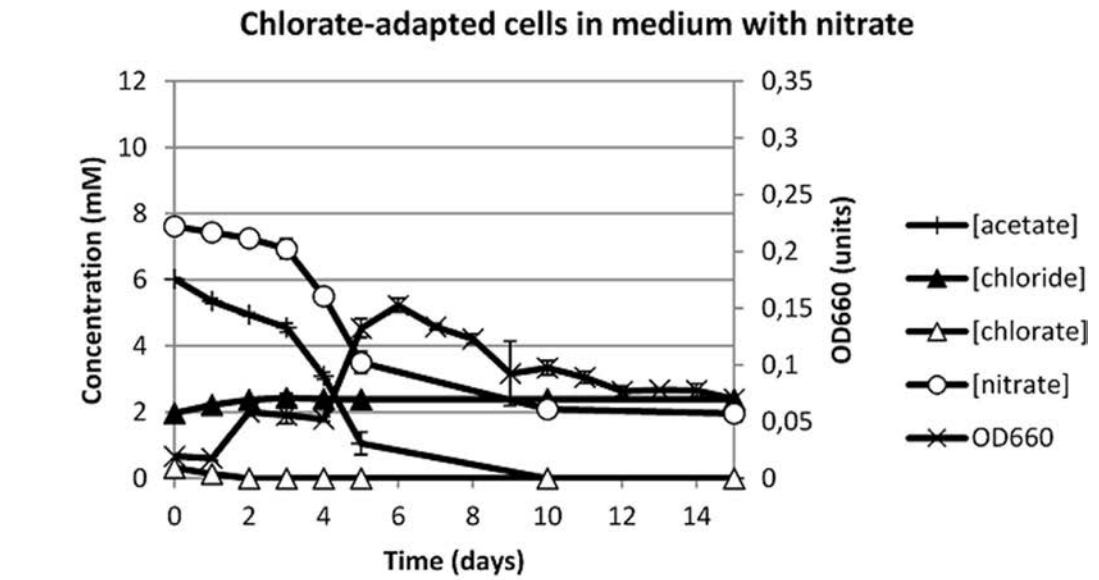


b)

Figure 1. Time course of OD660, and acetate, chlorate, chloride and nitrate concentration in minimal medium supplemented with acetate and chlorate and inoculated with cultures of *A. denitrificans* strain BC that are pre-grown with acetate and chlorate (**a**) or with acetate and nitrate (**b**).



a)



b)

Figure 2. Time course of OD660, and acetate, nitrate, nitrite, chlorate, and chloride concentration in minimal medium supplemented with acetate and nitrate and inoculated with cultures of *A. denitrificans* strain BC that are pre-grown with acetate and nitrate (**a**) or with acetate and chlorate (**b**).

Table 1. Properties of chlorate reductase, chlorite dismutase, nitrate reductase and nitrite reductase of *A. denitrificans* strains BC and K601^T. Data are shown for strain BC adapted to acetate and chlorate (chlorate), acetate and nitrate (nitrate) and/or acetate and oxygen (oxygen) and for strain K601^T adapted to acetate and nitrate. Enzyme activity (in U/mg protein) is given for the two strains of all enzymes and in all conditions tested. Protein abundance (in Log 10 label free quantification units) is provided for strain BC of all enzymes and in all conditions tested and gene expression (in Log 10 rel transcript number) for *clrA* and *narG* is given for strain BC in all conditions tested . nd is not detected

	Strain BC			Strain K601T
	Chlorate	Nitrate	Oxygen	Nitrate
Clr activity	0.28 ± 0.07	1.67 ± 0.08	0.83 ± 0.48	0.92 ± 0.08
ClrAB abundance	6.99/6.13	nd/nd	nd/nd	
clrA expression	-0.60 ± 0.68	-1.63 ± 0.24	-1.38 ± 0.13	
Cld activity	1.61 ± 0.39	0.41 ± 0.07	0.10 ± 0.15	0.01 ± 0.02
Cld abundance	7.78	nd	4.15	
Nar activity	0.01 ± 0.00	0.56 ± 0.00	0.26 ± 0.12	0.33 ± 0.01
NarGHI abundance	5.78/nd/nd/	7.00/5.15/5.55	6.93/5.09/5.75	
narG expression	-2.06 ± 0.22	-0.08 ± 0.12	-0.67 ± 0.13	
Nir activity	0.02 ± 0.00	0.06 ± 0.00	0.45 ± 0.28	0.04 ± 0.00
NirS abundance	5.93	7.00	5.78	

Two cytochrome *c* oxidases (Alide_3906 and Alide_2816/2817) were more abundant in nitrate- than in chlorate- or oxygen-grown cells (Fig. 4, S5). The cytochrome *c* oxidase encoded by Alide_2816 is not present in chlorate- and oxygen-adapted cells (Fig. S4). Furthermore, a cytochrome *c* oxidase accessory protein (Alide_3324) was more abundant in chlorate- than in nitrate- or oxygen-grown cells (Fig. 4, S4, S5). A cytochrome c_1 protein (Alide_0905) was more abundant in chlorate- and nitrate- than in oxygen-adapted cells (Fig. 4, S4, S5). Ubiquinol cytochrome *bc* reductase (Alide_0903/0904), cytochrome *c* oxidase (Alide_3325/3327), and three cytochrome *c* class I proteins (Alide_0450/0830/3075) are present in similar amounts in chlorate-, nitrate-, and oxygen-grown cells *(*Fig. 4, S4, S5).

Gene expression variations of narG and clrA in chlorate-, nitrate- and oxygen-adapted cells

The transcript levels of *narG* and *clrA* in chlorate-, nitrate-, and oxygen-adapted strain BC cells were analyzed using qPCR. In cells adapted to chlorate *narG* transcription was hardly detectable. *NarG* transcript levels were increased in nitrate-adapted compared to oxygen-adapted cells (Table 1). Furthermore, there was a strong increase in the transcript level of *clrA* in chlorate-adapted strain BC cells compared to cells adapted to nitrate and oxygen (Table 1).

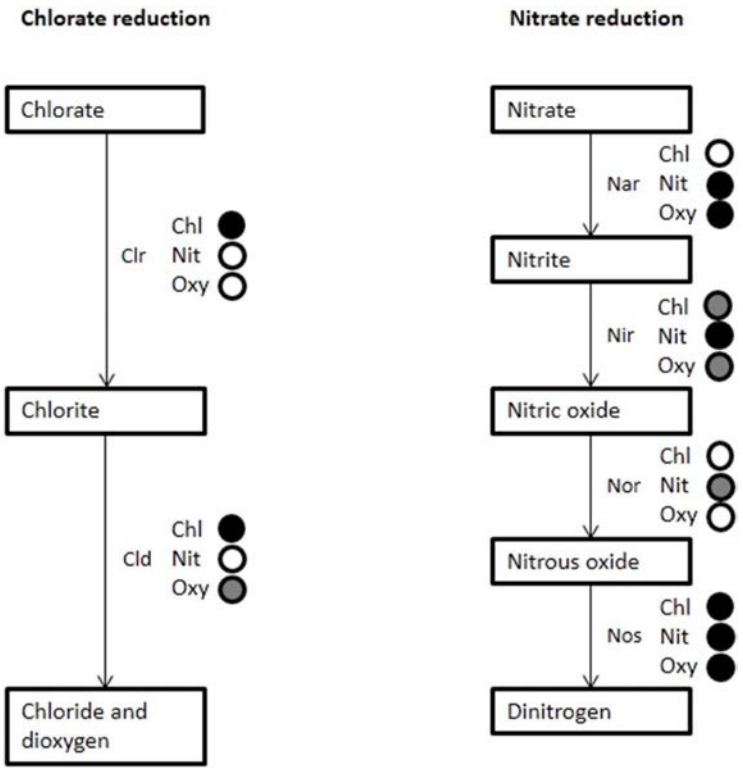


Figure 3. Overview of abundance of enzymes from the respiratory chlorate and nitrate reduction pathways of *A. denitrificans* strain BC. High (> 6 Log 10 label free quantification (LFQ) units, black filled circles), medium (4 – 6 Log 10 LFQ units, grey filled circles) and low (< 4 Log 10 LFQ units white filled circles) abundance of the enzymes in chlorate- (Chl), nitrate- (Nit) and oxygen- (Oxy) adapted cells is indicated and deduced from our proteomics data.

Discussion

Nitrate and chlorate reduction in A. denitrificans *strain BC*

This study focused on chlorate and nitrate reduction in *A. denitrificans* strain BC. Strain BC cells adapted to using chlorate as electron transporter that were transferred to nitrate showed an extended lag-phase and vice versa (Fig. 1, 2). Metabolic adaptation to chlorate and nitrate was shown by proteomics, transcript analysis and specific enzyme activity assays. Since a lag phase was observed with a change in electron acceptor, this adaptation is likely robust. The NarGHI nitrate reduction pathway is not active in chlorate-adapted cells (Table 1, Fig 4, S4, S5). In chlorate-adapted cells, *narG* transcript levels were very low and nitrate reductase was not active. Furthermore, proteome analysis indicated that the abundance of the alpha subunit NarG was at least ten fold lower compared to nitrate-adapted cells, while the beta and gamma subunits NarH and NarI were below the detection limit. We also found that the ClrAB chlorate reduction pathway was not active in nitrate-adapted strain BC cells (Table 1). Cell free extracts of nitrate-adapted cells, however, showed a high chlorate reductase activity. It is known that Nar-type nitrate reductases are able to efficiently reduce chlorate (Goksøyr 1951; Goksøyr 1952; Nakagawa and Yamashita 1986). Genes coding for the NarGHI complex are encoded on the genomes of strains BC and K601^T (Oosterkamp, Veuskens et al. 2011). Strain K601^T cannot grow using chlorate as electron acceptor (Weelink, Tan et al. 2008) and its genome lacks genes involved in the ClrAB chlorate reduction pathway (Oosterkamp, Veuskens et al. 2011). There was a relatively high Clr activity in nitrate adapted cells of strain K601^T (Table 1),

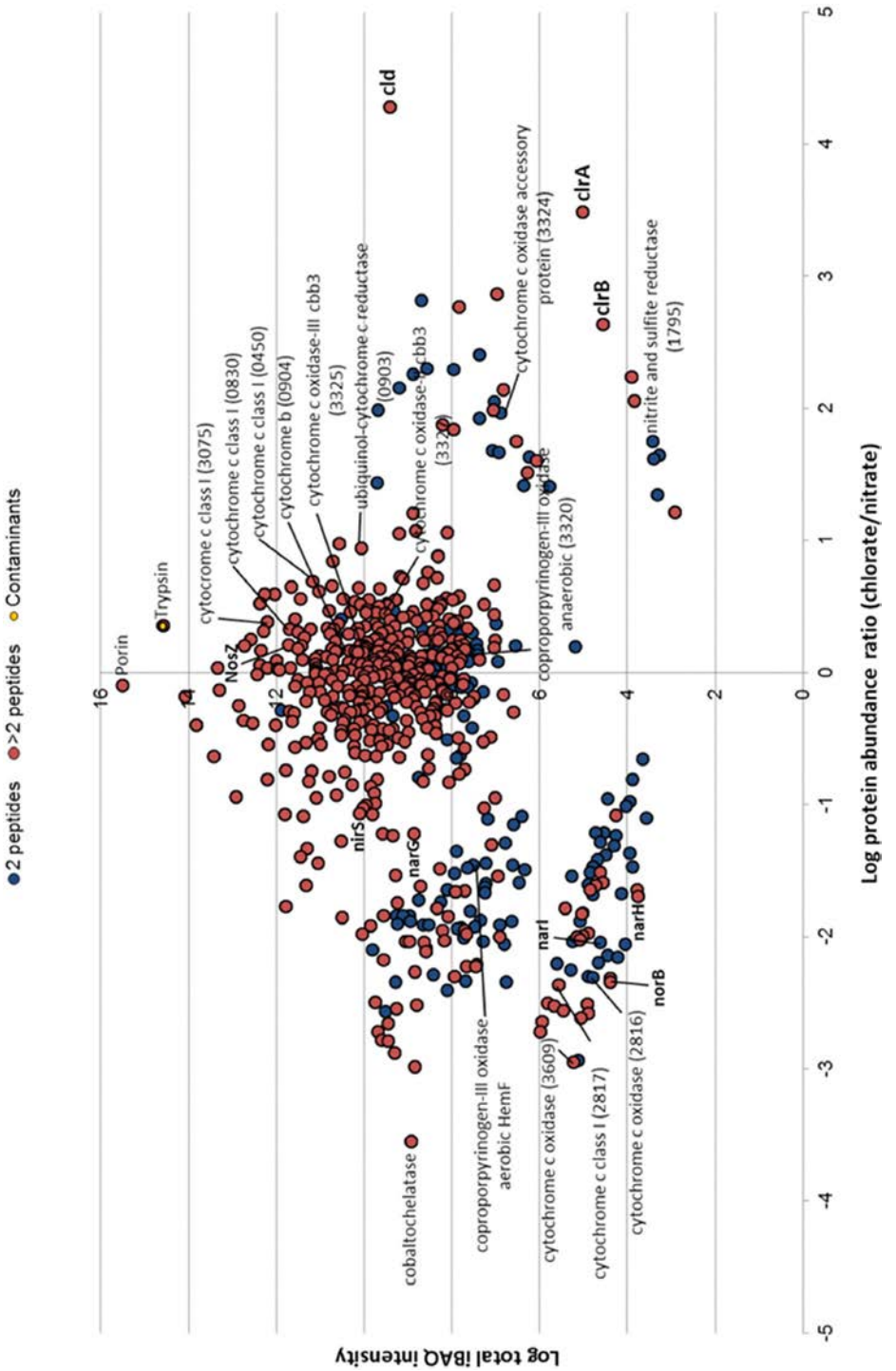


Figure 4. Whole-proteome analysis of chlorate- versus nitrate-grown *A. denitrificans* strain BC cells. The Log of the total intensity based absolute quantification (iBAQ) intensity is plotted against the total protein abundance ratio (in Log label-free quantification (LFQ)). Proteins of which two peptides were identified (blue filled circles) and of which more than two peptides were detected (red filled circles) and the contaminant trypsin (filled circle including yellow dot) are shown.

therefore it was concluded that the Nar-type nitrate reductase of strains BC and K601^T can also reduce chlorate (Table 1). Such direct interference of nitrate reductase with specific chlorate reductase activity of (per)chlorate-reducing microorganisms has not been described before. In a study of the (per)chlorate-reducing *Dechlorosoma sp.* strain KJ, cells adapted to nitrate had lower specific (per)chlorate reductase activity than nitrate reductase activity, while chlorate-adapted cells had higher (per)chlorate reductase activity than nitrate reductase activity (Xu, Trimble et al. 2004).

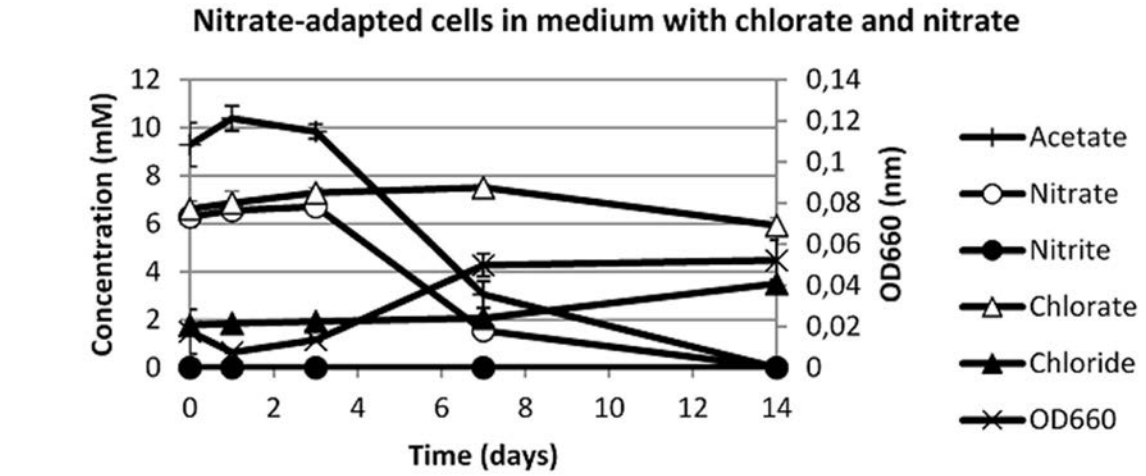
Chlorate and nitrate reduction in aerobically grown strain BC cells

Oxygen-adapted strain BC cells do not grow with chlorate as electron acceptor over the time interval that was investigated (14 days, Fig. S2). Accordingly, proteomics, transcript analysis and specific enzyme activity assays showed that the ClrAB chlorate reduction pathway is not active in oxygen-adapted cells (Table 1). These results are according to other studies with (per)chlorate-reducing microorganisms. Transcription of genes involved in chlorate reduction is low when *Ideonella dechloratans* grows under oxic conditions (Hellberg Lindqvist, Johansson et al. 2012). In the perchlorate-reducing bacteria *Dechloromonas agitata* and *Rhodocyclaceae* strain JDS4, genes involved in perchlorate reduction were down-regulated when grown aerobically (Bender, Rice et al. 2004; Bender, Shang et al. 2005; De Long, Kinney et al. 2010). Furthermore, several (per)chlorate-reducing bacteria grown with oxygen as electron acceptor exhibited low Cld activity (Malmqvist, Welander et al. 1991; Rikken, Kroon et al. 1996; Chaudhuri, O'Connor et al. 2002; Hellberg Lindqvist, Johansson et al. 2012). Cld protein abundance and activity are not in agreement in oxygen- and nitrate-adapted strain BC cells (Table 1). According to previous research (Mehboob, Wolterink et al. 2009), a bias in measuring Cld activity can occur when Cld is too poorly present.

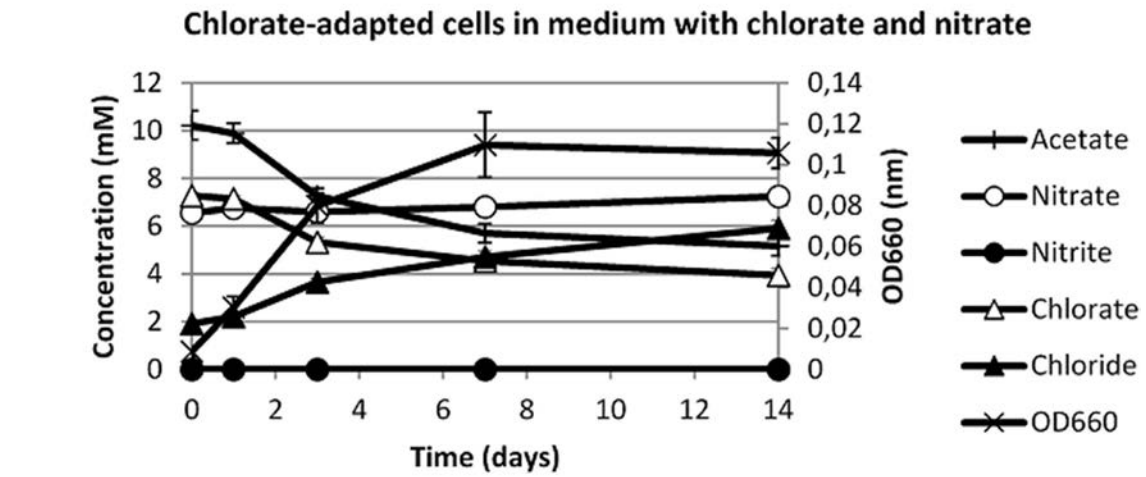
Oxygen may affect nitrate reduction (Sacks and Barker 1949; Skerman and MacRae 1957) and expression of Nar and Nir is inhibited by oxygen (Körner, Sofia et al. 2003). Furthermore, nitrite is toxic (Bollagh and Henniger 1978; O'Reilly and Colleran 2005; Chen, Liu et al. 2011). Nitrite accumulates when oxygen-grown strain BC cells use nitrate as electron acceptor (Fig. S2). Accordingly, the Nar-type nitrate reductase is active, while the NirS is less abundant in oxygen-adapted cells (Table 1). There was uncertainty in the enzyme activity measurements of the nitrite reductase, since the standard deviation was relatively high for oxygen-adapted cells. Furthermore, in these cells NirS abundance was lower while the specific Nir enzyme activity was higher compared to chlorate-adapted cells (Table 1). Cytochrome oxidases can interfere with nitrite reductase activity assays (Basu, Azarova et al. 2008). Accordingly, cytochrome oxidases (Alide_3325-3327) are abundant in oxygen-adapted cells (Figure 4, S4, S5). Overall, although enzyme systems involved in electron acceptor metabolism of strain BC are present, regulation of the metabolism in an environment with changing electron acceptor availability seems to be complex. Further studies with purified proteins involved in chlorate and nitrate reduction are needed to explain this complexity in more detail.

Acknowledgements

This research was supported and funded by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053 (www.stw.nl) of which M.J.O. is the principal receiver. Special thanks to Biqualys (www.biqualys.com) for generating the proteomics data. This project was conducted under the auspices of the graduate school WIMEK (Wageningen Institute for Environment and Climate Research, which is part of SENSE Research School for Socio-Economic and Natural Sciences of the Environment, www.wimek-new.wur.nl and www.sense.nl). Research of A.J.M.S. is supported by grants of ALW-TOP (project 700.55.343) and ERC (project 323009). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno, Côté et al. 2013) with the dataset identifier PXD000258.

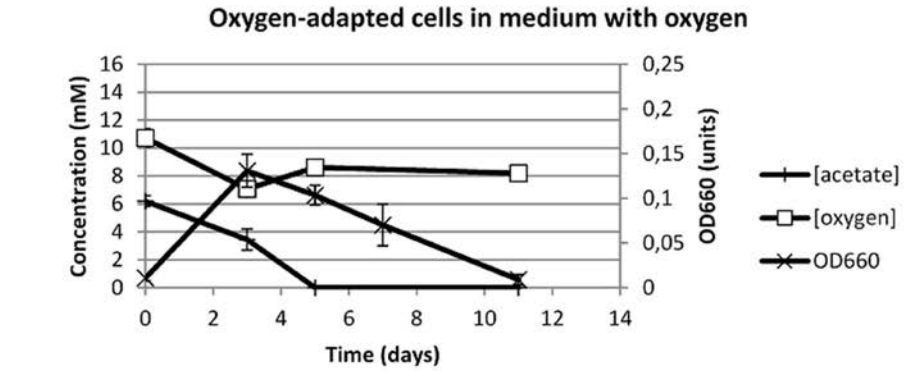


a)

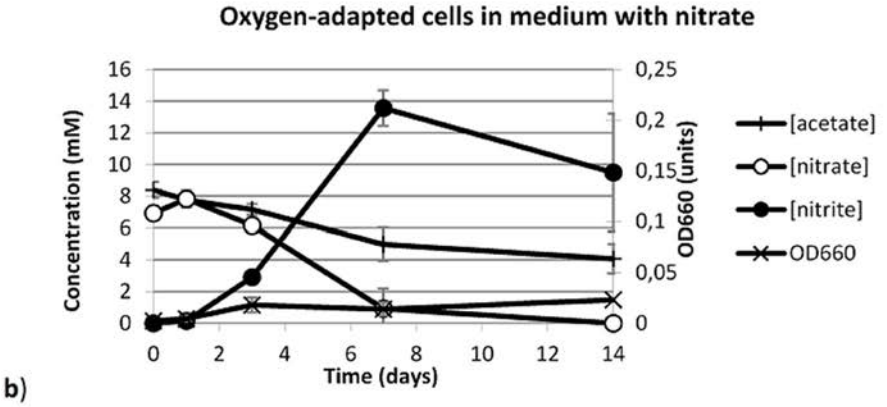


b)

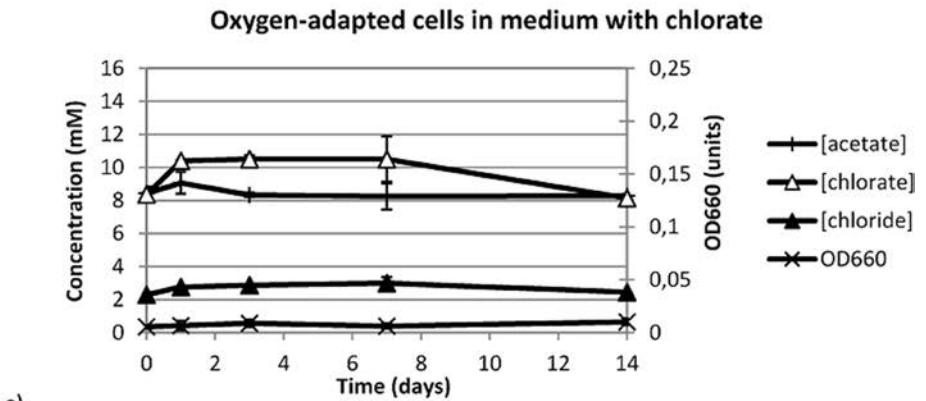
Figure S1. Time course of OD660 and acetate, nitrate, nitrite, chlorate, and/or chloride concentration in minimal medium supplemented with acetate, nitrate, and chlorate and inoculated with cultures of *A. denitrificans* strain BC that are pre-grown with acetate and nitrate (**a**), or chlorate (**b**).



a)

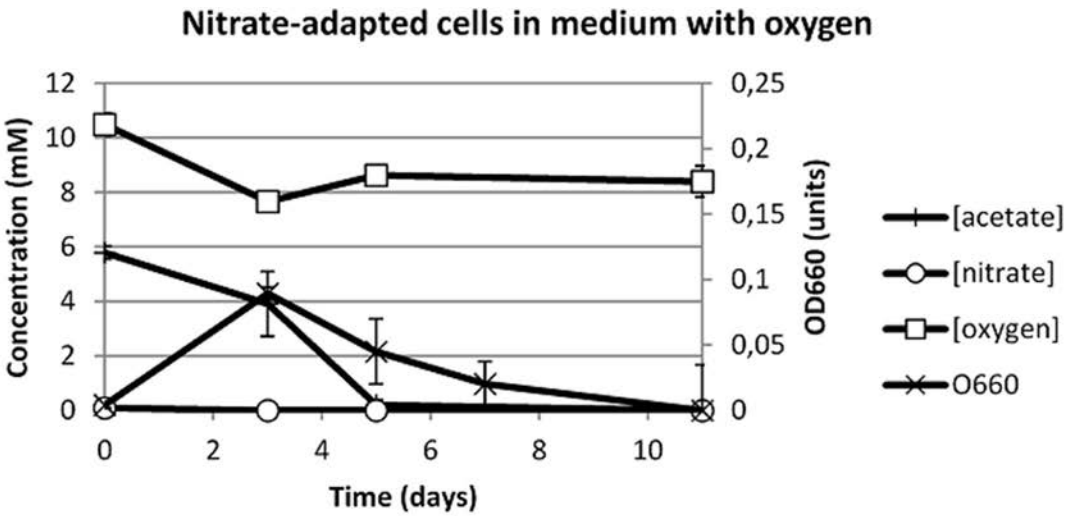


b)

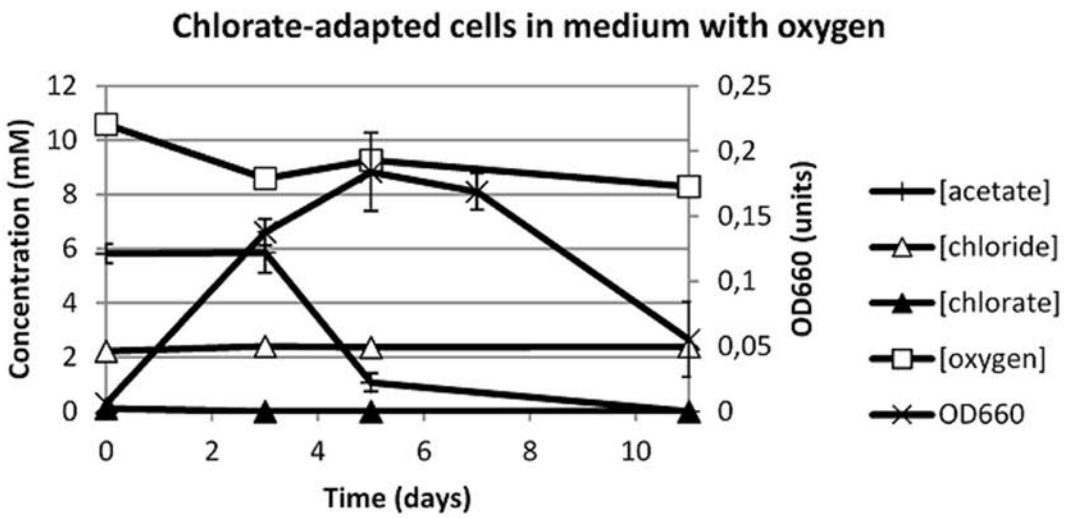


c)

Figure S2. Time course of OD660, and acetate, oxygen, nitrate, nitrite, chlorate, and/or chloride concentration in minimal medium inoculated with cultures of *A. denitrificans* strain BC that are pre-grown with acetate and oxygen and supplemented with acetate and oxygen (**a**), nitrate (**b**), or chlorate (**c**).



a)



b)

Figure S3. Time course of OD660, and acetate, oxygen, nitrate, nitrite, chlorate, and/or chloride concentration in minimal medium supplemented with acetate and oxygen and inoculated with cultures of *A. denitrificans* strain BC that are pre-grown with acetate and nitrate (**a**), or chlorate (**b**).

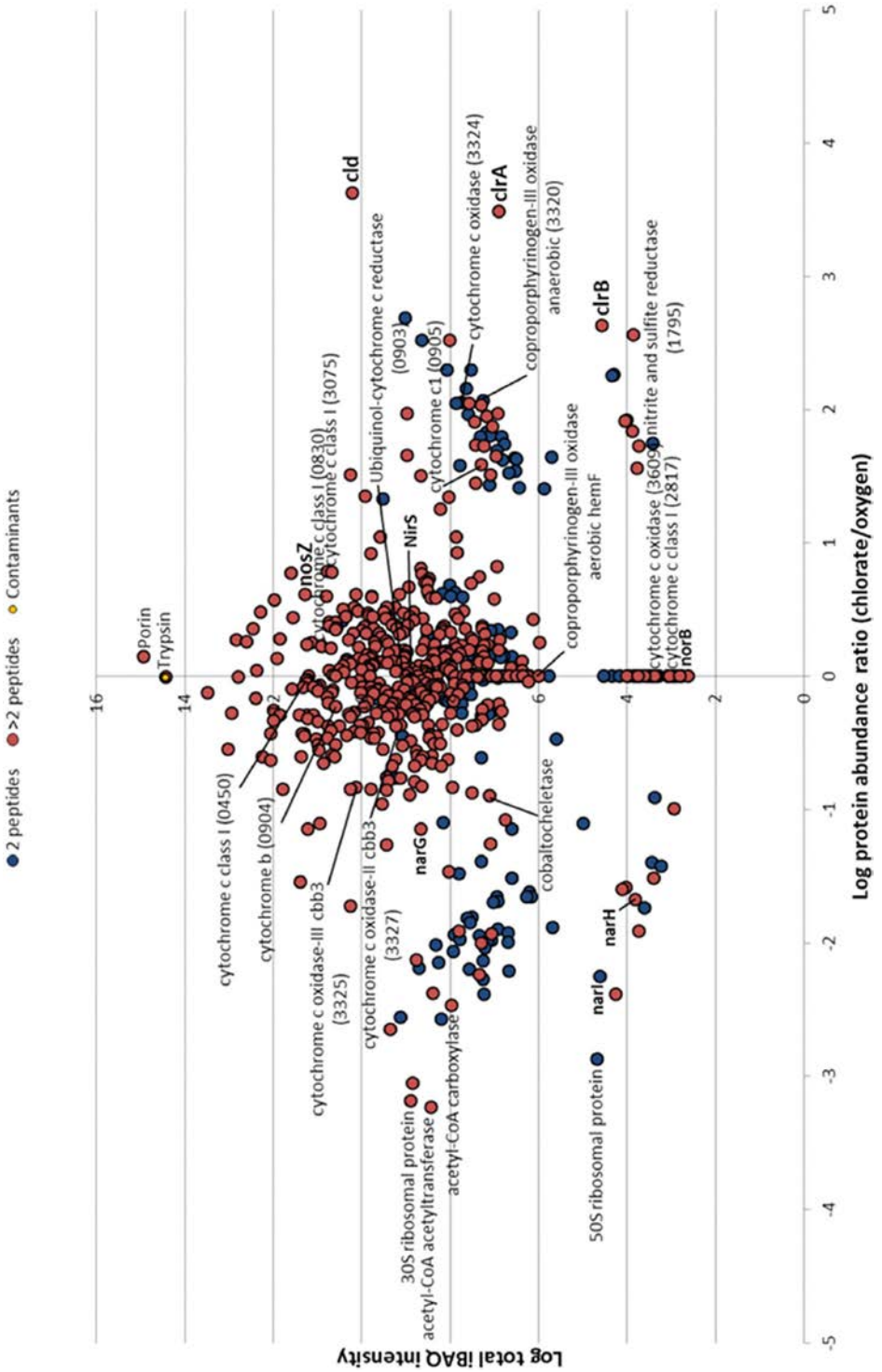


Figure S4. Whole-proteome analysis of chlorate- versus oxygen-grown *A. denitrificans* strain BC cells. The Log of the total intensity based absolute quantification (iBAQ) intensity is plotted against the total protein abundance ratio (in Log label-free quantification (LFQ)). Proteins of which two peptides were identified (blue filled circles) and of which more than two peptides were detected (red filled circles) and the contaminant trypsin (filled circle including yellow dot) are shown.

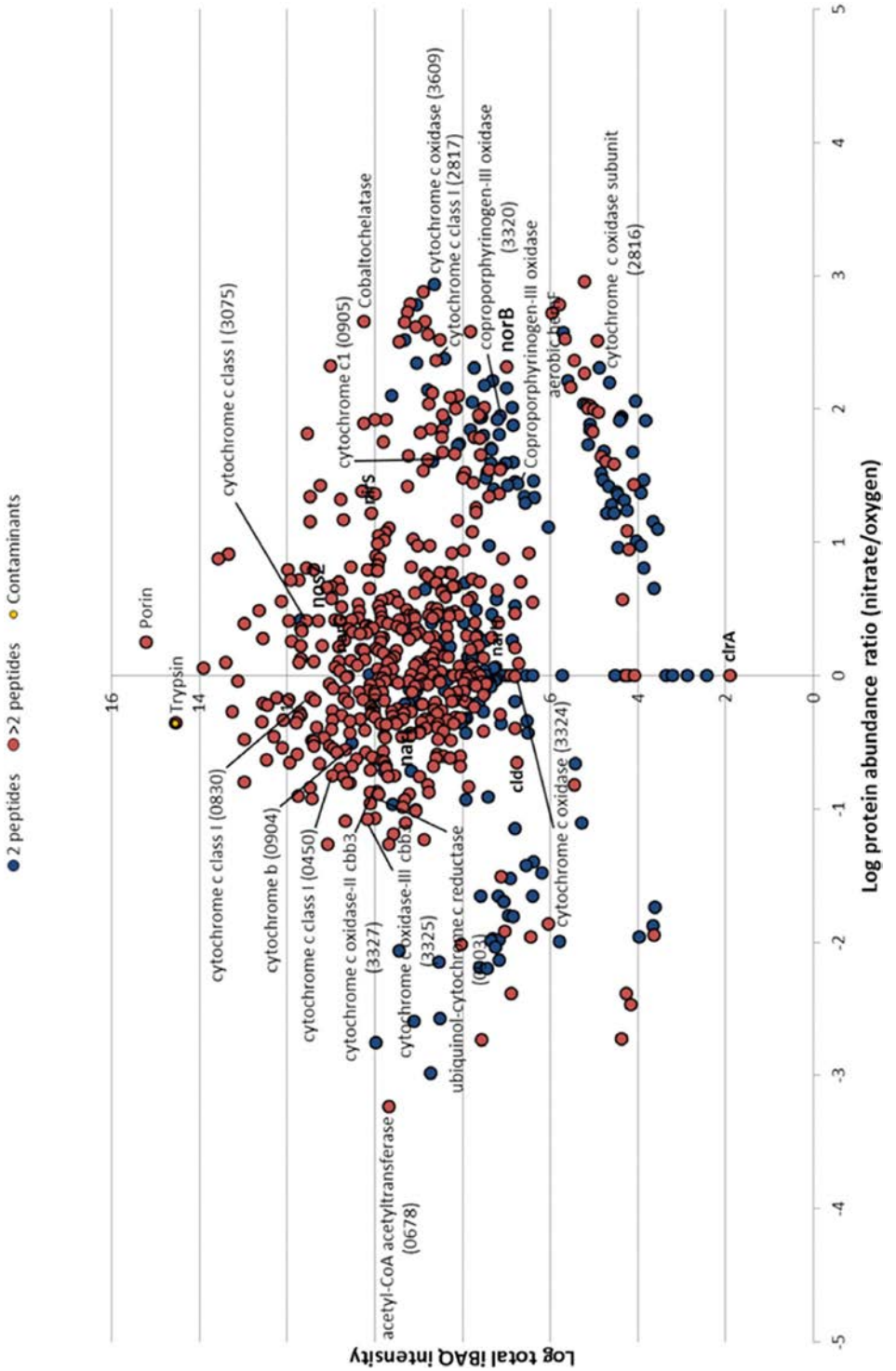


Figure S5. Whole-proteome analysis of nitrate- versus oxygen-grown *A. denitrificans* strain BC cells. The Log of the total intensity based absolute quantification (iBAQ) intensity is plotted against the total protein abundance ratio (in Log label-free quantification (LFQ)). Proteins of which two peptides were identified (blue filled circles) and of which more than two peptides were detected (red filled circles) and the contaminant trypsin (filled circle including yellow dot) are shown.

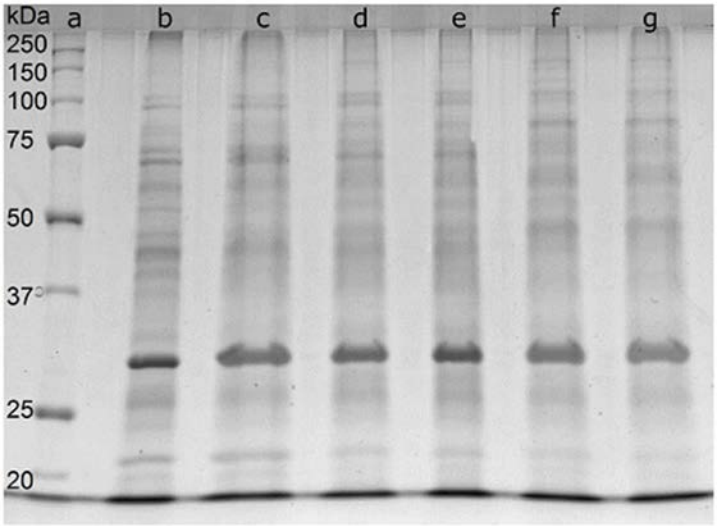


Figure S6. SDS-PAGE gel with cell free extracts of nitrate- (b and c), chlorate- (d and e) and oxygen-adapted (f and g) *A. denitrificans* strain BC cells. A size marker (a), with sizes of 250, 150, 100, 75, 50, 37, 25 and 20 kDa from top to bottom, is shown additionally.

Chapter 4

Chlorate-dependent transposon-mediated modification and deletion of *narG* in *Alicycliphilus denitrificans* strain BC

Authors and affiliations
Margreet J. Oosterkamp[1(a)], Peter J. Schaap[2], Caroline M. Plugge[1], Alfons J.M. Stams[1, 3]*
1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
2 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
3 Centre of Biological Engineering, Campus de Gualtar, University of Minho, 4710-057, Braga, Portugal
(a) Current address: Animal Sciences Laboratory, University of Illinois at Urbana-Champaign, 1207 West Gregory Drive, Urbana, Illinois 61801, USA.
* Correspondent: E-mail: fons.stams@wur.nl; Tel.: (+31) 317 483101; Fax: (+31) 317 483829.

Keywords
Transposon, IS3/IS911, *narG*, chlorate

Manuscript status
Thesis chapter

Abstract

Alicycliphilus denitrificans strain BC is a facultative anaerobic bacterium that uses chlorate and nitrate as electron acceptors for anaerobic growth. Cells adapted to nitrate grow with a lag-phase when transferred to chlorate and vice versa. Genes involved in nitrate reduction are located on the chromosome of strain BC and genes coding for chlorate reduction are located on a plasmid. The gene coding for the alpha subunit of nitrate reductase, *narG*, has a lower gene copy number in chlorate-adapted cells compared to nitrate-adapted cells. We found that, depending on the presence of chlorate, a transposon can insert in *narG*. Cells grown in presence of chlorate also can have a *narG* gene deletion. We propose that the transposon is causing inactivation of the *narG* gene and subsequently a deletion in the NarG locus. To our knowledge this is the first report of transposon-mediated gene-deletion that depends on environmental factors, such as here the nature of electron acceptors.

Introduction

The facultative anaerobic *Alicycliphilus denitrificans* strain BC can degrade benzene with chlorate as an electron acceptor. Strain BC is closely related to *A. denitrificans* strain K601^T that cannot reduce chlorate. The genes necessary for chlorate reduction are located on a plasmid, present in strain BC, but absent in strain K601^T, and genes required for nitrate reduction are located on the chromosomes of both strains (Oosterkamp, Veuskens *et al.* 2011). Nar, the nitrate reductase, can use chlorate as cosubstrate. In chapter 3 we described that cells adapted to nitrate had a lag-phase when transferred to medium with chlorate, and vice versa. In this study we found that gene copy numbers of *narG*, encoding the nitrate reductase alpha subunit, were lower in chlorate- than in nitrate-grown cells. The aim of this study was to get insight in this genetic effect. Initial experiments pointed to an important role of mobile elements.

Mobile genetic elements, such as phages, plasmids, conjugative transposons, integrons and genomic islands, can be spread between bacteria of different species or even different genera (Hacker and Kaper 2000). The flexible gene pool, the genes present in mobile genetic elements, are motility genes and genes that are not strictly necessary for basic metabolism and maintenance of cell integrity, but that offer advantages under particular environmental conditions such as when interacting with host cells, under antibiotic stress and in extreme environments (Hacker and Kaper 2000). Core genes encode proteins involved in basic cellular functions, such as translation and metabolism. The core gene pool and the flexible gene pool together form the main constituents of a bacterial genome (Hacker and Kaper 2000).

Genome evolution is a continuous process leading to changes in the organization and content of genomic information over time. Informational changes include point mutations, gene conversions, gene rearrangements, organizational changes include gene deletions and DNA transposition and insertion of alien DNA. In prokaryotes, this may eventually result in the divergence of populations and to the formation of new species. Transposons were initially discovered by Barbara McClintock and found to be able to change phenotypically significant mutations in maize cells (McClintock 1950). Due to their mobility, transposons are implicated as drivers of genome evolution (Kidwell and Lisch 2001; Feschotte and Pritham 2007). As such they can affect gene function. For example, transposition in a gene followed by misrepair after excision from this gene may result in gene modulation (Emanuel and Shaikh 2001; Ostertag and Kazazian Jr 2001; Kazazian Jr and Goodier 2002; Belancio, Hedges et al. 2008).

Transposons are divided in two classes, class I and class II (Wicker, Sabot *et al.* 2007), and MITEs (miniature inverted-repeat transposable elements) that have characteristics of both class I and II transposons (Casacuberta and Santiago 2003). Class I transposons comprise the retrotransposons. Retrotransposons integrate in a new position in the genome of their hosts by reverse transcription of an RNA intermediate. The three main groups of Class I transposons comprise the Long Terminal Repeats (LTRs), Long INterspersed Elements (LINEs) and Short INterspersed Elements (SINEs). Class II transposons are DNA transposons that do not use an RNA intermediate for proliferation (Shapiro 1979). Class I and II transposons do not always include genes encoding reverse transcriptase or transposase, some are depending on other transposons that do produce the necessary enzymes for proliferation. Therefore, they can be either autonomous or non-autonomous.

Transposase genes are the most abundant genes in genomes and metagenomes and the most ubiquitous in metagenomes (Aziz, Breitbart *et al.* 2010). Although transposons have been implicated in modulating gene function, the presence or absence of a transposon in a gene was, to our knowledge, not directly linked to environmental factors, such as the nature and availability of electron acceptors. This possibility was studied in *A. denitrificans* strain BC.

Material and methods

Inoculum and cultivation procedures

Alicycliphilus denitrificans strain BC (DSM 18852) was isolated in our laboratory (Weelink, Tan *et al.* 2008). Liquid cultures were prepared in 120-mL serum flasks, containing 40 mL anaerobic AW-1-sulfate medium with an N2/CO2 headspace (Weelink, Tan *et al.* 2007). Cultures were incubated at 30°C, in the dark. Acetate, chlorate and nitrate (sodium salts of analytical grade, from commercial sources) were added from sterile, anaerobic stock solutions (0.4 M) to give a concentration of 10 mM. To ensure genetic homogeneity of the cultures, liquid cultures of strain BC were plated on AW-1-sulfate agar medium with 1.2% agar MP (Difco, Becton Dickinson Microbiology Systems, Sparks USA) (Weelink, Tan *et al.* 2007). Acetate (10 mM) was used as carbon and energy source. Either chlorate (10 mM) or nitrate (10 mM) was added as electron acceptor. Colonies were picked and transferred to liquid medium. This procedure was repeated three times and performed in an anaerobic glove box.

Molecular techniques

PCR and quantitative PCR amplification of genes involved in chlorate and nitrate reduction

DNA isolation was performed with the Fast DNA spin kit for soil (MP Biomedicals LLC., CA, USA). 2-mL samples of *A. denitrificans* strain BC cultures were pelleted and resuspended in 500 µL supernatant, these samples were treated as described by the kit manufacturer.

At least 10 ng isolated genomic DNA was used as template in a PCR mix containing 1x green GoTaq reaction buffer (Promega, Madison, WI, USA), 200 µM NTPs (Fermentas), 1.25 u GoTaq polymerase (Promega, Madison, WI, USA) and 10 pmol of the primers (synthesized by Biolegio, Nijmegen, The Netherlands). The primers were designed based on genes involved in chlorate and nitrate reduction that are present in the genome sequence of strain BC (Oosterkamp, Veuskens *et al.* 2011) (Table 1). The PCR program was performed at 95°C for 2 min, with 35 cycles of 95°C for 40 sec, 60°C for 30 sec and 72°C for 1 min and with 72°C for 5 min followed by cool-down to 12°C. Samples were used or stored at -20°C.

Quantitative PCR amplification was performed with universal 16S rRNA primers for bacteria and specific primers targeting the genes coding for NarG and ClrA (synthesized by Biolegio, Nijmegen, The Netherlands). Universal 16S rRNA primers for bacteria were 16S rRNA-bact-1369-F and 16S rRNA-prok-1492-R (Suzuki, Béjà *et al.* 2001). Specific primers for *narG* and *clrA* were used (AdeBC-narG-F, AdeBC-narG-R, AdeBC-clrA-F and AdeBC-clrA-R, Table 1). Primers targeting *narG* and *clrA* were validated using MIQE guidelines (Bustin, Benes *et al.* 2009). Reactions were performed in the Bio-rad CFX96 machine and using the corresponding software package (Bio-rad Laboratories, Hercules, CA, USA). 250 pg/µL and 2.5 pg/µL of cDNA and 5 pmole of primers was used per reaction, prepared as described before (Worm, Stams *et al.* 2011). The program was as follows, 98°C for 5 minutes, 45 cycles of 95°C for 1 minute, 56 (16S rRNA)/60°C (narG, clrA) for 30 seconds and 72°C for 40 seconds. Furthermore, PCR products were analyzed using a melting curve from 65 to 90°C. Log relative copy and transcript numbers were calculated a with 16S rRNA as a reference and with a default Cq value of 35 in case no fluorescence was detected.

Table 1. Primers designed and used in this study.

Primer name	Primer sequence (5' to 3')
AdBC-ClrC-F	GCC AAC ACA GTG ATG AAG GA
AdBC-ClrC-R	ACA TTG ACG ACG TTG ACA GG
AdBC-ClrD-F	CTG ACT GAA AAT GGG CTG GT
AdBC-ClrD-R	AAG GGA CAT TGT GGA AGT GC
AdBC-ClrB-F	AGC CCA ACG TCT TTT ACG TG
AdBC-ClrB-R	CAT ACG CCG TAC CTG TCC TT
AdBC-ClrA-F	GGT CCG CAT TTT CAA TGA CT
AdBC-ClrA-R	CCG ACG TGT ACT TGA TGT GG
AdBC-Cld-F	AAA ACG CTG ATT GGT GGA AC
AdBC-Cld-R	CAG CGC ACG TGA TAC TTG TT
AdBC-NarI-F	CTG GAT CGA CCA TTT CCT GT
AdBC-NarI-R	TCG CTC TTC CAC GTG TAC TG
AdBC-NarJ-F	GAG GAG CGC TAC GTC GAT AC
AdBC-NarJ-R	CTC GTA GGT CTG CGT CAG GT
AdBC-NarY-F	GCC AGT TCG AGA ACA CCT TC
AdBC-NarY-R	TCT TCC TCG CGC TTG TAG AT
AdBC-NarG-F	GCC AGC AGT TCT ACC AGG AC
AdBC-NarG-R	GTT GTC GCT GTA CGT GCT GT
AdBC-NarL-F	AGA TGA TGG GCA AGC TGG T
AdBC-NarL-R	GTG GAT CTT CAC CGT GGT CT
AdBC-NarX-F	CCA GCA GGA ATG GAA GAT GT
AdBC-NarX-R	GAG CAC CAG CAG ATA GCT CA
AdBC-NirN-F	CCT CAA CCT GGT CAA GAC CTA C
AdBC-NirN-R	GGC CTG TTT GTC GTA GGA GAT
AdBC-NirJ-F	GTT TCG CAT CAG CCA ATA CA
AdBC-NirJ-R	TAG CAA TGC TTG CAG GTC AG
AdBC-NirH-F	ACT ACC GCC TGG GCT ACA C
AdBC-NirH-R	CAT GGC GAA AAG GTT GTA GG
AdBC-NirD-F	AAC GCC ATG ACG GTG TTC
AdBC-NirD-R	TGC ACC ATG CAG TAC AGG TT
AdBC-NirF-F	GAG GTG CTG GTC GTC GAT AC
AdBC-NirF-R	ACC GAG AAG TTG ACC CAC AC
AdBC-NirC-F	CAA GCC TCC CGA ATA CCT G
AdBC-NirC-R	GGC TTG GCT CCT CAT TTC TT
AdBC-NirS-F	AGC AGT ACG CCT TCA AGG AA
AdBC-NirS-R	CCC TTG TCC AGG TTG TTG AT
AdBC-asNirB-F	CAG ACA TCG ACC AAG CTC AA
AdBC-asNirB-R	TCC TTG AGC ACC AGC TTC TT
AdBC-asNirD-F	AGG TGT TCG CCC TGC TC
AdBC-asNirD-R	GCA CAG GCC TAT CGT CCA
AdBC-asNapA-F	GGG ACA CAT CAA GCT CCT GT
AdBC-asNapA-R	TGG TGA CCG TAC CTT CCT TC
AdBC-asNarK-F	AGC TTC CTG CCC ACC TAC TT
AdBC-asNarK-R	AAG CAC AGC ATG AAC AGC AG

DNA sequencing

PCR fragments were purified using the DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA) and prepared according to the company's instructions. PCR products were sequenced in collaboration with the company Baseclear (Leiden, The Netherlands).

Results

Amplification and analysis of genes involved in chlorate and nitrate reduction

A. denitrificans strain BC was grown with acetate as electron donor and oxygen, nitrate or chlorate as electron acceptor. Primers targeting genes involved in nitrate and chlorate reduction were used for PCR analysis of genomic DNA from strain BC (Figure 1). PCR products were obtained with all primer sets. The *narG* fragment obtained from cells grown with chlorate (lane 10) was, however, much larger than expected (about 1500 bp versus 200 bp expected).

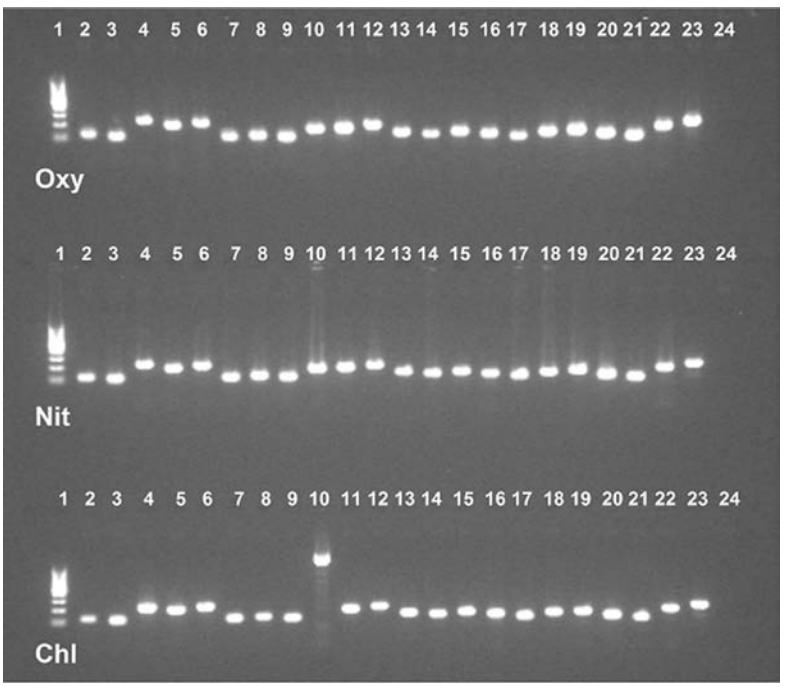


Figure 1. Image of 1.5 % agarose gel used to visualize PCR products of strain BC adapted to oxygen (Oxy), nitrate (Nit) and chlorate (Chl) using primers targeting genes involved in chlorate or nitrate reduction and nitrate assimilation. The lanes show a 100 bp marker (1) and the PCR products of the reaction targeting *clrC* (2), *clrD* (3), *clrB* (4), ***clrA* (5)**, *cld* (6), *narI* (7), *narJ* (8), *narY* (9), ***narG* (10)**, *narL* (11), *narX* (12), *nirN* (13), *nirJ* (14), *nirH* (15), *nirD* (16), *nirF* (17), *nirC* (18), *nirS* (19), assimilatory *nirB* (20), assimilatory *nirD* (21), *napA*-like (22), *narK*-like (23), no template control (24).

Quantitative PCR amplification showed that the *narG* gene copy number was reduced in chlorate-adapted strain BC cells compared to nitrate- and oxygen-adapted cells (Figure 2). Furthermore, the *clrA* gene copy number was higher in chlorate-adapted cells than in nitrate- and oxygen-adapted cells.

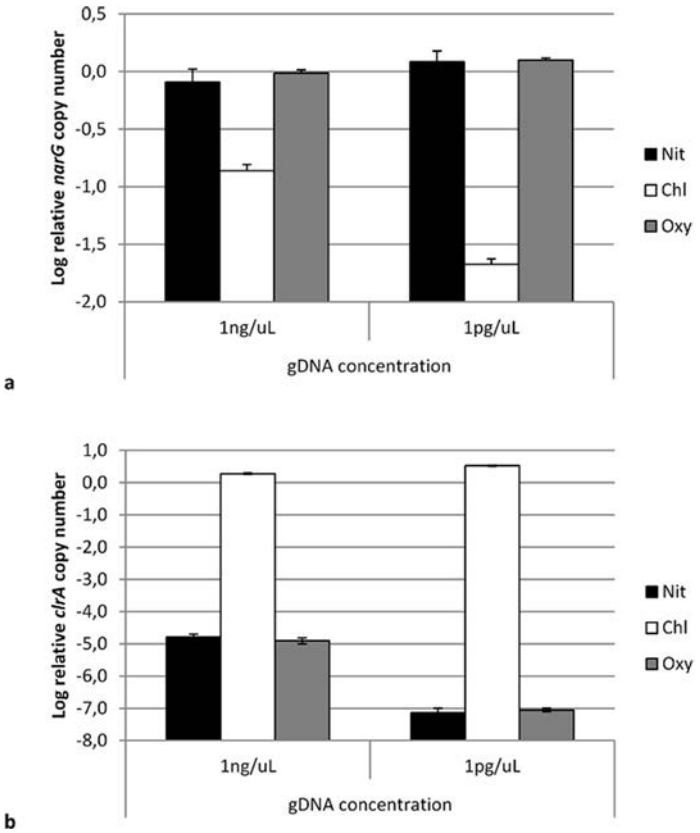


Figure 2, Log relative copy number of *narG* (**a**) and *clrA* (**b**) in *A. denitrificans* strain BC grown with nitrate (nit), chlorate (chl) or oxygen (oxy).

Sequence analysis indicated that a genetic element had inserted in the *narG* gene of chlorate-adapted cells. The 1324 bp insert has inverted repeats at the 5'- and 3' -termini and contains two genes, coding for an integrase and a transposase (Figure 3).

In the whole-genome sequence of strain BC the integrase and transposase encoding genes have twelve copies. There is synteny, all of them are present on the chromosome, but none nearby the NarG encoding gene (Alide_0508) (Table 2).

5′-**TCAGATGA**ACCGCCCCGGGTATCGAGGAGGCCGGTTGGTTTAAGTTGACACCTTTGTCGAGGTGTCGCTGGC
GGCGAGTTGCCTCCAGTAGTTTGCCTCAGCTTCTGCCGGAGGGATGCCCCCAATCGGCGTGAGCAATCGGACGTG
GTTGAACCAGTGCACCCATTGCAGGGTGGCCAGTTCCACGGATTCCCTGGTCTTCCAAGGTCCCCGGCGGTGAAT
CAACTCGGCCTTGTACAGACCGTTGATGGTCTCGGCCAGCGCGTTGTCATAGCTGTCTCCCCTGCTGCCCACCGA
AGGTTGTATGCCCGCCTGGTCCAAGCGTTCGGTGTAGCGTATGGAAACGTACTGACTGCCCCTATCGGAATGGTG
CGTCAATGCATGAGCCGCAGGCTGACGGTCGTACAAGGCCTGCTCCAGGGCATCGAGCACGAAGTCCGTCTGCAT
GCTGCGACTGACGCGCCAGCCCACGATGCGCCGAGCGTACACATCCACCACAAAGGCCACATACAACCAGCCTTG
CCAGGTGGAGACATAGGTGAAGTCCGACACCCATAGCTCGTTGGGACGGCTGGCATGGAAGTGCCGGTTGACATG
ATCCAGCGGGCACGGGACCGAGGTATCCGGCGTGGTGGTGCGCACCGCCCTGCCACGGCGTGCCCCTTGCAAGCC
CATGGCACGCATCAGACGCTCGACCGTGCAGCGCGCCACCACGATGCCCTCGCGGTTCATCTGCAGCCAGACCTT
GTCGGCTCCGTAGACCTGCCAGTTGGCGTGCCACACGCGCTGAATGTCGGCCTTCAAACCCTCGTCACGCTGGGC
GCGTGCACTGCGCAGTTGCGCGTTGCGTTGCCGGGCTGCGTGGCGCCAGTAACACGACGGGGCCATCTGCAGCGC
CCGGCAGATGGGCTCGACCCCGTAGTCATCACGGTGGCGGTCGATGTAGGCCT**TCAC**GACTTGAGTCGGCGGTCG
AGCTCCGCCTGCGCGAAAAACGCGCTGGCCGTCTTCAGGATGTCGTTGGCCCGGCGCAATTCTTTGACCTCACGC
TCCAGTACCTTGATGCGCAGGGCGTCTGCAGTGGTGGTGCCGGGGCGCTGGCCGCTGTCGACCTCGGCCTTCTTG
ACCCAGTCGTTCAAGGTCTGTGGCACGCAGCCAATCTTGGGGGCAATCGATTCAATCGCTGCCCACAGCGACGGG
TAGTCGGCTCGGTGCTCCTGCACCATGCGCACGGCGCGCTCGCGCACTTCCGGGGAGAACTTCGGTGATTTGTTC
ATGGCTCCATCTTCTCAAGTGTTGGAGCCTCCTCAAAACCCGGGGCGGT**TCA**-3′

Figure 3. Nucleotide sequence of the 1324bp insert in *narG* of chlorate-grown *A. denitrificans* strain BC. The insert encodes an integrase (red) and an IS3/IS911 family transposase (blue), transcription start and end of the integrase and transposase encoding genes, respectively, are overlapping (bold and underlined), border nucleotides (bold) and inverted repeats (underlined) were found at the 5'- and 3'-termini.

Table 2. Copies of the integrase and transposase genes located on the *narG* insert in the *A. denitrificans* strain BC genome sequence. A neighboring functional gene with the protein that it encodes is included.

Integrase gene	Transposase gene	Neighbor integrase gene	Neighbor transposase gene
Alide_0725	Alide_0726	Pseudo gene	Pseudo gene
Alide_0921	Alide_0922	Hypothetical protein encoding gene	Hypothetical protein encoding gene
Alide_1440	Alide_1439	Hypothetical protein encoding gene	Pseudo gene
Alide_1482	Alide_1481	Pseudo gene	Integrase gene
Alide_1531	Alide_1530	ABC-transporter gene	LexA repressor/DNA repair protein encoding gene
Alide_1822	Alide_1821	Hypothetical protein encoding gene	Integrase gene
Alide_1837	Alide_1838	Helicase gene	Pseudo gene
Alide_1874	Alide_1875	Pseudo gene	Pseudo gene
Alide_2126	Alide_2127	tRNA gene	Efflux transporter gene
Alide_2711	Alide_2712	Hypothetical protein encoding gene	DNA topoisomerase III gene
Alide_2879	Alide_2880	Pseudo gene	Integrase-like gene
Alide_3769	Alide_3768	Integrase gene	Pseudo gene

Presence of an insert in narG ***in chlorate- and nitrate-adapted cells***

Growth experiments and genetic studies were performed to show that the *narG* insert is depending on the presence of chlorate. In all genetic analyses performed with cells adapted to nitrate, *narG* did not contain an insert. Strain BC was grown with acetate as electron donor and chlorate, nitrate or oxygen as electron acceptor, and then cultures were transferred regularly to fresh medium for several years. Chlorate-adapted cells from strain BC that were harvested in the year 2008, contained both *narG* with and without insert and cells harvested in 2009, 2010 or 2011 only *narG* with insert. Furthermore, nitrate-adapted cells harvested in the years 2008, 2009, 2010 or 2011 had no insert in *narG* (Figure 4).

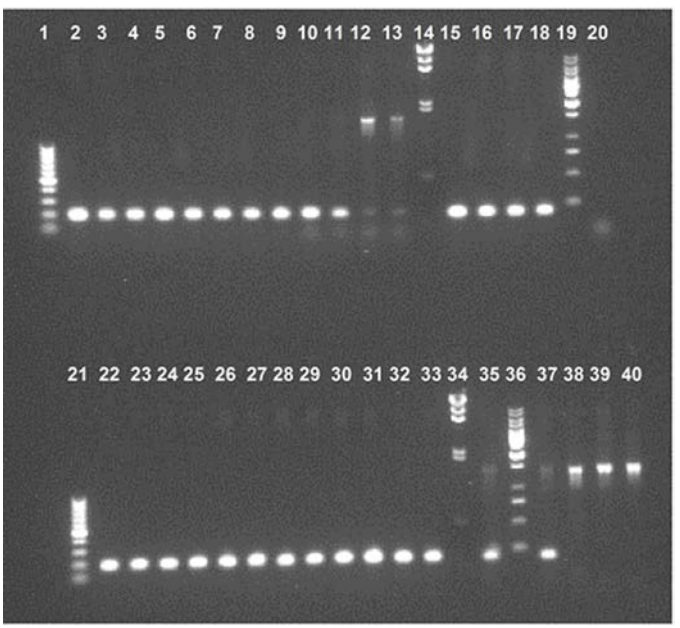


Figure 4. Image of 1.5 % agarose gel used for *narG* PCR amplification of nitrate- and chlorate-adapted cultures and cultures of nitrate- or chlorate adapted cells transferred to medium with chlorate or nitrate, respectively. The lanes contain a 100 bp marker (1, 21), a Lambda/HindIII marker (14, 34), a 1kb marker (19, 36). Furthermore, in the upper part fragments of the following cultures are shown: chlorate-adapted cultures transferred to nitrate from the first (2, 3), second (4, 5) and third (6, 7) transfer and of the subsequent first (8, 9), second (10, 11) and third (12, 13) transfer back to chlorate. Nitrate-adapted cultures from 2008 (15), 2009 (16), 2010 (17) and 2011 (18) and a no template control (20) are also shown. In the lower part, fragments of the following cultures are displayed: nitrate-adapted cultures transferred to chlorate from the first (22, 23), second (24, 25) and third (26, 27) transfer to chlorate and from the first (28, 29), second (30, 31) and third (32, 33) transfer back to nitrate. Chlorate-adapted cultures from 2008 (35, 37), 2009 (38), 2010 (39) and 2011 (40) are also shown.

The nitrate-adapted cultures were transferred in duplicate to medium with chlorate for three times successively and, subsequently, back to medium with nitrate for three successive transfers. The chlorate-adapted cultures were treated similarly but were transferred to medium with nitrate and back to chlorate, respectively. Subsequently, PCR amplification was performed (Figure 4) and when chlorate-grown cells were transferred to nitrate and subsequently to chlorate, the insert was observed after three transfers to chlorate (lanes 12 and 13). In all other cells tested, the insert in *narG* was not observed.

From colonies of strain BC genetically homogeneous chlorate-adapted strain BC cells were grown. PCR analysis did not show integration of the insert in *narG* in these cells. Instead,

no *narG* fragment was obtained from genetically homogeneous chlorate-adapted cells (Figure 5). Furthermore, these cells did not grow easily in medium with nitrate and no *narG* fragment performed with cell material collected from these experiments was observed (Figure 5). From genetically homogeneous nitrate-adapted cells, *narG* fragments without insert were obtained (Figure 5). Similar fragments were observed in *narG* PCR analysis with homogeneous nitrate-adapted cells that were transferred to chlorate (Figure 5).

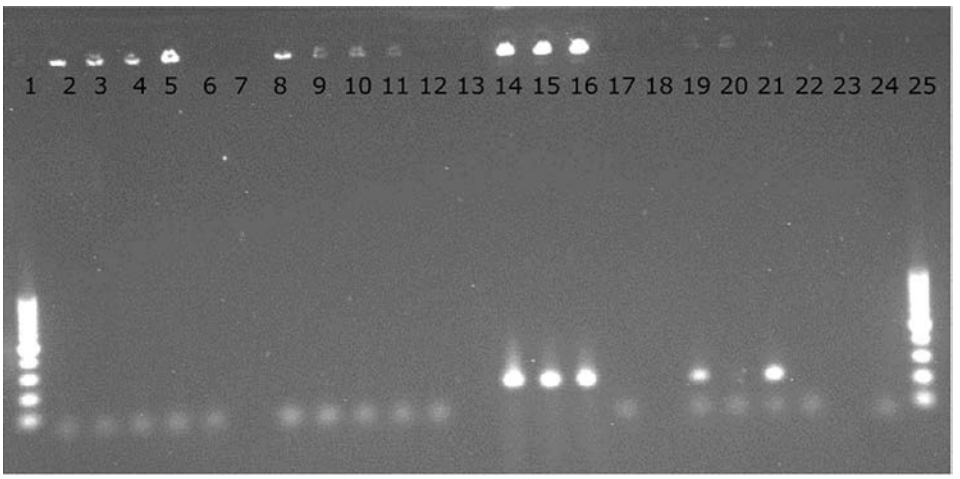


Figure 5. Image of 1.5 % agarose gel used for *narG* PCR amplification of nitrate- and chlorate-adapted cultures and cultures of nitrate- or chlorate adapted cells transferred to medium with chlorate or nitrate, respectively. The lanes contain a 100 bp marker (1, 25) and fragments of the following cultures: chlorate-adapted cells (2, 3, 4, 5, 6), chlorate-adapted cells transferred to nitrate (8, 9, 10, 11, 12), nitrate-adapted cells (14, 15, 16, 17) and nitrate-adapted cells transferred to chlorate (19, 20, 21, 22). A no template control (24) and lanes left empty (7, 13, 18, 23) are also shown.

Discussion

To our knowledge, reversible targeted integration of a mobile genetic element into a functional gene as a genetic switch in response to changes in environmental conditions has not been reported previously. Nitrate-adapted *A. denitrificans* strain BC cells never contained *narG* with insert, but this insert was present in cells transferred to chlorate (Figure 4). Previously it was described that strain BC cells adapted to chlorate do not grow easily with nitrate as electron acceptor and vice versa (Chapter 3). Growth difficulties of chlorate-adapted cells transferred to nitrate may result from modification of *narG* in these cells.

Although preparing genetically homogeneous cultures using colony-picking is not always conclusive (Korolev, Xavier *et al.* 2011), genetic variation might at least be limited. From the genetically homogeneous chlorate-adapted cultures *narG* fragments could not be obtained anymore, likely because (one of) the sites that are targeted by the primer set annealing to *narG* were removed (Figure 5). Genetically homogeneous cultures adapted to nitrate did not grow easily with chlorate (Chapter 3). The *clrA* gene is located on a plasmid (Oosterkamp, Veuskens *et al.* 2011). Since the *clrA* gene is less present in nitrate-adapted cells (Figure 2) there was likely a loss of this plasmid in nitrate-adapted cells.

The *narG* insert was present at 12 loci of the whole-genome sequence of strain BC, but not in the *narG* gene (Table 2). Based on the integration loci of the transposon in the genome sequence, the transposon was not present at loci of which the genes are functionally related (Table 2). Therefore, gene regulation or inactivation by this insert is not restricted to

only one specific function or enzymatic pathway. Despite the fact that this transposon seems to integrate into random genes, integration in *narG* was observed in chlorate-adapted cells, but not in nitrate-adapted cells of strain BC.

Two proteins are encoded by the *narG* insert, a transposase and an integrase involved in mobilization and integration of genetic material. The transposase is of the IS3/IS911 family. Little is known about the insertion specificity of this family of transposases. The gene coding IS911 is present next to DNA sequences that resemble their inverted repeats (IRs). Furthermore, the helix-turn-helix motif of transposase is involved in binding of transposase to these IRs (Polard, Seroude *et al.* 1994; Rousseau, Loot *et al.* 2007). Transposition involves a closed circular DNA intermediate (Rousseau, Tardin *et al.* 2010). Recently, it was shown that the transposase proliferates more actively when the transposase is encoded on the transposon DNA sequence. Furtermore, the transposase needs to connect the ribosome to the IRs for proliferation, indicating that there is no unrestrained mobilization of IS911 elements (Duval-Valentin and Chandler 2011). Further research needs to show if and how the mechanism of IS911 transposition can be directly coupled to regulation of gene function.

Acknowledgements

This research was funded by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053.

Chapter 5

Proteomics of nitrate-dependent acetone degradation by *Alicycliphilus denitrificans* strain BC

Authors
Margreet J. Oosterkamp[1(a)], Sjef Boeren[2], Caroline M. Plugge[1], Peter J. Schaap[3] and Alfons J.M. Stams[1]*

1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
2 Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.
3 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
(a) Current address: Animal Sciences Laboratory, University of Illinois at Urbana-Champaign, 1207 West Gregory Drive, Urbana, Illinois 61801, USA.
* Correspondent: E-mail: fons.stams@wur.nl; Tel.: (+31) 317 483101; Fax: (+31) 317 483829.

Keywords
Acetone, anaerobic, nitrate, acetoacetate, beta-hydroxybutyrate

Manuscript status
Thesis chapter

Abstract

Alicycliphilus denitrificans strain BC, like reported for *Alicycliphilus denitrificans* strain K601^T, is able to degrade acetone anaerobically with nitrate as electron acceptor but not with chlorate or aerobically. The proteomes of acetone and nitrate-grown cells and acetate and nitrate-grown strain BC cells were compared to reveal the biochemical pathway and the proteins involved in the degradation of acetone. In strain BC acetone is proposed to be degraded via acetoacetate, acetoacetyl-CoA and acetyl-CoA as intermediates. This pathway is similar to the one present in other microorganisms that degrade acetone anaerobically. Like in these microorganisms, in strain BC acetone carboxylase is involved in initiation of acetone degradation. Furthermore, an AMP-dependent synthetase and ligase is proposed to be involved in conversion of acetoacetate to acetoacetyl-CoA and an acetyl-CoA acetyltransferase in the conversion of acetoacetyl-CoA to two acetyl-CoA. Our proteome data indicated that an aldehyde dehydrogenase is important for acetone degradation in strain BC. We suggest that this enzyme functions as a beta-hydroxybutyrate dehydrogenase, that catalyses surplus acetoacetate conversion to beta-hydroxybutyrate and the reverse reaction. beta-hydroxybutyrate is one of the keton bodies and may also function as carbon and energy storage for strain BC.

Introduction

Acetone (or dimethylketone, or 2-propanone) is a compound that is produced in biological solvent production but also by mammals (George, Johnson *et al.* 1983; Ensign, Small *et al.* 1998; Kalapos 2003). It is a common organic solvent that is used in nail-polish remover or as disinfectant. There is a need to biodegrade the compound when it is a burden to the environment, due to the disposal by pharmaceutical industries (Balasubramanian, Philip *et al.* 2011). Acetone is volatile and has been found in the upper troposphere where it may contribute to the production of hydrogen radicals (HO and HO_2) and to nitrogen oxide and ozone cycling (Singh, Kanakidou *et al.* 1995).

Acetone is known to be degraded by aerobic as well as anaerobic microorganisms. Several pathways for microbial acetone metabolism have been described. Aerobically, but not anaerobically, acetone can be converted to 1-hydroxyacetone by oxygenases in the pathway: acetone → 1 hydroxyacetone → methylgloxal → pyruvate (Taylor, Trudgill et al. 1980). Pyruvate is further metabolized in the tricarboxylic acid (tca) cycle. Another aerobic acetone degradation pathway, which is present in *Gordonia sp.* strain TY-5 was reported to involve oxidation of acetone to methyl acetate by acetone monooxygenase and a subsequent mono-oxygenase mediated conversion of methyl acetate to acetate and methanol (Kotani, Yurimoto et al. 2007). Acetate is degraded further in the tca cycle, while the fate of methanol is unknown.

Acetone can also be first carboxylated to acetoacetate (acetone + CO_2 + ATP + $2H_2O$ → acetoacetate + AMP + $2P_i$ + $3H^+$). Acetoacetate is activated to acetoacetyl-CoA and then converted to 2 acetyl-CoA (Platen, Temmes et al. 1990). The initial activation of acetone is catalyzed by acetone carboxylase. This enzyme was described to be involved in anaerobic photosynthetic degradation of acetone, and in acetone degradation using nitrate or sulfate as electron acceptor (Siegel 1957; Taylor, Trudgill *et al.* 1980; Bonnet-Smits, Robertson *et al.* 1988; Platen and Schink 1989; Birks and Kelly 1997; Sluis, Larsen *et al.* 2002; Dullius, Chen *et al.* 2011).

It is known that the carboxylation of acetone is a thermodynamically unfavorable reaction ($\Delta G^{0\prime}$ = +17.1 kJ/mole) that is ATP -dependent (Ensign, Small *et al.* 1998; Schühle and Heider 2012). In the denitrifying bacterium BunN, the ATP-dependent decarboxylation of acetoacetate could be demonstrated (Platen and Schink 1990). This was proposed to be a partial reaction of the acetone carboxylase (Janssen and Schink 1995). Recently, *Alicycliphilus denitrificans* strain KN Bun08 was isolated and the acetone carboxylase of this strain and of the type strain, *A. denitrificans* strain $K601^T$ were purified and characterized (Dullius, Chen et al. 2011). We studied acetone degradation in *A. denitrificans* strain BC and performed proteome analysis to obtain more insight in the enzymes involved in acetone degradation.

Materials and Methods

Physiological studies and culture preparation

A. denitrificans strain BC was grown using anaerobic phosphate-bicarbonate-buffered (pH 7.3), AW1-sulfate medium (Weelink, Tan *et al.* 2007). Cultures were incubated without agitation at 30°C in the dark.

Initial experiments to test growth of strain BC with acetone as electron donor and nitrate, chlorate or oxygen as electron acceptor, were performed using 40-mL cultures in 120-mL serum flasks. The conditions were as described previously (Weelink, Tan *et al.* 2007) with exceptions as follows. The concentration of acetone varied (8, 4 or 2 mM) and acetate was added in low concentration (1 mM) to stimulate growth. Nitrate and chlorate were added to

final concentrations of 10 mM, and 3 mmol/L of oxygen was added to the headspace of the flasks from a sterile pure oxygen stock. 5% of inoculum adapted to growth with acetate and either nitrate, chlorate or oxygen was added to the flasks. Negative controls (without inoculum) were included. Cultures were incubated for six days.

For more detailed physiological analysis, strain BC was grown in triplicate with acetone (10 mM) and nitrate (10 mM) as electron acceptor and cultures with acetate (10 mM) and nitrate (10 mM) as electron acceptor were used for comparison. Negative controls (no inoculum) were also included. 5% of inoculum adapted to growth with acetate and nitrate was used in the cultures. Cultures were grown in 120-mL serum as described above. Cultures were sampled at 0, 2, 5, and 8 days of incubation for OD and HPLC analysis. 1.5-mL culture samples were stored at -20°C. Prior to storage of samples, the OD at 660nm was measured with a Hitachi spectrophotometer (Hitachi, Tokyo, Japan). To determine biomass yields, cells of the cultures were harvested in 50-mL Greiner tubes (Greiner Bio-One, Frickenhausen, Germany) and centrifuged in Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany). Pellets were dried in a SpeedVac concentrator (Savant Instruments, Holbrook, NY, USA) and used for dry weight determination.

To obtain cells for proteome analysis, 80-mL cultures in 250-mL flasks closed with butyl rubber stoppers and aluminum screw caps were prepared as described previously (Weelink, Tan *et al.* 2007). Cells were grown with acetone or acetate (10 mM) and nitrate (10 mM) and 5% inoculum (cells used as inoculum were adapted to acetone or acetate and nitrate). Cultures were incubated for ten days.

Analytical procedures

Gas chromatography

Oxygen was measured by headspace analysis using a gas chromatograph (GC-14B; Shimadzu, Kyoto, Japan) with a packed column (Molsieve 13x 60/80 mesh, 2 m x 2.4 mm; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The column, detector, and injector temperatures were 100, 150, and 90°C, respectively. Argon was used as carrier gas (flow rate 30 $mLmin^{-1}$). 0.4 ml headspace was injected in the gas chromatograph.

Liquid chromatography

The anions chlorate, nitrate, chloride, and nitrite were measured by suppressor mediated ion chromatography (Dionex, Breda, the Netherlands) with a conductivity detector. The chromatograph was equipped with an IonPac AS9-SC column (Dionex). A solution containing 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ is pumped through the system at a flow rate of 1 mL $min.^{-1}$. Mannitol (10 mM) was added to samples for stabilization, and KF (1 mM) was used as internal standard. Acetate was analyzed by liquid chromatography. The chromatograph was equipped with a column for detection of organic acids (Merck organic acid column 300-6,5: Polyspher OA HY). The eluent used was 10 mN sulfuric acid at a flow rate of 0.8 mL/min. The internal standard was 10 mM crotonate.

Microorganisms

Alicycliphilus denitrificans strain BC (DSM 18852) was isolated in our laboratory (Weelink, Tan *et al.* 2008).

Chemicals

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

Phylogenetic studies

Phylogenetic comparison of acetone carboxylase alpha subunit of A. denitrificans *strain BC*

The acetone carboxylase alpha subunit of strain BC was found to be encoded by Alide_1503. The amino acid sequence of this subunit was used for blast searching (Altschul, Madden et al. 1997) and the most related proteins (11 leaves) were selected and used for clustalW alignment and phylogenetic tree construction (www.ebi.ac.uk/Tools/msa/clustalw2 and /Tools/phylogeny). The phylogenetic tree was calculated using the neigbour-joining clustering method and in nexus tree format. Using these data, a tree was constructed in TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

The phylogenetic tree was constructed using the protein sequences YP_002890441.1 of *Thauera sp* MZ1T, ZP_08503988.1 of *Methyloversatilis universalis* FAM5, YP_004389269.1 of *Alicycliphilus denitrificans* K601^{T}, YP_004126150.1 of *Alicycliphilus denitrificans* BC, ABE73732.1 of *Azoarcus communis*, YP_004681502.1 of *Cupriavidus necator* N-1, YP_293345.1 of *Ralstonia eutropha* JMP134, YP_586242.1 of *Cupriavidus metallidurans* CH34, BAL22908.1 of *Azoarcus sp.* KH32C, YP_284245.1 of *Dechloromonas aromatica* RCB, and YP_159681.1 of *Aromatoleum aromaticum* EbN1.

Proteomics

Separation of proteins from cell free extracts

Cell free extracts of strain BC grown with acetate and acetone with nitrate as electron acceptor were prepared for whole-proteome analysis. The cell free extracts were prepared as described previously (Wolterink, Jonker *et al.* 2002) and stored in 2-mL Low Binding tubes (Eppendorf, Hamburg, Germany) at -20°C. A Bio-rad protein-assay (Bio-rad Laboratories, Hercules, CA, USA) was used according to the manufacturer's instructions to determine the protein concentration of cell extracts. Bovine serum albumin was used as protein standard. 25 µg of protein was loaded on a 10% SDS-polyacrylamide separation gel (pH 8.8) with a stacking gel (pH 6.8) using the mini-protean 3 cell (Bio-rad Laboratories, Hercules, CA, USA). An empty lane was left between each loaded lane and the electrophoresis procedure was according to the manufacturer's instructions. Gels were stained using Coomassie Brilliant Blue R-250 as indicated in the protocol of the mini-protean 3 system. Gels were scanned and Quantity One software (Bio-rad Laboratories, Hercules, CA, USA) was used to calculate the intensity of each of the lanes (whole lanes) loaded with cell free extract. The ratio of intensity of the lanes to the lane with the highest intensity was calculated and used to prepare a gel in which these intensities were similar.

In-gel digestion of proteins

SDS-PAGE separated proteins from strain BC grown with acetate with chlorate, nitrate, or oxygen as electron acceptor, were subjected to in-gel digestion. Each of the used gel lanes was cut into four slices using a clean scalpel and on a clean microscope slide. Slices were further processed to pieces of about 1 mm^2 and the gel pieces obtained were put in 1.5-mL Low Binding tubes (Eppendorf, Hamburg, Germany) and reduced, alkylated and trypsin digested as previously described (Rupakula, Kruse *et al.* 2013). The supernatant obtained was used for LC-MS/MS analysis.

Liquid chromatography with tandem mass spectrometry

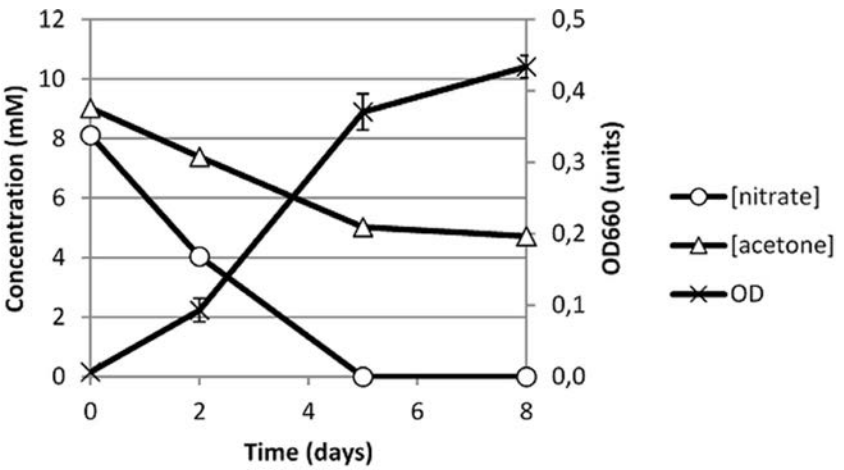
Protein digests obtained from cells of *A. denitrificans* strain BC grown with acetate with chlorate, nitrate, or oxygen as electron acceptor, were analyzed on LC-MS/MS (at Biqualys, Wageningen, The Netherlands). The procedure is as described previously (Lu, Boeren *et al.* 2011). MS/MS spectra were analyzed using MaxQuant software (maxquant.org) and a protein

database of *A. denitrificans* strain BC obtained from the European Bioinformatics Institute (www.ensemblegenomes.org) as described before (Peng, van Lent *et al.* 2012).

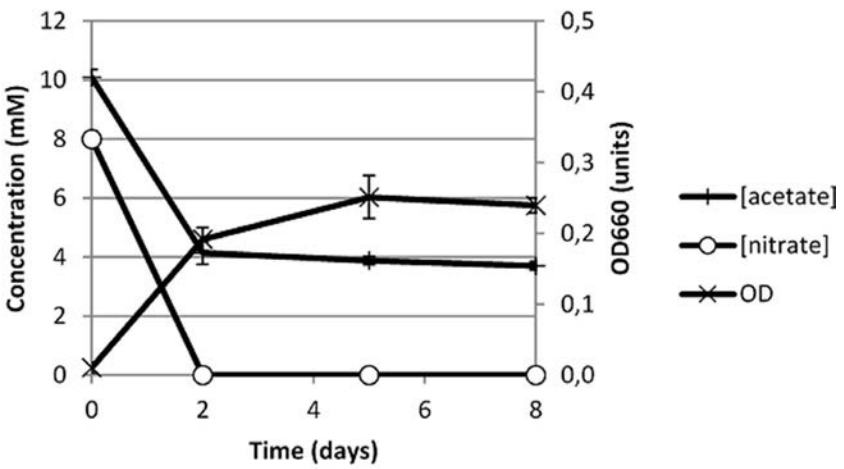
Results

Nitrate-dependent acetone-degradation in A. denitrificans

A. denitrificans strain BC was grown with acetone or acetate as electron donor and with nitrate, chlorate and oxygen as electron acceptors. Acetone was degraded with nitrate, but not with chlorate and oxygen.Subsequent experiments with acetone and nitrate and acetate and nitrate showed that higher OD values were reached with acetone, but that conversion of acetone was slower thanconversion of acetate (Figure 1).



a



b

Figure 1. Time course of *A. denitrificans* strain BC growing with acetone (**a**) and acetate (**b**). Nitrate was used as electron acceptor and optical density (OD at 660nm) as a measure for growth.

The optical density in acetone cultures was about twice as high compared to acetate cultures (Table 1). In cultures grown with acetone, the dry weight biomass yield was about three times higher (38.9 gram biomass mole acetone^{-1}) than in cultures grown with acetate (12.9 gram biomass mole acetate^{-1}).

Table 1. Growth of *A. denitrificans* strain BC with acetone and acetate as electron donor. Nitrate was used as electron acceptor.

culture	Δculture density	Δe^- donor	Δe^- acceptor	Dry weight biomass (g) per mole e^- donor
	t 0-8 (OD units)	t 0-8 (mM)	t 0-8 (mM)	
Acetone	0.427±0.016	4.30±0.23	8.11±0.15	38.9
Acetate	0.229±0.016	6.39±0.16	8.00±0.29	12.9

Proteomics of acetone-degradation in A. denitrificans *strain BC*

A. denitrificans strain BC was grown with acetate and nitrate and acetone and nitrate. Cell free extracts of these cultures were used for proteome analysis. As can be seen in Figure 2, the three acetone carboxylase subunits acxABC (encoded by Alide_1502-1504) were abundant in cells grown with acetone. Given the protein abundance ratio, this enzyme is about 4000 fold more abundant in acetone-grown cells compared to acetate-grown cells (Table 2). Acetone carboxylase of *A. denitrificans* is a hexamer with an α_2-β_2-γ_2-structure (Dullius, Chen *et al.* 2011). In the genome of strain BC, Alide_1504 was annotated as acetone carboxylase gamma subunit (Oosterkamp, Veuskens et al. 2011). According to previous research (Dullius, Chen *et al.* 2011), Alide_1502 is similar to the beta subunit and Alide_1503 to the alpha subunit of acetone carboxylase from *A. denitrificans*. In the genome of strain BC, these genes are annotated as hydantoinase/oxoprolinase and hydantoinase b/oxoprolinase, respectively. These proteins belong to the same protein family as acetone carboxylases (Pfam01968).

Acetone carboxylase converts acetone to acetoacetate, which is further degraded to acetoacetyl-CoA and then to 2 acetyl-CoA (Platen, Temmes et al. 1990). In acetone-grown cells of strain BC, an AMP-dependent synthetase/ligase (Alide_4154) was 65 fold more abundant than in acetate-grown cells (Figure 2, Table 2). This protein might function as an AMP-forming acetoacetate-CoA ligase. An acetyl-CoA acetyltransferase (Alide_0678) that can convert acetoacetyl-CoA to two acetyl-CoA was found to be 68 fold more abundant in acetone-grown cells (Figure 2, Table 2).

An aldehyde dehydrogenase (Alide_4113) was 32810 fold more abundant in acetone–grown cells (Figure 2, Table 2). The function of this enzyme is not fully clear. We speculate that this enzyme may function as a beta-hydroxybutyrate dehydrogenase, that converts acetoacetate to beta-hydroxybutyrate. This compound is one of the ketone bodies that can be stored and converted back to acetoacetate by the same enzyme.

Other proteins that were abundantly present in acetone-grown compared to acetate-grown cells are an extracellular ligand-binding ABC transporter (Alide_1344-1347), regulators (Alide_0251+0468+2994+3200+3351) and transporters (Alide_0395+0650+2304), see Table 2.

Proteins that were more abundant in acetate-grown cells include peptidoglycan glycosyltransferase, cobaltochelatase, protein channel protein, ribosomal protein (Figure 2, Table 2).

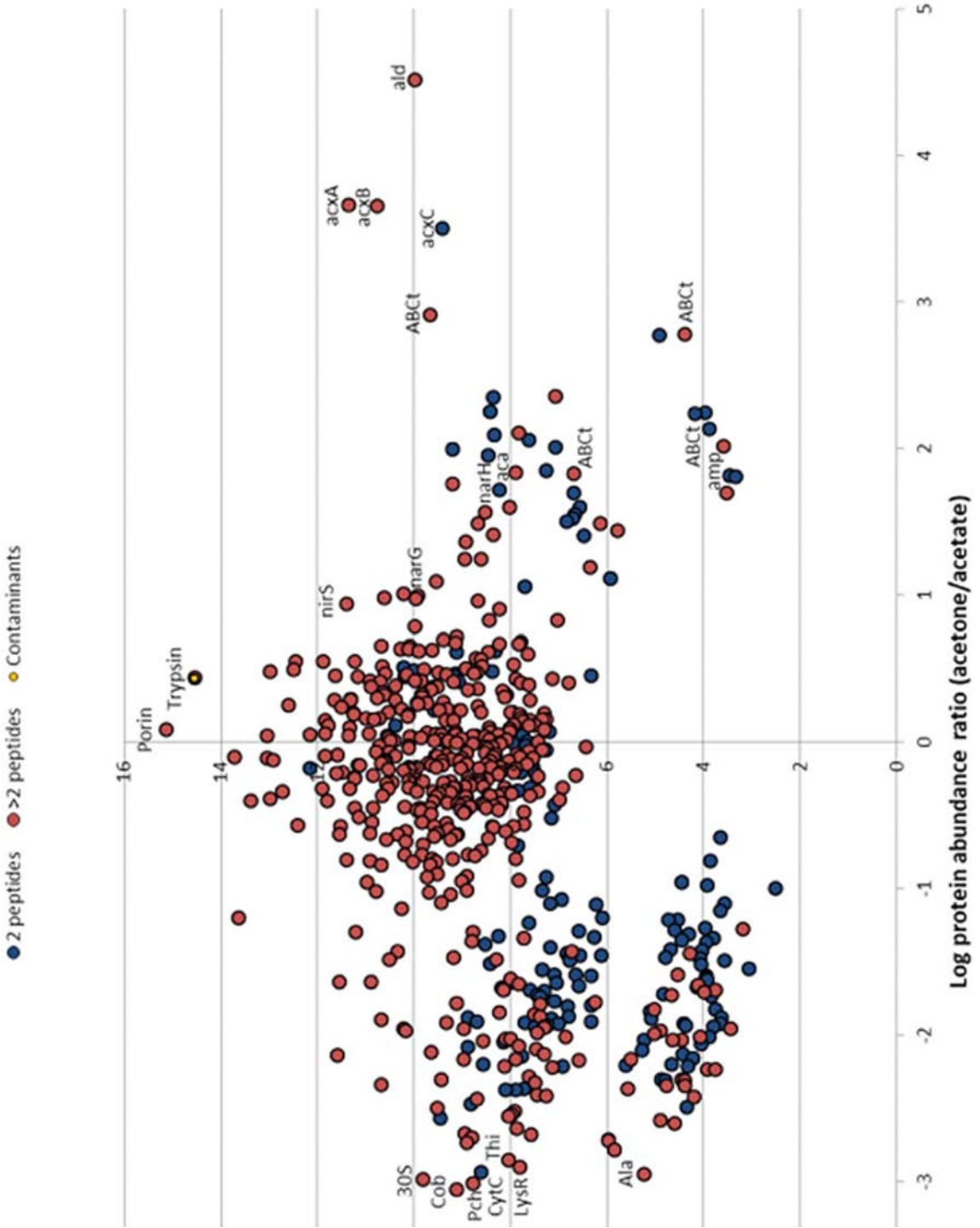


Figure 2. Protein abundance in *A. denitrificans* strain BC grown with acetone and nitrate and grown with acetate and nitrate. Proteins were detected by LC-MS/MS and quantified using MaxQuant software. The log total iBAQ intensity is potted against the log protein abundance ratio of acetone to acetate. Acetone carboxylase (acxABC), aldehyde dehydrogenase (ald), ABC transporter (ABCt), acetyl-CoA acetyltransferase (aca), AMP-dependent synthetase/ligase (amp), nitrate reductase (narGH), nitrite reductase (nirS) and trypsin are indicated.

Table 2. Most abundant proteins in *A. denitrificans* strain BC grown with acetone and nitrate compared to grown with acetate and nitrate. Cut-off was set to log protein abundances <-2.8 and >1.8. The log total iBAQ intensity and log protein abundance ratio are as obtained from MaxQuant analysis. Uniprot numbers and protein descriptions are obtained from the EBI protein database.

Protein description	Gene #	Uniprot #	Log protein abundance ratio	Log total iBAQ intensity	Δabundance acetone to acetate (fold)
Aldehyde dehydrogenase	Alide_4113	E8TW43	4.516	9.989	32810
Acetone carboxylase A	Alide_1503	E8TTA6	3.663	11.357	4603
Acetone carboxylase B	Alide_1502	E8TTA5	3.652	10.764	4487
Acetone carboxylase C	Alide_1504	E8TTA7	3.501	9.419	3170
ABC transporter extracellular ligand	Alide_1346	E8TRK6; E8U068	2.910	9.669	813
ABC transporter extracellular ligand	Alide_1347	E8TRK7	2.782	4.395	605
Helix-turn-helix domain regulator	Alide_0251	E8TTT1	2.770	4.923	589
Chemotaxis sensory transducer	Alide_2994	E8TWT0	2.353	7.075	225
Efflux transporter	Alide_2304	E8U1Q1	2.346	8.361	222
LemA family protein	Alide_0447	E8TVL0	2.252	8.424	179
Protein of unknown function	Alide_0553	E8TX56	2.245	3.972	176
Response regulator receiver	Alide_0468	E8TWA3	2.238	4.184	173
ABC transporter extracellular ligand	Alide_1344	E8TRK4	2.132	3.886	136
Chemotaxis sensory transducer	Alide_3351	E8U0M0	2.108	7.823	128
Cytb containing protein	Alide_3380	E8U0P9	2.088	8.340	122
Basic membrane lipoprotein	Alide_1365	E8TRM5	2.054	7.611	113
Dead/deah box helicase domain protein	Alide_1216	E8TQ43	2.014	3.579	103
Heat-shock chaperone protein	Alide_0582	E8TX83	2.008	7.073	102
Ribosomal interface protein	Alide_4171	E8TW99	1.994	9.205	99
Sporulation domain-containing protein	Alide_4085	E8TVE4	1.954	8.466	90
ABC transporter extracellular ligand	Alide_1345	E8TRK5	1.846	7.261	70
Acetyl-CoA acetyltransferase	Alide_0678	E8TY47	1.831	7.892	68
Flagellin domain protein	Alide_3863	E8TSX0	1.830	6.696	68
AMP-dependent synthetase/ligase	Alide_4154	E8TW82	1.815	3.458	65
PAS sensor protein	Alide_3200	E8TZA1	1.804	3.326	64
Thiazole biosynthesis family protein	Alide_1783	E8TWL1	-2.855	8.031	-716
LysR family transcriptional regulator	Alide_2752	E8TU78	-2.905	8.602	-804
Cytochrome-c oxidase	Alide_3609	E8TQH0	-2.938	5.231	-867
Alanine racemase	Alide_3409	E8U0S8	-2.953	9.823	-897
Ribosomal protein s6	Alide_3124	E8TYE8	-2.988	8.788	-973
Proton channel subunit	Alide_2521	E8TRP0	-3.014	9.118	-1033
Cobaltochelatase	Alide_1228	E8TQL5	-3.054	10.632	-1132
Peptidoglycan glycosyltransferase	Alide_2254	E8U152	-3.635	5.231	-4315

Comparison of acetone metabolism-related genes in A. denitrificans *strains BC and K601^T*

The key-enzymes of acetone metabolism in strain BC are acetone carboxylase, AMP-dependent synthetase/ligase and acetyl-CoA acetyltransferase. There is an identical copy of the gene encoding the acetone carboxylase beta subunit of strain BC (Alide_1502) in strain K601^T (Alide2_3424). There is also a homologue of the gene coding for the acetone carboxylase alpha subunit of strain BC (Alide_1503) in strain K601^T (Alide2_3423). These genes have a one nucleotide difference that leads to an amino acid difference of T(K601^T)/A(BC) of amino acid 553. This amino acid is not present in the conserved sequence of the alpha subunit. There is also a one nucleotide difference between the acetone carboxylase gamma subunit encoding gene of strain BC (Alide_1504) and its homologue in strain K601^T (Alide2_3422), but this does not result in an amino acid difference at protein level.

In strain BC, Alide_4154 codes for the AMP-dependent synthetase/ligase. This gene and its homologue in strain K601^T, Alide2_4495, are identical. The gene coding for acetyl-CoA acetyltransferase in strain BC, Alide_0678, has three amino acid differences at positions 47, 187, and 219 at protein level and ten nucleotide differences at gene level compared to the homologous gene in strain K601^T (Alide2_0638). The amino acids that differ between the strains are not present at the active site of the enzyme.

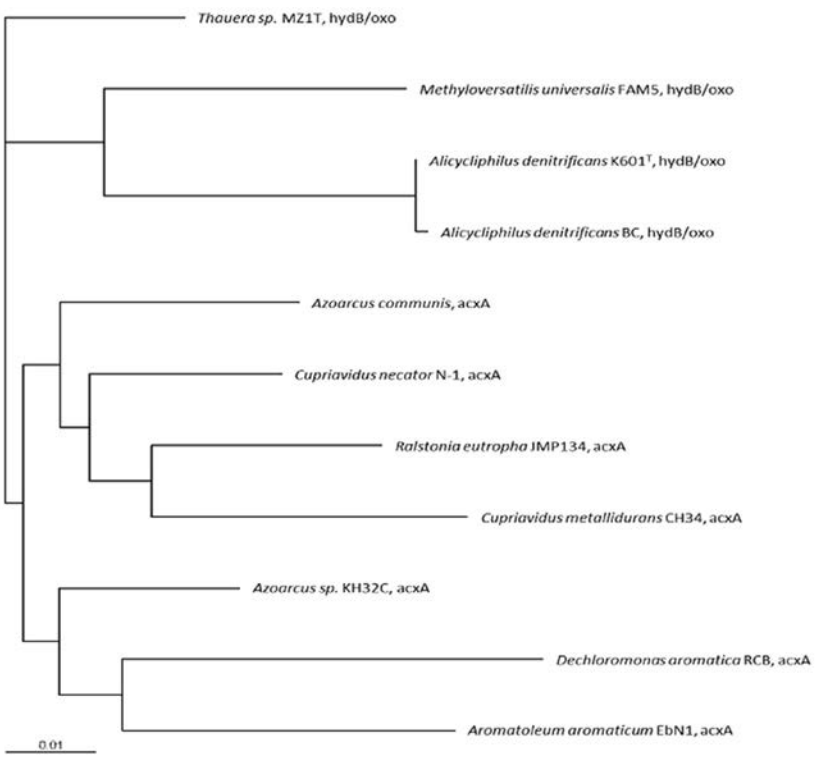


Figure 3. Phylogenetic tree of proteins most closely related to the acetone carboxylase alpha subunit of *A. denitrificans* strain BC.

Phylogenetic comparison of acetone carboxylase alpha subunit of **A. denitrificans** *strain BC*

The catalytic subunit of the acetone carboxylase (alpha subunit) of strain BC was used for phylogenetic comparison. Proteins that were most closely related were from beta-Proteobacteria of *Azoarcus, Thauera, Ralstonia, Dechloromonas, Cupriavidus* and *Methyloversatilis* species (Figure 3).

M. universalis strain FAM5^T is known to degrade acetone (Kalyuzhnaya, De Marco *et al.* 2006). *Thauera* sp MZ1T, however, has not been described to oxidize this compound, but the strain was previously described to contain acetone carboxylase genes (Lajoie, Layton *et al.* 2000; Rosier, Leys *et al.* 2012). Of the other strains of which proteins were found to be closely related to the acetone carboxylase alpha subunit of *A. denitrificans* strain BC, *A. communis* is not known to degrade acetone (Reinhold-Hurek, Hurek *et al.* 1993). *C. necator* strain N-1 is also not known to oxidize this compound, however, another *Cupriavidus* strain, *C. metallidurans* strain CH34, is known to degrade acetone (Makkar and Casida 1987; Rosier, Leys *et al.* 2012). *R. eutropha* strain JMP134 (also known as *Cupriavidus pinatubonensis* strain JMP134) is known to possess acetone carboxylase genes (Rosier, Leys *et al.* 2012). Furthermore, *D. aromatica* strain RCB and *A. aromaticum* strain EbN1 were also described to have acetone carboxylase genes (Rosier, Leys *et al.* 2012). Finally, *Azoarcus sp.* strain KH32C is not known to metabolize acetone (Tago, Ishii *et al.* 2011).

Discussion

Acetone carboxylase is involved in anaerobic degradation of acetone (Taylor, Trudgill et al. 1980; Bonnet-Smits, Robertson et al. 1988; Platen and Schink 1989; Birks and Kelly 1997; Sluis, Larsen et al. 2002; Dullius, Chen et al. 2011). This enzyme is described to convert acetone to acetoacetate with ATP. Other intermediates in the degradation pathway of acetone are acetoacetyl-CoA and acetyl-CoA, which are degraded further in the citric acid cycle. Previously, *A. denitrificans* strains KN Bun08 and K601^T were shown to degrade acetone with nitrate as electron acceptor (Dullius, Chen et al. 2011). We found that *A. denitrificans* strain BC is also capable of this and performed proteome analysis to study the enzymes involved.

The dry weight biomass yield of *A. denitrificans* strain BC grown with acetone was found to be 38.9 gram biomass per mole acetone and biomass yield with acetate was 12.9 gram biomass per mole acetate. In other strains biomass yields with acetone and acetate were in the same range, although the yield with acetone is the highest in strain BC. The biomass yield was 19.5, 27.8 and 28.2 gram biomass per mole acetone for *A. denitrificans* strain KN Bun08, *Paracoccus pantotrophus* and *Paracoccus denitrificans*, respectively, and 9.2, 13.7 and 11.5 gram biomass per mole acetate, respectively (Dullius, Chen *et al.* 2011). *Pseudomonas* strain BunN oxidized acetone with nitrate as electron acceptor with a biomass yield of 27.3 gram biomass per mole acetone (Platen and Schink 1989).

Based on proteome analysis, proteins of strain BC that are involved in acetone degradation include all subunits of the acetone carboxylase, acxABC, (Alide_1502-1504). Acetone carboxylase is involved in the conversion of acetone to acetoacetate. In the following degradation step, acetoacetate-CoA ligase is involved. CoA transferases, such as acetoacetate-CoA ligase and acetyl-CoA synthetase, require binding of AMP to induce a conformational change in the active site that allows the binding of CoA to the substrate (Jogl and Tong 2004). Likely, the AMP-dependent synthetase/ligase (Alide_4154) that is more abundant in acetone-grown cells functions as acetoacetate-CoA ligase. An acetyl-CoA acetyltransferase (Alide_0678) is also involved in acetone degradation in strain BC. Acetyl-CoA acetyltransferases can convert acetoacetyl-CoA to acetyl-CoA. The genes involved in acetone degradation in strain BC have homologues in strain K601^T, these are Alide2_3422-3424 for acetone carboxylase, Alide2_4495 for 3-oxoacid-CoA transferase, and Alide2_0638 for acetyl-CoA acetyltransferase. Based on this, the acetone degradation pathway of strain BC and K601^T is proposed to be similar and shown in Figure 4.

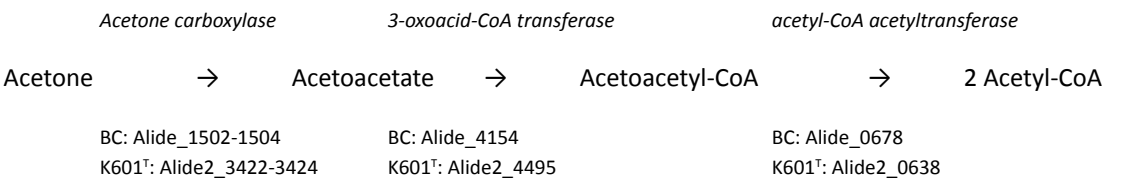


Figure 4. The anaerobic acetone degradation pathway of *A. denitrificans.* The enzymes involved in the pathway are indicated above in italics and the genes coding for these enzymes in *A. denitrificans* strains BC (BC) and K601^T (K601^T) are indicated below (Alide_geneID and Alide2_geneID).

The proteomics experiments also showed that aldehyde dehydrogenase is involved in acetone degradation in strain BC. This enzyme can function as beta-hydroxybutyrate dehydrogenase and catalyse conversion of acetoacetate to beta-hydroxybutyrate and the reverse reaction. In *Azotobacter vinelandii* aldehyde dehydrogenase was implicated to be involved in this conversion (Gama-Castro, Núñez *et al.* 2001). This allows bacteria to store carbon and energy and it also is an explanation for the high deviation of the actual and theoretical biomass yield of strain BC when grown with acetone and nitrate.

Further phylogenetic studies using the amino acid sequence of the acetone carboxylase alpha subunit of *A. denitrificans* strain BC pointed out that it was closely related to Betaproteobacteria of *Azoarcus, Thauera, Ralstonia, Dechloromonas, Cupriavidus* and *Methyloversatilis* species (Figure 2). *M. universalis* strain FAM5^T and *Thauera sp.* strain MZ1T were found to contain proteins that were most closely related to the protein of strain BC. *M. universalis* strain FAM5^T is known to metabolize single-carbon compounds and *Thauera sp.* strain MZ1T is a known wastewater bacterium (Allen, Welch *et al.* 2004; Kittichotirat, Good *et al.* 2011). Both have not been linked to acetone degradation, but most likely they can convert this compound. Previous genome analyses revealed that multiple bacteria possess an acetone carboxylase operon and consequently are potentially able to metabolize acetone. Many of these bacteria, including *A. denitrificans* strain BC can be found in soil or in contact with soil (e.g., by plant symbiosis) and belong to *Proteobacteria* and especially *Betaproteobacteria*.

Acknowledgements

This research was funded by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053.

Chapter 6

Isolation and characterization of the benzene-degrading *Pseudomonas stutzeri* strain BN

Authors and affiliations
Margreet J. Oosterkamp[1], Flávia Talarico Saia[1], Jueeli Vaidya[1], Bas van Dongen[1], Luuk Gollenbeek[1], Peter J. Schaap[2], Jan Gerritse[3], Caroline M. Plugge[1] and Alfons J. M. Stams[1]*

1 Wageningen University, Agrotechnology and food sciences group, Laboratory of microbiology, Dreijenplein 10, 6703HB, Wageningen, The Netherlands
2 Wageningen University, Agrotechnology and food sciences group, Laboratory of systems and synthetic biology, Dreijenplein 10, 6703HB, Wageningen, The Netherlands
3 Deltares, Princetonlaan 6, 3584CB, Utrecht, The Netherlands
* Corresponding author: E-mail: fons.stams@wur.nl; Tel.: (+31) 317 483101; Fax: (+31) 317 483829.

Keywords
Aromatic hydrocarbon, *Pseudomonas stutzeri*, benzene, anaerobic degradation, denitrification

Manuscript status
Thesis chapter

Abstract

A facultative anaerobic bacterium that degrades benzene with nitrate and oxygen as electron acceptors was isolated. Strain BN was isolated from the microbial community of a chemostat that was operated with benzene and nitrate for more than eight years. Strain BN was identified as a *Pseudomonas stutzeri*, based on its physiological and phylogenetic characteristics. Strain BN degrades benzene, toluene, ethylbenzene and xylene isomers with nitrate as electron acceptor and benzene, ethylbenzene and xylene isomers also with oxygen. We obtained the draft genome sequence of strain BN. Based on this draft genome, degradation with nitrate as electron acceptor involves anaerobic degradation in which the most likely mechanisms to initiate degradation are carboxylation to benzoates and/or hydroxylation to phenols, using pathways involving a putative benzene carboxylase and phenol hydroxylase, respectively. The phenol hydroxylase likely also functions in aerobic aromatic hydrocarbon degradation in strain BN. Other genes involved include genes encoding the *meta*- and *ortho*-routes of catechol-degradation. A putative nitric oxide dismutase that produces oxygen from nitric oxide could not be identified in the draft genome of strain BN, suggesting that anaerobic degradation pathways are used with nitrate as electron acceptor.

Introduction

Aerobic BTEX degradation is a well-known process in which oxygenases, enzymes that introduce oxygen atoms in the aromatic nucleus, are involved and catechol (or a catechol-derivative) is a central intermediate (Gibson and Parales 2000; Suenaga, Koyama et al. 2009; Pérez-Pantoja, Gonzáles et al. 2010). Aerobic degradation can occur with oxygen, chlorate (Weelink, Tan et al. 2008; Mehboob, Junca et al. 2009) and nitrate (Zedelius, Rabus et al. 2011) as electron acceptor.

The anaerobic benzene degradation pathway has been less well studied than degradation of toluene, ethylbenzene and xylene isomers (Weelink, van Eekert *et al.* 2010). The central aromatic intermediate in anaerobic BTEX degradation is benzoyl-CoA (Heider and Fuchs 1997; Harwood, Burchhardt et al. 1998).

A chemostat was operated with benzene and nitrate for more than eight years. This chemostat was inoculated with samples of benzene polluted soil from The Netherlands. Stable Isotope Probing studies showed that Peptococcaceae were dominant and Rhodocyclaceae as well as Burkholderiaceae were associated with benzene degradation in the microbial community of the chemostat (Zaan, Talarico Saia et al. 2012). Here we describe the isolation of a benzene-degrading nitrate-reducing *Pseudomonas stutzeri,* strain BN, from this chemostat. The phylogeny, physiology and genome of this strain were studied.

Materials and Methods

Microorganism

Pseudomonas stutzeri strain BN was isolated from a chemostat at Deltares (Utrecht, The Netherlands) fed with benzene and nitrate for more than eight years.

Chemicals

All compounds were of analytical grade and purchased from commercial sources. When needed, compounds were added as sodium salts. Compounds used as electron donors or acceptors were added from sterile aqueous stocks. Gaseous compounds were sterilized in closed serum flasks before addition.

Isolation and culture conditions

Biofilm samples from a chemostat were used as inoculum for batch experiments. The chemostat was opened under increased N_2/CO_2 flushing. Biofilm samples were grown in 50 mL AW-1 medium with either sulphate (AW-1 sulphate), or cysteine (AW-1 cysteine) (Wolterink, Jonker et al. 2002; Weelink, Tan et al. 2007) at 25°C. Initially, they were grown with benzene (20-65 µM) and nitrate (5 mM). After two weeks, cultures were transferred to medium containing benzene (20 µM) or acetate (10 mM) as electron donor and nitrate (5 mM) or oxygen (2 mmole L^{-1}) as electron acceptor. Finally, dilution series were made (10^{-1} until 10^{-10} for AW-1 sulphate and 10^{-1} until 10^{-6} for AW-1 cysteine). Cultures of the dilution series were supplemented with benzene (20 µM) and nitrate (5 mM).

Other batch experiments were performed in 120-mL flasks with a total culture volume of 40 mL (AW-1 sulphate medium with 10% v/v inoculum) as described previously (Weelink, Tan et al. 2007). Cultures were incubated at 25°C without agitation.

For genome sequencing, cultures were prepared in two 1 L-serum bottles that contained 480 mL AW-1 sulfate medium (Weelink, Tan et al. 2007) with 5% inoculum. The medium contained 10 mM acetate and 10 mM nitrate.

Denaturing gradient gel electrophoresis (DGGE) analysis

To isolate genomic DNA, a 2.0-mL sample was centrifuged for 3 min. at max. speed in a tabletop centrifuge (Eppendorf, Hamburg, Germany). Subsequently, 1.5 mL of supernatant was removed and the pellet was resuspended in the remaining supernatant. The cell suspension (0.5 mL) was added to a Lysing Matrix E Tube of the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) and the procedure was according to the manufacturer's instructions. The DNA was quantified using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Genomic DNA samples were used for 16S rRNA gene amplification by PCR using universal bacterial 968F-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and 1401R (5'-CGG TGT GTA CAA GAC CC-3') primers (Nubel *et al*., 1996). The primers were synthesized by Biolegio (Biolegio, Nijmegen, The Netherlands). PCR mixtures consisted of at least 10 ng isolated genomic DNA and 1x PCR Green reaction buffer, PCR nucleotide mix (0.2 mM each dNTP), 10 pmol of each primer, and 1.25 u GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR water (Promega, Madison, WI, USA) was added to obtain a final volume of 50 µl. PCR amplifications were performed with an Arktik PCR thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR was run for 2 min at 94°C, 35 cycles of 94°C for 30 sec, 56°C for 40 sec, and 72°C for 1 min and 5 min at 72°C with final cool-down to 10°C. PCR samples were directly used or stored at -20°C. 5 µL of the PCR samples was analyzed on a 1.5 % agarose gel with a 100 bp marker (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Fragments of 450-500 bp were purified using a DNA clean and concentrator kit (Zymo Research, Orange, CA, USA) and used for DGGE analysis.

Depending on the band intensity in the agarose gel, 10-22.5 µL PCR sample was loaded on a DGGE gel. The DGGE gel was run in the Dcode system (Bio-rad Laboratories, Hercules, CA, USA). DGGE analysis was performed using a polyacrylamide gel with a 30-60 % denaturation gradient as described previously (Muyzer, de Waal et al. 1993; Heilig, Zoetendal et al. 2002). DNA fragments were visualized using silver staining (Sanguinetti, Dias Neto et al. 1994).

Genome sequencing

After three days of incubation at 30°C, cells were harvested using 500-mL Sorvall buckets with screw caps that were centrifuged using the SLA3000 rotor in a Sorvall RC-6 plus centrifuge (Sorvall, Thermo Fisher Scientific, Waltham, MA, USA). Cell pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). High molecular weight genomic DNA was isolated using the protocol for bacterial genomic DNA isolation using CTAB recommended by the U.S. Department Of Energy, Joint Genome Institute (DOE JGI, Walnut Creek, CA, USA, www.jgi.doe.gov). DNA concentration was measured using Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and DNA integrity and quality was determined by loading the genomic DNA (5 µL) on a 1 % agarose gel with size and concentration markers according to the instructions of DOE JGI.

At least 20 µg genomic DNA was sent to Baseclear (Baseclear, Leiden, The Netherlands) for paired-end whole genome sequencing (75 bp). The genomic DNA was sequenced to a > 25× depth using Roche 454 pyrosequencing with GS FLX Titanium chemistry. Sequence reads were assembled into contigs using the Newbler assembler. The draft genome was automatically annotated using the RAST tool (Aziz, Bartels et al. 2008).

Cloning and 16S rRNA sequence analysis

To confirm purity of the strain, we used genomic DNA for cloning and sequencing. The DNA was isolated using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) as described for DGGE analysis. Subsequently, a PCR was performed to amplify the 16S rRNA gene using universal 27F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1510R (5'-TAC GGC TAC CTT GTT ACG ACT T-3') primers (Suzuki and Giovannoni 1996; Dojka, Hugenholtz et al. 1998). The primers were synthesized by Biolegio (Biolegio, Nijmegen, The Netherlands). PCR reactions were performed in 0.2-mL PCR tubes that contained PCR mixtures consisting of at least 10 ng isolated genomic DNA and 1x PCR Green reaction buffer, PCR nucleotide mix (0.2 mM each dNTP), 10 pmol of each primer, and 1.25 u GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR water (Promega, Madison, WI, USA) was added to obtain a final volume of 50 µL. PCR amplifications were performed with an Arktik PCR thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). Pre-denaturation was performed for 2 min at 94°C followed by 35 cycles of 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1.5 min. Finally, a post-elongation step of 5 min at 72°C was executed after which samples were cooled down until 10°C. PCR samples were directly used or stored at -20°C. 5 µl of the PCR samples was loaded on a 1.5% agarose gel (Mupid-exU gel casting set, ABC Scientific, Los Angeles, CA, USA). A 100 bp marker (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) was loaded additionally. If fragments of about 1500 bp could be observed (using Sybr Safe staining (Invitrogen, Madison, WI, USA) and the Gel doc EQ (Bio-rad Laboratories, Hercules, CA, USA)), PCR products were purified using a DNA clean and concentrator kit (Zymo Research, Orange, CA, USA) and used for ligation.

The purified 16S rRNA gene fragments were used for ligation with the pGEM-T easy ligation kit (Promega, Madison, WI, USA), which was performed according the manufacturer's instruction. The ligation mix obtained was used for cloning into competent *Escherichia coli* DH5α cells (Invitrogen, Madison, WI, USA), according to the manufacturer's guidelines. Positive (white) colonies were picked and transferred to wells of a 96-wells plate that contained 1 mL of LB broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) covered with a gas permeable seal. Cells were grown at 37°C overnight and cell lysates were prepared by mixing 10 µL cell culture with 40 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in wells of a 96-wells plate, this was heated to 94°C for 10 min in a PCR machine (Biometra, Goettingen, Germany).

Cell lysates were used for PCR with universal T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-TAT TTA GGT GAC ACT ATA G-3') primers. The primers were synthesized by Biolegio (Biolegio, Nijmegen, The Netherlands). PCR reactions were performed in 0.2-mL PCR tubes that contained 1 µL cell lysate and 1x PCR Green reaction buffer, PCR nucleotide mix (0.2 mM each dNTP), 10 pmol of each primer, and 1.25 u GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR water (Promega, Madison, WI, USA) was added to obtain a final volume of 25 µl. PCR amplifications were performed with a PCR thermal cycler (Biometra, Goettingen, Germany). Pre-denaturation was performed for 2 min at 94°C followed by 35 cycles of 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1.5 min. Finally, a post-elongation step of 5 min at 72°C was executed after which samples were cooled down until 10°C. PCR samples were directly used or stored at -20°C. 5 µl of the PCR samples was loaded on a 1.5% agarose gel (Mupid-exU gel casting set, ABC Scientific, Los Angeles, CA, USA). A 100 bp marker (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) was loaded additionally. If fragments of at least 1500 bp could be observed (using Sybr Safe staining (Invitrogen, Madison, WI, USA) and the Gel doc EQ (Bio-rad Laboratories, Hercules, CA, USA), PCR products were used to prepare pools. These pools were subjected to DGGE analysis as described. Pools that contained all fragments observed in previous DGGE analyses of the bacterial strain, were selected. Samples from these pools were purified using a DNA clean and concentrator kit (Zymo Research, Orange, CA, USA), quantified using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and sent for sequencing using T7 and SP6 primers. Sequencing was performed in collaboration with Baseclear (Baseclear, Leiden, The Netherlands) and preparations were performed according to the company's instructions.

Phylogenetic analysis of strain BN

The 16S rRNA gene and amino acid sequences of the phenol hydroxylase alpha subunit, benzoate 1,2-dioxygenase alpha subunit and probable NO dismutases predicted from the draft genome of strain BN were used for blast searching (Altschul, Madden et al. 1997). Sequences were selected and used for clustalW alignment and phylogenetic tree construction (www.ebi.ac.uk/Tools/msa/clustalw2 and /Tools/phylogeny). Trees were calculated using the neighbour-joining clustering method and in nexus tree format and were constructed in TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Physiological characterization of strain BN

Determination of substrate spectrum

Growth of cells with various compounds was tested and compounds were added to 10 mM end concentration, unless indicated otherwise. The following electron donors were tested: acetate, lactate, succinate, propionate, malate, formate, glucose, alanine, proline, ethanol, acetone, benzene (50 µM), toluene (50 µM), ethylbenzene (50 µM), xylene isomers (20 µM of each isomer) dihydrogen (2 mmole L^{-1}) and methane (2 mmole L^{-1}). The following electron acceptors were used: nitrate, nitrite, chlorate, sulphate and oxygen (9 mmole L^{-1} in headspace).

Tests were performed in duplicate batches and growth was monitored by visual inspection of turbidity. Optical density at 660 nm was determined using the Hitachi-2100 spectrophotometer (Hitachi, Tokyo, Japan). Electron acceptor use was measured by HPLC and GC analysis. For HPLC analysis, 1.5-mL culture sample was extracted and stored at -20°C.

The API 20 NE test was performed according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France) including the oxidase test (bioMérieux, Marcy l'Etoile, France).

Determination of optimal temperature and pH

Duplicate batches were incubated at 4, 15, 20, 30, 37, 45, and 55°C to determine optimal temperature for growth. Acetate (10 mM) and nitrate (10 mM) were used as electron donor and acceptor. For determination of the optimal pH, duplicate batches were prepared with pH 4.7, 5.1, 6.0, 6.9, 7.9 and 8.8 (using phosphate buffer).

Growth was monitored by visual inspection of turbidity and by measuring electron acceptor use by HPLC and GC analysis. For HPLC analysis, 1.5-mL culture sample was extracted and stored at -20°C.

Analytical techniques

Liquid chromatography

Electron acceptors were measured by suppressor mediated ion chromatography using an Ionpac AS9-SC analytical column (250 mm length and 4 mm diameter) and an Ionpac AG9-SC guard column (50 mm length and 4 mm diameter) (Dionex, Breda, The Netherlands) with a conductivity detector. The oven temperature was 30°C and the effluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ (1 mL min^{-1}). Potassium bromide (1 mM) was used as internal standard and mannitol (10 mM) for stabilization.

Gas chromatography

Benzene, toluene, ethylbenzene and xylene isomers were monitored on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a CP-SIL5 CB column of 30 m length and 0.25 mm internal diameter (Agilent Technologies, Amstelveen, The Netherlands) and a flame ionization detector. Column, detector and injector temperatures of 80, 300 and 250°C, respectively, were used and the dinitrogen gas and air mixture (30 mL min^{-1}) was used as carrier gas. 0.4 mL headspace samples were expanded to 0.8 mL prior to injection.

Other gaseous compounds were measured using the GC-14B (Shimadzu, Kyoto, Japan) that is equipped with a packed column (Molsieve 13X, 60-80 mesh, 2 m x 3 mm, Varian, Middelburg, The Netherlands) and a thermal conductivity detector. Column, detector and injector temperatures of 100, 100, and 150°C were used and argon (30 mL min^{-1}) was used as carrier gas. 0.4 mL headspace sample was injected to the GC.

Results

Isolation and characterization of a benzene-degrading bacterium

A stable enrichment that degraded benzene with nitrate as electron acceptor was obtained from serial dilutions inoculated with biofilm material from a chemostat operated with benzene and nitrate. The cells were transferred to medium with glucose, malic acid, acetate and with both benzene and acetate as electron donors and nitrate as electron acceptor and, with acetate and oxygen. The DGGE banding pattern of cultures grown with these different electron donor and acceptor combinations were identical (Figure 1). This indicates that the bottles contain a pure culture, which is designated as strain BN.

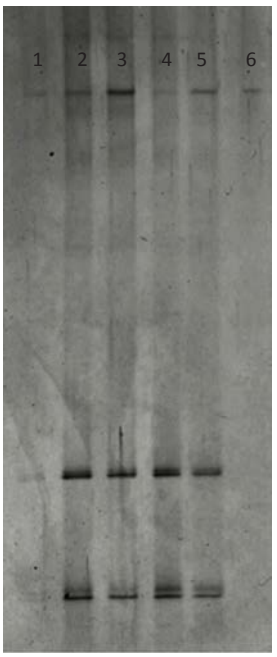


Figure 1. DGGE analysis of cultures that actively degraded benzene with nitrate. Cultures were transferred to medium containing glucose and nitrate (1), malic acid and nitrate (2), acetate and nitrate (3), acetate and oxygen (4), benzene, acetate and nitrate (5) and to medium without electron donor and acceptor (6).

Strain BN grows at temperatures higher than 15 and lower than 55°C and a pH higher than 6.0 and lower than 8.8. Around neutral pH, in pH 7.4 to 7.7, OD values obtained did not differ substantially (values were around OD 0,100, not shown).

An API 20NE strip test showed that strain BN uses nitrate as electron acceptor and, furthermore, that it converts nitrate to dinitrogen. The strain grows with D-glucose, but not with L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine and D-maltose. The strain does not ferment D-glucose. Potassium gluconate, capric acid, adipic acid, malic acid and trisodium citrate are degraded under aerobic conditions but not phenylacetic acid. Aerobic degradation of the amino acid arginine was not observed. The strain does not contain urease or β-galactosidase and it hydrolyses gelatin, but not esculin. The strain is an indole-negative and oxidase-positive bacterium. Hydrolysis of gelatin and degradation of adipic acid was observed after 48 instead of 24 hours. The API 20NE strip profile of the strain is most similar to that of *Pseudomonas stutzeri*. But, different from strain BN, *P. stutzeri* species are generally able to degrade D-maltose and unable to degrade trisodium citrate (according to the API 20NE guidelines). Cloning and sequencing of the 16S rRNA gene shows that the gene is closely related to 16S rRNA genes from other *P. stutzeri* strains (Figure 2).

Strain BN is able to degrade benzene with nitrate as electron acceptor over long time periods in batch cultures, (Figure 3). In these cultures, nitrate (7 mM) was present in excess. The ratio benzene:nitrate was 1:1.5, since for 0.11±0.01 mM benzene oxidized, 0.16 ± 0.06 mM nitrate was reduced. Furthermore, 0.26 ± 0.15 mM nitrite was produced. Nitrite did not accumulate in cultures in which nitrate was present in a lower concentration (1 mM). The ratio of benzene:nitrate in these cultures was 1:2.5 (Figure 4a). Strain BN uses benzene, ethylbenzene and xylene isomers as substrates with nitrate and oxygen as electron acceptor. Furthermore, it degrades toluene in presence of nitrate (Table 1). As a mixture of xylenes was fed to strain BN, it is not clear if the strain uses all xylene isomers. Finally, strain BN can use nitrate, nitrite and oxygen, but not chlorate and sulfate as electron acceptor (Table 2).

Strain BN draft genome composition

The 4.53 Mb draft genome of strain BN was assembled in 97 scaffolds with an average length of 47 kb. Furthermore, in total 3954 open reading frames were predicted (Table 3). 52 tRNA sequences are encoded by the genome. rDNA operons could not be found in the draft genome, but these are present in the total genomic DNA because we were able to amplify the 16S rRNA encoding gene from that.

Resistance against viral attack

The draft genome of strain BN encodes 8 CRISPR-associated proteins (Brouns, Jore et al. 2008). Despite these systems, genes originating from bacteriophages (encoding seven integrase and 16 phage-related proteins) have integrated in the genome.

Signal transduction and regulation of transcription

Genes involved in specific regulatory functions were discovered in the genome of strain BN, such as six genes involved in the response to nitrate/nitrite, two in nitrogen fixation, two in response to heavy metals, three in phosphate transport, seven in motility, three in RNA polymerase sigma factor Rpo regulation, one in carbon storage, 34 in chemotaxis, two in aerotaxis and one in osmolarity. Signal transduction proteins are also encoded by the genome, ten genes are coding for two-component signal transduction proteins, four genes for sigma-54 specific and one for heme-regulated regulatory proteins, four genes are coding for cAMP-dependent protein kinases, 29 for histidine kinase and eleven for TonB-dependent receptors. Among the transcriptional regulators are genes of the AraC (20), LysR (25), GntR (ten) TetR (nine), MerR (five), LuxR (five), XRE (two), ArsR (four), AsnC (three), Crp/Fnr (one), and IclR (four) protein families.

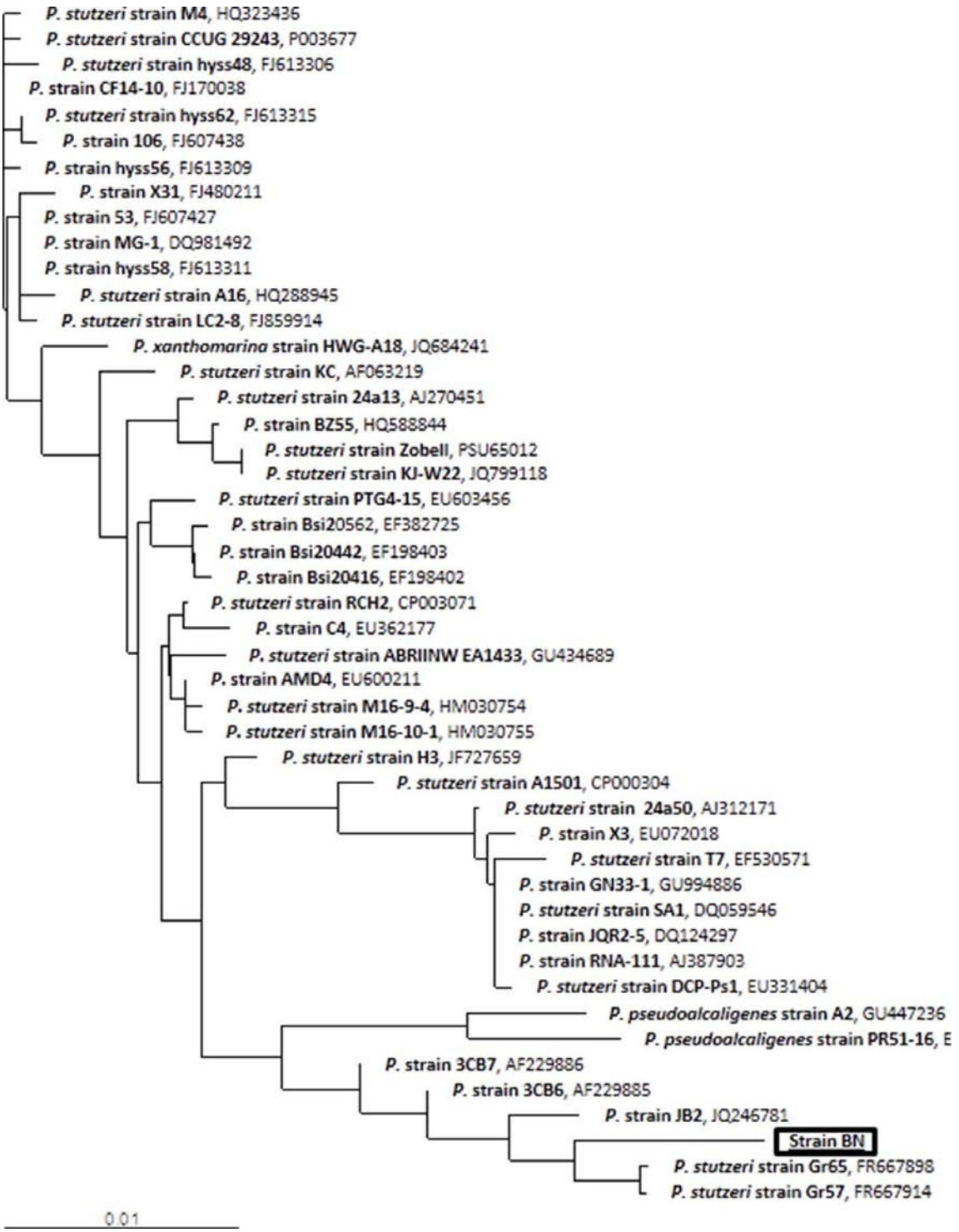


Figure 2. Phylogenetic analysis of the 16S rRNA gene of strain BN. The phylogenetic relationship of the strain BN gene (in frame) to 16S rRNA genes from other *Pseudomonas* strains is indicated (strain, in bold, and GenBank accession number are shown, for strain BN the sequence obtained from a clone library is used), the bar represents 1% sequence divergence.

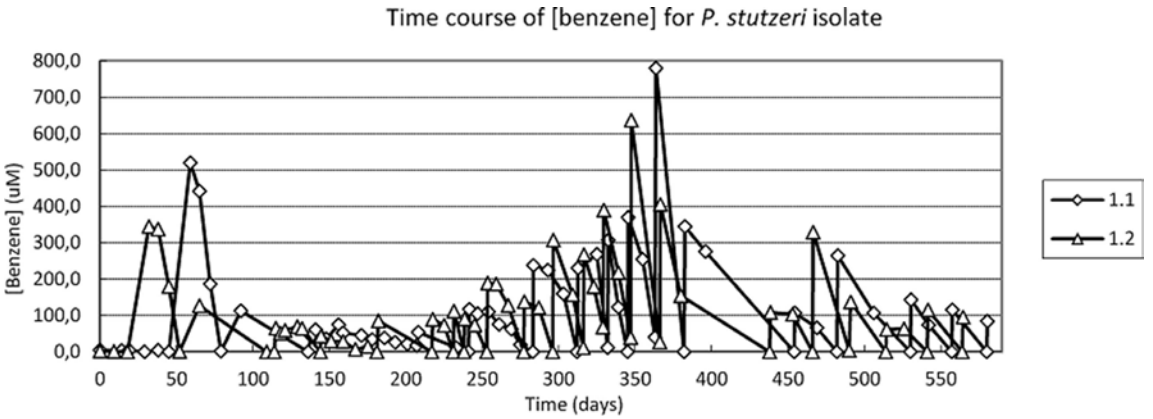
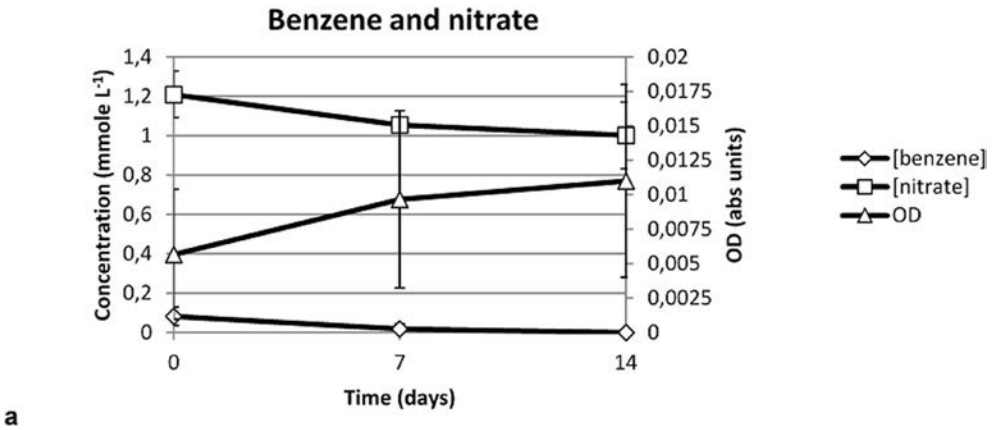
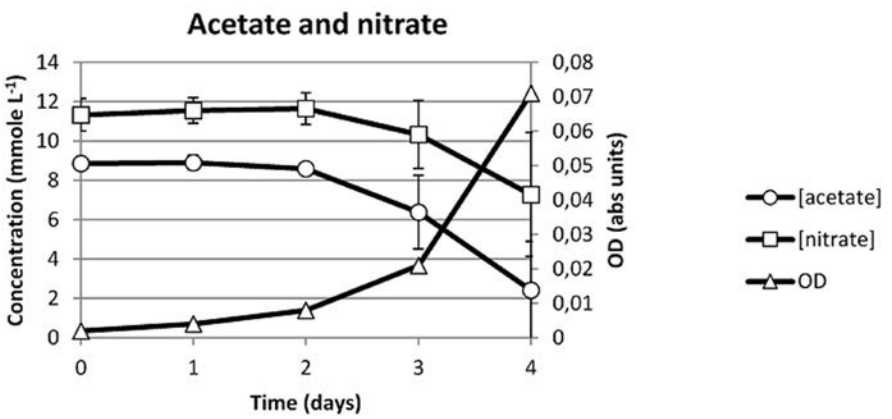


Figure 3. Time courses of benzene concentration in two cultures (1.1 and 1.2) of *P. stutzeri* strain BN. Benzene was re-added when depleted.



a



b

Figure 4. Growth of *P. stutzeri* strain BN with benzene (**a**) and acetate (**b**) and with nitrate as electron acceptor. Time courses of benzene, acetate and nitrate concentration are plotted to the primary axis and the time course of optical density at 660 nm (OD) is plotted to the secondary axis.

Table 1. Electron donor use of *P. stutzeri* strain BN. Electron donors were tested with nitrate, oxygen, or no electron acceptor and in the table is indicated if the electron donors were used in these conditions.

Electron donor	Nitrate	Oxygen	No electron acceptor
Acetate	+	+	-
Lactate	+	+	-
Succinate	+	+	-
Propionate	+	+	-
Malate	+	ND	-
Formate	-	-	-
Glucose	+	+	-
Alanine	-	+	-
Proline	+	+	-
Ethanol	+	+	-
Acetone	+	+	-
Benzene	+	+	-
Toluene	+	-	-
Ethylbenzene	+	+	-
Xylene isomers	+	+	-
Dihydrogen	-	-	-
Methane	-	ND	-

+: used as electron donor, -: not used as electron donor, ND: not determined

Table 2. Electron acceptor use of the *P. stutzeri* strain BN. Electron acceptors were used with acetate or benzene as electron donor and in the table is indicated if the electron acceptors were used in these conditions. No electron acceptor was used as negative control.

Electron acceptor	Acetate	Benzene
Nitrate	+	+
Nitrite	+	ND
Chlorate	-	-
Sulfate	-	ND
Oxygen	+	+
No electron acceptor	-	-

+: used as electron donor, -: not used as electron donor, ND: not determined

Table 3. General features of the draft genome of *P. stutzeri* strain BN.

Genome features	Strain BN
Size (bp)	4 531 861
Scaffolds	97
Average size (bp)	46 720
Median size (bp)	28 330
G+C content (mol%)	65.5
Coding sequences	3 954
Coding (%)	69.21
Average size (bp)	791
Median size (bp)	686
Assigned function (%)	78.40
rDNA operons	0
tRNAs	52

According to its genome sequence, strain BN has proteins responding to heat (eight genes), cold (seven genes) and osmotic (one gene) shock. Furthermore, it has universal stress proteins (four genes), sigma B stress response regulators (five genes), thioredoxin (seven genes), thioredoxin reductase (two genes), organic hydroperoxide resistance protein (two genes), catalase (four genes), superoxide dismutase (two genes), non-heme chloroperoxidase (one gene), paraquat oxidative stress response proteins (three genes), peroxidase (two genes), thiol peroxidase (one gene), glutathionine peroxidase (one gene), cytochrome *c*551 peroxidase (two genes), cytochrome *c* oxidase (see the description of the respiratory pathways of strain BN), rubredoxin (one gene), and ferredoxin (ten genes).

Efflux pump proteins that offer resistance to toxic compounds and allow efflux of other compounds are present in the genome of strain BN, such as RND superfamily (nine genes), multidrug (five genes), major facilitator (three genes), drug/metabolite (seven genes), auxin (two genes), cobalt/zinc/cadmium (four genes), potassium (four genes) and amino acid (four genes) efflux proteins. ABC transporter proteins (100 genes) are encoded on the genome, among these are proteins involved in nitrate (four genes), sugar (four genes), lipopolysaccharide (two genes), multidrug (five genes), lead/cadmium/zinc/mercury (three genes), sodium/bicarbonate (one gene), potassium (two genes), magnesium (one gene), ferric iron (three genes), sulfate (four genes), zinc (seven genes), molybdenum (four genes), hemin (two genes), spermidine/purescine (nine genes), hydroxymethylpirimidine (five genes), urea (nine genes), amino acid (six genes) and organosulfonate (five genes) transport. Others are TRAP (15 genes), TegT family (one gene), LysE family (two genes), Tol biopolymer (ten genes) transporters or are specific for nitrate/nitrite (three genes), ammonium (five genes), benzoate (four genes), C4-dicarboxylate (twelve genes), tricarboxylate (ten genes), short-chain (one gene) and long-chain (one gene) fatty acids, ferric siderophores (five genes), malonate (one gene), D-glycerate (one gene), metals (three genes), vitamin B12 (two genes), heme (three genes), magnesium/cobalt (one gene), nitrate/sulfonate/bicarbonate (one gene), sodium/phosphate (two genes), sodium/sulphate (one gene), phosphate (four genes), phosphonate (eight genes), iron (four genes), iron(III) dicitrate (two genes), sulphate (one gene), sodium/protons (one gene), amino acids (29 genes) or oligopeptides (six genes). Finally, two Rnf cluster electron transport complexes (ten genes in total) are encoded on the genome.

Electron donor spectrum of strain BN

Aerobic aromatic compound degradation

Genes encoding a multicomponent phenol hydroxylase (peg.2199-2204) and a protein involved in regulation of the phenol hydroxylase (peg. 2205) are present in the genome of strain BN. Phylogenetic analysis showed that the phenol hydroxylase alpha subunit was not similar to enzymes from other *P. stutzeri* strains of which the genomes are known (Figure 5). Genes of other subunits from the enzyme are located in the same scaffold as the alpha subunit and phylogenetic analysis of the predicted amino acid sequences showed similar results as obtained from analysis of the alpha subunit (not shown). Sequences from *Cupriavidus metallidurans* CH34 (YP_583480.1) and *Pseudoxanthomonas spadix* BD-a59 (YP_004929579.1) were annotated as toluene hydroxylase. Since toluene hydroxylases also oxidize phenols, these enzymes are similar to phenol hydroxylases.

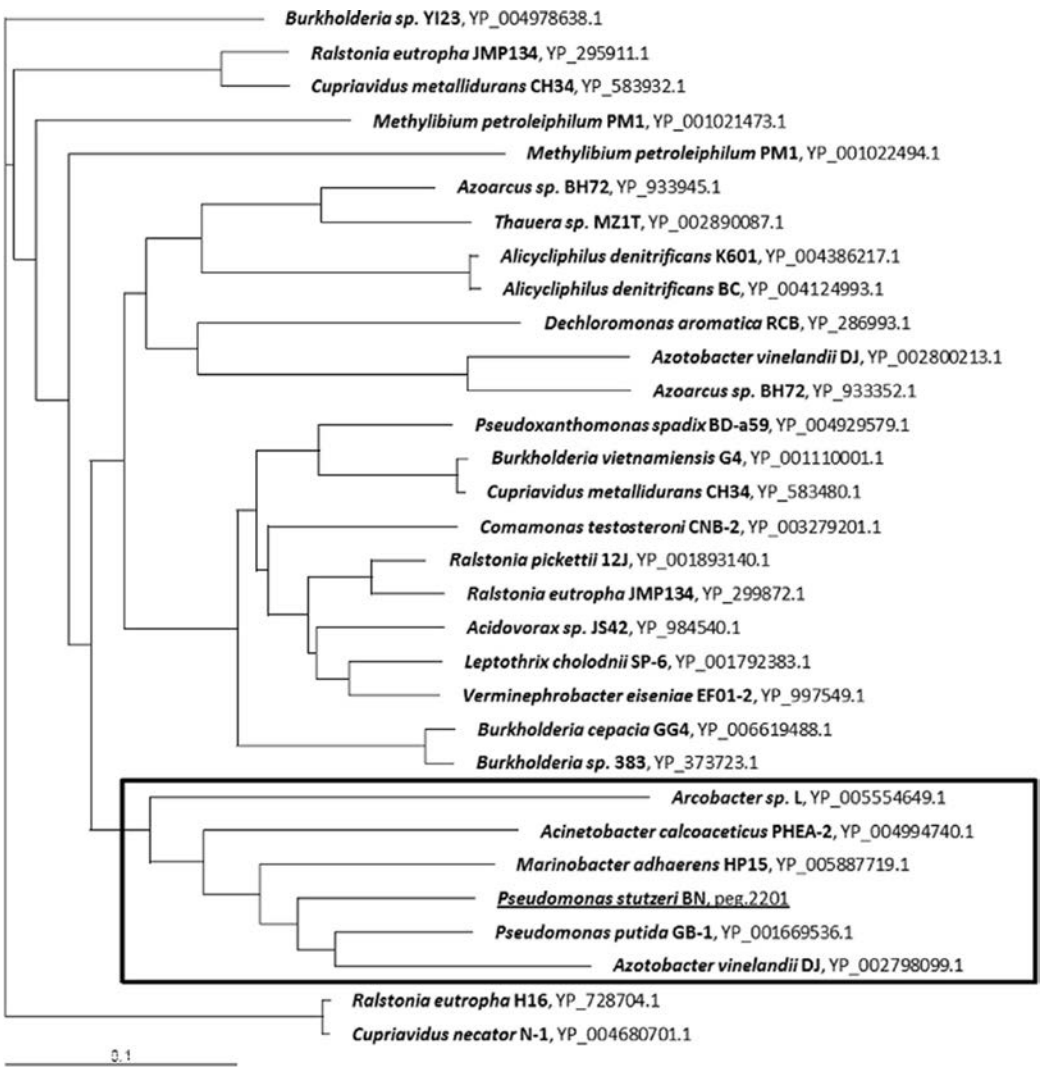


Figure 5. Phylogenetic analysis of the phenol hydroxylase alpha subunit of *P. stutzeri* strain BN. The phylogenetic relationship of the strain BN subunit (underlined) to phenol hydroxylase alpha subunits from other strains is indicated (strain, in bold, and RefSeq accession number are shown, for strain BN a locus tag is used), the bar represents 10% sequence divergence.

All genes involved in the *meta*-pathway of catechol degradation (Suenaga, Koyama et al. 2009) are present in the genome (Figure S1). Catechol 2, 3-dioxygenase (peg.2197) catalyzes the cleavage of catechol to 2-hydroxymuconic semialdehyde. Genes involved in the catechol degradation pathway are located in close proximity to the genes coding for phenol hydroxylase. Strain BN possesses the oxalocrotonate branch of catechol degradation. In this pathway 2-hydroxymuconic semialdehyde is converted to 2-hydroxyhexa-2,4-diene-1,6-dioate, which is converted via 2-oxohex-3-ene-1,6-dioate, 2-oxopent-4-dienoate, 4-hydroxy-2-oxopentanoate and acetaldehyde to acetyl-CoA. The enzymes involved are 2-hydroxymuconic semialdehyde dehydrogenase (peg.2196), 4-oxalocrotonate tautomerase (peg.2190), 4-oxalocrotonate decarboxylase (peg.2191), 2-oxopent-4-enoate hydratase (peg.2194), 4-hydroxy-2-oxovalerate aldolase (peg.2192) and an acetaldehyde dehydrogenase (peg.2193). The hydrolytic branch for toluene degradation is also encoded on the genome of strain BN. Toluene degradation is performed via 3-methylcatechol to 2-hydroxy-6-oxo-2, 4-heptadienoate. This compound is converted to 2-oxohex-3-ene-1, 6-dioate by 2-hydroxymuconic semialdehyde hydrolase, which was found in the genome of strain BN (peg.2195).

Catechol can also be degraded via the *orto*-cleavage pathway. The enzymes involved in this pathway are catechol 1,2-dioxygenase (peg.2019), muconate cycloisomerase (peg.2021), muconolactone isomerase (peg.2020), 3-oxoadipate enol-lactone hydrolase (peg.3028), 3-oxoadipate-CoA transferase (peg.3031), and 3-oxoadipyl-CoA thiolase (peg.3029).

Based on the genome sequence, strain BN also degrades other aromatic compounds aerobically. Benzoate 1, 2-dioxygenase (peg.2024-2026) converts benzoate to 1, 2-dihydroxycyclohexa-3,5-diene-1-carboxylate. This latter compound can be converted to catechol by 1, 2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase (peg.2023). The alpha subunit of benzoate 1, 2-dioxygenase was found to be related to subunits of other *P. stutzeri* strains (Figure 6). Phylogenetic analysis of the other subunits from the enzyme showed similar results (not shown). Not all enzymes in the phylogenetic tree are benzoate 1, 2-dioxygenases. Benzoate and toluate 1, 2-dioxygenase are evolutionary related (Neidle, Hartnett *et al.* 1991; Harwood and Parales 1996). *P. putida* (NP_542871.1), *P. putida* BIRD-1 (YP_005930402.1), *P. fluorescens* Pf-5 (YP_260958.1), *P. aeruginosa* UCBPP-PA14 (YP_790719.1) and *P. aeruginosa* PAO1 (NP_251208.1) are toluate 1,2-dioxygenase subunit alpha and these enzymes are not sharing a branch on the phylogenetic tree. Furthermore, two proteins of *P. putida* that code for toluene 1, 2-dioxygenase alpha subunits (YP_709342.1 and YP_709317.1), share a phylogenetic branch with the toluate 1,2-dioxygenase of *P. putida* (NP_542871.1). However, benzoate and toluate 1, 2-dioxygenases are not related to toluene 1, 2-dioxygenases (Neidle, Hartnett et al. 1991). The salicylate-5-hydroxylase alpha subunit of *R. solanacearum* Po82 (YP_006029697.1) is also included in the phylogenetic tree. This subunit is related to benzoate 1, 2-dioxygenase subunit alpha of *R. solanacearum* CFBP2957 (YP_003745762.1).

Ring-cleaving dioxygenase (related to biphenyl-2, 3-diol 1,2-dioxygenase III) and acyl-CoA dehydrogenase genes (peg.2628-2629) are present in the genome. Another gene coding for biphenyl-2, 3-diol 1,2-dioxygenase III, that converts biphenyl-2,3-diol to 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate is present in proximity to a gene that can encode a 2, 5-dichloro-2, 5-cyclohexadiene-1, 4-diol dehydrogenase (Nagata, Ohtomo et al. 1994), which converts gamma-hexachlorocyclohexane, and a gene coding for 2-hydroxymuconic semialdehyde hydrolase, involved in catechol degradation (peg.3040-3042). Furthermore, a cluster of genes likely involved in the *meta*-cleavage of hydroxyphenyl-propionate was found (peg.3059-3063). These genes encode the enzymes 2, 3-dihydroxyphenylpropionate 1, 2-dioxygenase, 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate hydrolase, 2-oxopent-4-enoate hydratase, acetaldehyde dehydrogenase, acetylating and 4-hydroxy-2-oxovalerate aldolase.

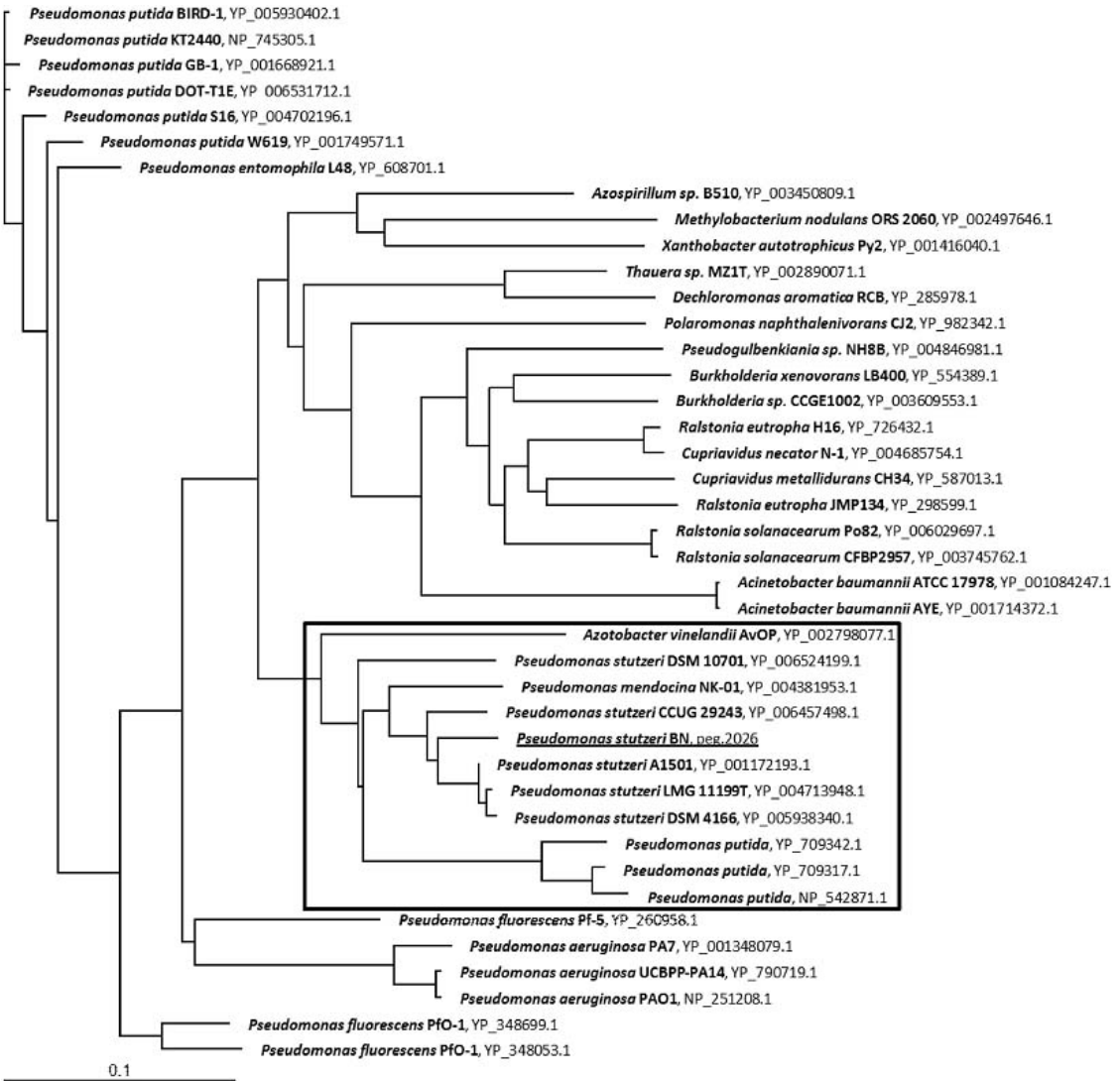


Figure 6. Phylogenetic analysis of the benzoate 1,2-dioxygenase alpha subunit of *P. stutzeri* strain BN. The phylogenetic relationship of the strain BN subunit (underlined) to oxygenase subunits from other strains is indicated (strain, in bold, and RefSeq accession number are shown, for strain BN a locus tag is used), the bar represents 10% sequence divergence.

Anaerobic degradation of aromatic compounds

Strain BN degrades benzene, toluene, ethylbenzene and xylene isomers with nitrate. A mechanism for activation of anaerobic degradation of these aromatic compounds is carboxylation. There is a gene encoding a putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (peg.1928). Genes related to this enzyme have been described as putative benzene carboxylase subunit (Abu Laban, Selesi et al. 2010). No other genes encoding putative aromatic compound carboxylase subunits are present in the genome of strain BN. Benzoates, produced from aromatic compound carboxylation, can be converted to benzoyl-CoA by a benzoate-CoA ligase. There is a 4-hydroxybenzoyl-CoA thioesterase family protein (peg.3093) that might catalyze this conversion.

Enzymes involved in anaerobic degradation of aromatic compounds are also present in the anaerobic toluene degradation pathway (Kube, Heider et al. 2004). Benzylsuccinate synthase is a key-enzyme in this pathway. However, genes coding for enzymes involved in this pathway were not found in the genome of strain BN.

Finally, in the genome of strain BN we found genes coding for phenol hydroxylase (peg.2199 to 2204), which can convert aromatic hydrocarbons to phenols. Subsequently, phenols can be carboxylated to form hydroxybenzoates. This step could be performed, for example, by the gene annotated as a 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (peg.1928). A putative 4-hydroxybenzoyl-CoA thioesterase encoding gene (peg.1789) can be involved in the conversion of hydroxybenzoates to hydroxybenzoyl-CoA. Furthermore, the genome contained genes encoding a three component periplasmatic aromatic aldehyde oxidoreductase or 4-hydroxybenzoyl-CoA reductase (peg.1848-1850), which can convert hydroxybenzoyl-CoA to benzoyl-CoA.

Degradation of other electron donors

Strain BN degraded acetate (10 mM) with nitrate (10 mM) as electron acceptor. The cultures did not accumulate nitrite and the ratio acetate:nitrate was 1:0.6 (Figure 4b). Strain BN also degraded other carboxylic acids, except formate, and glucose as growth substrates with nitrate or oxygen as electron acceptor (Table 1). The genome of strain BN contains all genes of the tricarboxylic acid cycle (Figure S2). Typical propionate degrading enzymes were not found in the draft genome of strain BN. Formate dehydrogenase is encoded by the draft genome (peg.165-168, 1050, 1051, 1418) but not used for formate degradation coupled to growth under the conditions used, because this was not observed in our experiments.

The amino acid alanine was degraded with oxygen as electron acceptor and proline with oxygen and nitrate. Alanine transaminase (peg.680) and glutamate dehydrogenase (peg.594 (NAD), 1845 (NADP) encoding genes are important for alanine degradation. The genome of strain BN encodes a bifunctional proline dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase gene (peg.1650) for proline degradation to glutamate. Glutamate dehydrogenase is involved in conversion of glutamate that can be produced in degradation of these amino acids. Strain BN also degraded ethanol and acetone with oxygen and nitrate. Alcohol dehydrogenase genes of strain BN (peg.218, 265, 417, 412, 1064, 1790, 2907, 3053, 3316, 3510) were found that are involved in the initial step of ethanol degradation. No acetone carboxylase gene was found in the draft genome of strain BN, but acetoacetyl-CoA synthase (peg.1137) and acetyl-CoA:acetoacetyl-CoA transferase (peg.1113) encoding genes were found and these genes can be involved in the acetone degradation pathway.

Respiratory pathways of strain BN

Nitrate, nitrite and oxygen, but not chlorate and sulfate were used as electron acceptor by strain BN (Table 2). The genome of strain BN encodes high affinity cbb3-type terminal cytochrome *c* oxidase (peg.71-76) that are involved in micro-aerophillic respiration. Furthermore, cox-type cytochrome *c* oxidase encoding genes were found (peg.242, 244-245). These cox-type cytochrome *c* oxidases are terminal oxidases with low oxygen affinity that are involved in aerobic metabolism.

The genome of strain BN contains genes encoding periplasmic nitrate reductase (peg.3729-3733) and a gene coding for respiratory nitrate reductase delta subunit (peg.2673) but no other genes coding for respiratory nitrate reductase subunits. Nitrate reductase catalyses the conversion of nitrate (NO_3^-) to nitrite (NO_2^-). Nitrite is converted to nitric oxide (NO) by nitrite reductase (peg.3814, 3816, 3819-3825) and nitric oxide reductase (peg.1827-1828, 1830, 3817-3818, 3827-3828, 3832) converts nitric oxide to nitrous oxide (N_2O). The strain BN genome also encodes N_2-forming nitrous oxide reductase (peg.3801-3806) catalyzing the final step of respiratory nitrate reduction.

Recently, putative nitric oxide dismutases, which form O_2 and N_2 from NO were described (Ettwig, Speth et al. 2012). Potential nitric oxide dismutase is related to nitric oxide reductase subunit B and *cbb*$_3$-type cytochrome *c* oxidase. Sequences of the latter enzymes from strain BN were compared to potential nitric oxide dismutase (Figure 7). The enzymes from strain BN did not share a phylogenetic branch with potential nitric oxide dismutases.

The strain BN genome encodes nitrogenase genes (peg.3633, 3634, 3635, 3640, 3641, 3651, 3653, 3654, 3655, 3659, 3660, 3661, 3668, 3678, 3685, 3688) involved in nitrogen fixation.

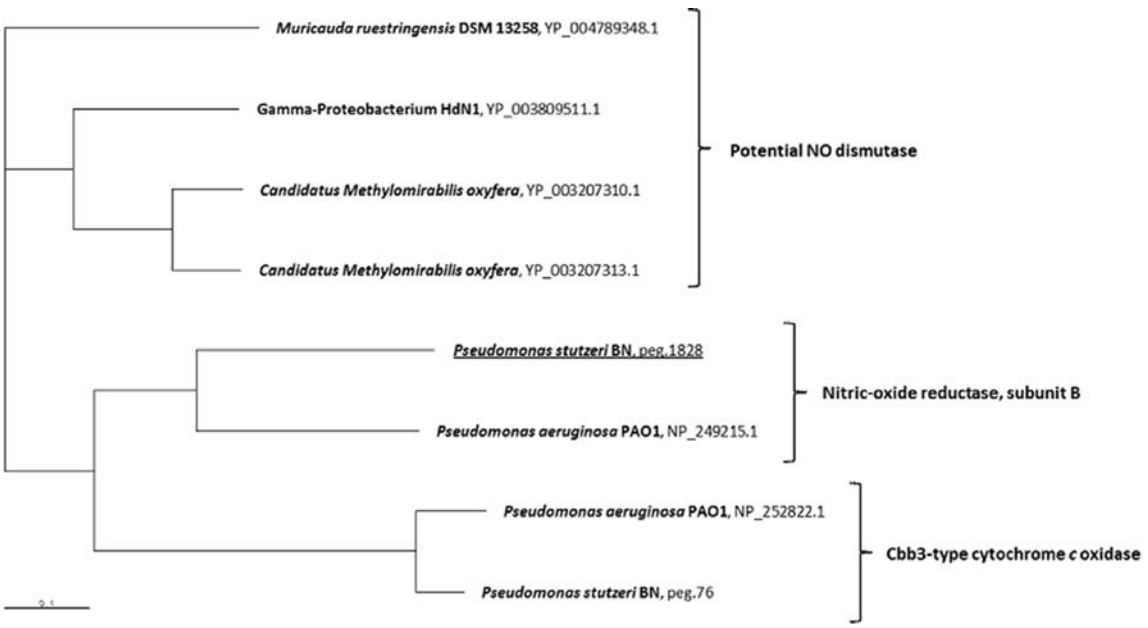


Figure 7. Phylogenetic relation of potential NO dismutase of *P. stutzeri* strain BN to selected NO reductase and *cbb*$_3$-type cytochrome *c* oxidase subunits. The nitric-oxide reductase B subunit of strain BN is underlined and, of all subunits used, the corresponding strain, in bold, and RefSeq accession number are shown (for strain BN a locus tag is used). The bar represents 10 % sequence divergence.

Discussion

The majority of microorganisms involved in benzene degradation coupled to nitrate reduction in the chemostat community belong to the classes Peptococcaceae, Burkholderiaceae and Rhodocyclaceae (Zaan, Talarico Saia et al. 2012). However, strain BN that we isolated, was a *Pseudomonas stutzeri*. *Pseudomonas* strains were not abundantly present in the chemostat community. Although *P. stutzeri* is a well-known species, there is little information on its involvement in anaerobic benzene degradation (Lalucat, Bennasar et al. 2006). This research gives insight in anaerobic and aerobic aromatic compound degradation of strain BN and the phylogeny and physiology of the strain.

Strain BN degraded BTEX under anaerobic conditions using nitrate as electron acceptor. Previously, oxygen produced from nitrate reduction has been proposed to be involved in degradation of alkanes (Zedelius, Rabus et al. 2011). As there is no potential nitric oxide dismutase gene in the genome of strain BN (Figure 7), such a mechanism does not occur for conversion of BTEX and uses anaerobic degradation pathways. According to the draft genome sequence of strain BN, carboxylation to benzoates or hydroxylation to phenols are possible mechanisms for anaerobic aromatic compound degradation by strain BN.

We found a gene coding for a putative aromatic compound carboxylase (peg.1928) and benzoate-CoA ligase (peg.3093). Carboxylation of aromatic compounds has been suggested for degradation of aromatic compounds, such as polycyclic aromatic compounds, acetophenone, phenanthrene and benzene (Davidova, Gieg et al. 2007; Abu Laban, Selesi et al. 2010; Jobst, Schühle et al. 2010; Mouttaki, Johannes et al. 2012). The source of the carboxyl-group of the intermediate benzoates is not clear. Studies on benzene carboxylation suggest that the carboxyl-group stems from transformation products of benzene (Caldwell and Suflita 2000) or from bicarbonate buffer of the medium (Kunapuli, Griebler et al. 2008). Genes of strain BC that are possibly involved in hydroxylation of aromatic compounds are code for and include phenol hydroxylase (peg.2199-2204), 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (peg.1928), 4-hydroxybenzoyl-CoA thioesterase (peg.1789) and a three component periplasmatic aromatic aldehyde oxidoreductase or 4-hydroxybenzoyl-CoA reductase (peg.1848-1850). Studies on anaerobic benzene degradation using hydroxylation in *Dechloromonas aromatica* strain RCB did not reveal the source of the molecular oxygen introduced in the aromatic ring, but did show that hydroxyl free radicals are important for benzene hydroxylation (Chakraborty and Coates 2005). Other studies using anaerobic toluene-degrading *Pseudomonas* strains suggest that the oxygen is derived from water (Altenschmidt and Fuchs 1991). Detailed proteome analysis will give more insight in the proteins involved in anaerobic aromatic hydrocarbon degradation in strain BN.

Aerobic BTEX degradation is a property of several *Pseudomonas* strains and oxygenases are key-enzymes in this process (Gibson and Parales 2000; Iwai, Kurisu et al. 2007; Iwai, Kurisu et al. 2010; Pérez-Pantoja, Gonzáles et al. 2010). These enzymes introduce oxygen atoms in the aromatic ring. This evokes ring-fission and activates aromatic compound degradation. Benzene, ethylbenzene and xylene isomers were degraded by strain BN under aerobic conditions, but not toluene (Table 1). It is unclear if all xylene isomers are degraded, since degradation was tested with a mix of the isomers. In the genome of strain BN, genes encoding phenol hydroxylase but no genes coding for toluene/*o*-xylene monooxygenase were found. Therefore, a toluene/*o*-xylene monooxygenase might be essential for conversion of toluene and possibly also for *o*-xylene. Further experiments, such as proteomics, need to be performed to show this. In *P. stutzeri* strain OX1 phenol hydroxylase as well as toluene/*o*-xylene monooxygenase are present and this *P. stutzeri* does degrade toluene and *o*-xylene (Cafaro, Izzo et al. 2004). Phenol hydroxylase of strain BN and strain OX1 are not closely related, but the general mechanism might be similar. The phenol hydroxylase of strain BN was closely related to the enzyme of *P. putida* strain GB-1, (Figure 5). The genome of this *P. putida* strain also does not contain a toluene/*o*-xylene monooxygenase encoding gene (NC_010322.1). It is not reported if this strain can degrade BTEX.

The identity of the isolated strain, *P. stutzeri* strain BN, was confirmed using API 20NE tests. Further physiological tests showed that it is a mesophile with a pH optimum around neutral, which is common for *P. stutzeri* (Lalucat, Bennasar et al. 2006). The substrate use of strain BN was similar to the substrate use of other *P. stutzeri*. D-maltose was not used as electron donor by strain BN. The draft genome contains a gene encoding amylomaltase (peg.392), but it was not active under the conditions tested. Furthermore, citrate is degraded by strain BN according to the API 20NE strip test. Accordingly, the genome encodes genes involved in the tricarboxylic acid cycle. Citrate degradation is not a common property of *P. stutzeri* strains, but occurs in some strains (Rosselló-Mora, Lalucat et al. 1994; Lalucat, Bennasar et al. 2006).

Overall, both physiological experiments and the draft genome sequence of this isolated *P. stutzeri* strain indicate that it is capable of anaerobic and aerobic degradation of aromatic compounds. Further experiments need to be performed to get more insight in the enzymatic pathways involved.

Acknowledgements

This research was supported by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053 and under the auspices of the graduate school WIMEK (Wageningen Institute for Environment and Climate Research, which is part of SENSE Research School for Socio-Economic and Natural Sciences of the Environment, www.wimek-new.wur.nl and www.sense.nl.

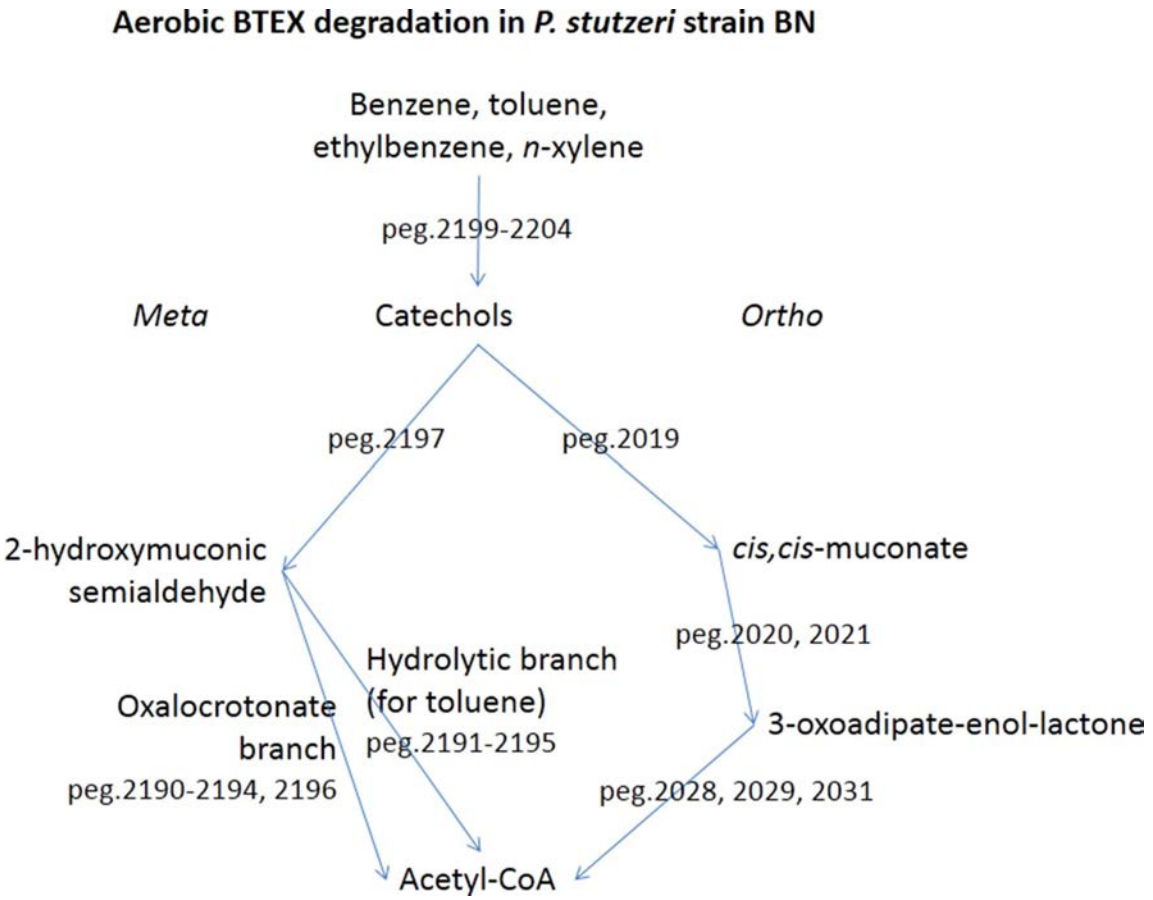


Figure S1. Schematic representation of aerobic degradation pathways of benzene, toluene, ethylbenzene and xylene isomers (BTEX) of *P. stutzeri* strain BN based on its draft genome. Identifiers of genes coding for the enzymes involved are indicated.

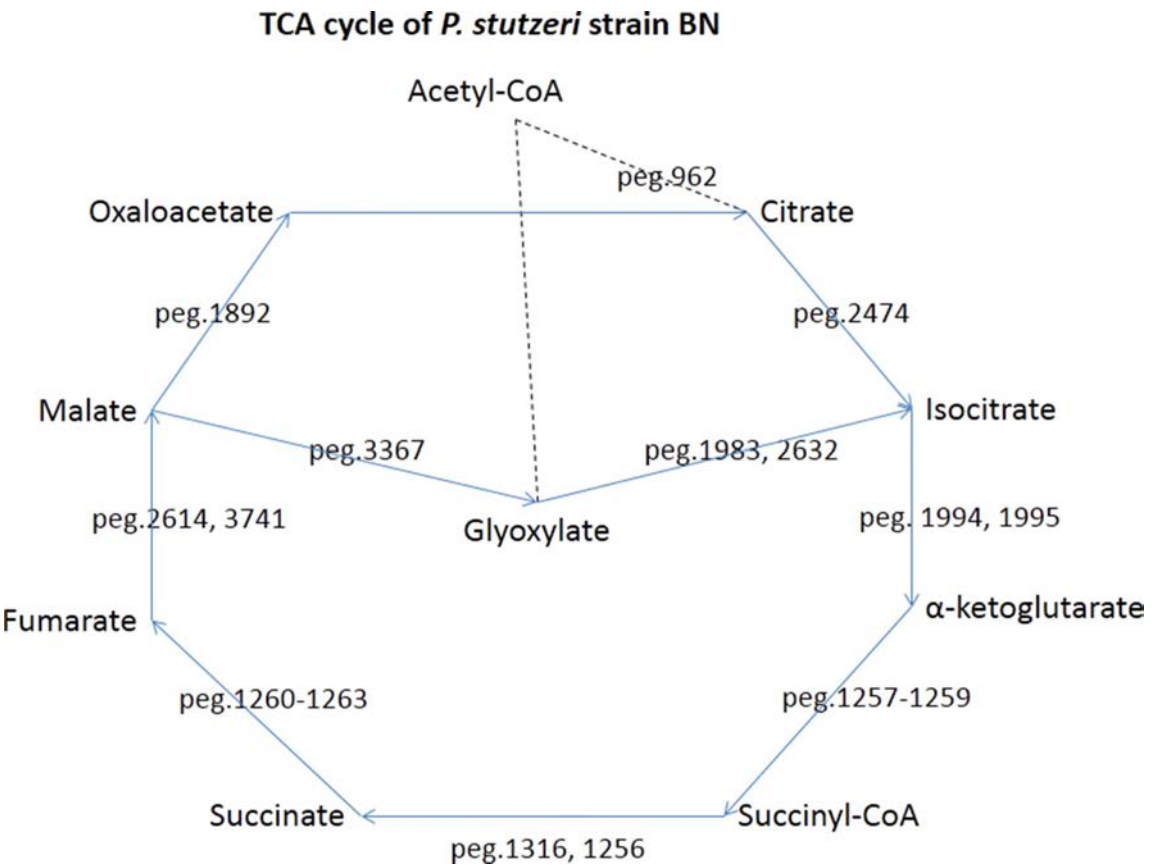


Figure S2. Overview of the tricarboxylic acid (TCA) cycle of *P. stutzeri* strain BN based on the draft genome of the strain. Identifiers of genes coding for the enzymes involved are indicated.

Chapter 7

Genome-based metabolic potentials of the anaerobic, monoaromatic compound-degrading bacterium *Georgfuchsia toluolica* strain G5G6^T

Authors and affiliations
Margreet J. Oosterkamp[1(a)#], Neslihan Taş[2(b)#], Martijn Staats[2(c)], Peter J. Schaap[3], Caroline M. Plugge[1], Wilfred F. M. Röling[2] and Alfons J. M. Stams[1*]

1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
2 Department of Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV, Amsterdam, The Netherlands.
3 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands.
(a) Current address: Laboratory of Animal Sciences, Energy Biosciences Institute, Institute of Genomic Biology, 1207 W Gregory Dr, Urbana, IL 61801, USA
(b) Current address: Department of Ecology, Earth Sciences Division, Lawrence Berkeley National Laboratory, One Cyclotron Road, MS 70A-3317, Berkeley, CA 94720, USA
(c) Current address: RIKILT, PO box 230, 6700 AE Wageningen, The Netherlands
# These authors contributed equally.
* Correspondent. E-mail: fons.stams@wur.nl; Tel.: (+31) 317 483101; Fax: (+31) 317 483829.

Keywords
Genome, metagenome, toluene, anaerobic, Banisveld aquifer*, Georgfuchsia toluolica*, strain G5G6^T

Manuscript status
Thesis chapter

Abstract

Georgfuchsia toluolica strain G5G6^T is a strictly anaerobic bacterium that degrades toluene and ethylbenzene with nitrate, iron(III) or manganese(IV) as electron acceptors. It was isolated from an iron-reducing aquifer polluted with monoaromatic compounds. Genome analysis of strain G5G6^T was performed to get insight into the physiological potentials of strain G5G6^T and of its putative function in the aquifer. The draft genome of strain G5G6^T encodes a relatively low number of genes involved in regulation and transport, which may limit the metabolic properties of the strain. The strain was known not to degrade sugars. We found that it can degrade the sugar ribose and besides the ribose transporter, no other sugar transporter proteins were encoded by the genome sequence of strain G5G6^T. The G5G6^T genome sequence also harboured genes typically contributing to anaerobic toluene degradation, including the enzyme benzylsuccinate synthase. The observed degradation of ethylbenzene likely proceeds via a pathway initiated by ethylbenzene dehydrogenase. Strain G5G6^T is not known to grow aerobically or microaerophilically. However, oxygenases, such as toluene 4-monooxygenase involved in aerobic degradation of toluene, are also encoded on the genome of strain G5G6^T. Furthermore, genes coding for an aerobic-type cytochrome oxidase were found. Nitrite reductase and nitrous oxide reductase encoding genes were not detected in the genome of strain G5G6^T, while Nar-type nitrate reductase and nitric oxide reductase were found to be present. Proteins likely involved in iron(III) and manganese(IV) reduction included a metal-reducing protein A, and two decaheme cytochrome *c* proteins. Genes of strain G5G6^T involved in degradation of aromatic compounds, ethanol and carboxylic acids and in nitrate reduction were also present in the aquifer metagenome, both in the pollution plume and the clean groundwater outside of the plume. This indicates that strain G5G6^T is widespread and an important pollutant-degrading bacterium in the aquifer.

Introduction

The Banisveld aquifer is an iron-reducing environment polluted with landfill leachate that contains low concentrations of benzene, toluene, ethylbenzene and xylene isomers (BTEX). Iron-reducing. *Geobacter* sp. are abundant in this aquifer (Röling, van Breukelen *et al.* 2001; Staats, Braster *et al.* 2011). *Georgfuchsia toluolica* strain G5G6^T was isolated from this aquifer and is the first iron-reducing, toluene-degrading strain that does not belong to the *Geobacter* family (Weelink, Van Doesburg et al. 2009). *G. toluolica* strain G5G6^T uses nitrate, iron(III), and manganese(IV) as electron acceptors and monoaromatics, such as toluene, ethylbenzene, phenol, cresol, benzaldehyde and hydroxybenzoate as electron donors. Furthermore, it does not grow on common substrates like glucose, lactate, acetate and hydrogen (Weelink, Van Doesburg *et al.* 2009).

The anaerobic toluene degradation pathway is evolutionary well conserved and involves benzylsuccinate synthase (Bss), an enzyme that catalyses fumarate addition to toluene (Leuthner, Leutwein *et al.* 1998). The resulting benzylsuccinate is subsequently converted to benzoyl-CoA in a series of conversions catalyzed by enzymes encoded by the *bbs* cluster of genes and benzoyl-CoA is further reduced to carbon dioxide (Leuthner and Heider 2000). *G. toluolica* strain G5G6^T contains the gene encoding the alpha-subunit of Bss (*bssA*) (Weelink, Van Doesburg *et al.* 2009).

A cultivation-independent molecular diversity analysis of groundwater derived from the aquifer, revealed that 89 % of retrieved *bssA* sequences were closest affiliated with those of *G. toluolica* strain G5G6^T and these *bssA* sequences were found throughout the aquifer, suggesting the significance of this strain in the anaerobic degradation of toluene and xylene (Staats, Braster *et al.* 2011). *G. toluolica*-like 16S rRNA sequences have also been detected among iron-reducing toluene-degrading enrichments from a tar-oil-contaminated aquifer in Germany (Pilloni, von Netzer *et al.* 2011). In microcosm studies using inoculum from a wide range of sources from the USA no sequences closely related to strain G5G6^T were found with augmentation of nitrate, sulfate or methane, but these sequences were found when iron was added (Sun, Sun *et al.* 2013), suggesting that microorganisms closely related to *G. toluolica* strain G5G6^T thrive best under iron-reducing conditions.

In order to reveal the genome properties and metabolic potentials of this apparent specialist of monoaromatics degradation under iron-reducing conditions, we obtained the whole-genome sequence of strain G5G6^T and compared the Banisveld metagenome to the genome of strain G5G6^T.

Materials and Methods

Strain G5G6^T and its cultivation

Georgfuchsia toluolica strain G5G6^T (DSM 19032) was isolated from the Banisveld landfill (near Boxtel, The Netherlands) (Weelink, Van Doesburg *et al.* 2009). The strain was grown in the dark, stationary and at 30° C in mineral liquid medium supplemented with toluene and nitrate as described previously (Weelink, Van Doesburg *et al.* 2009). Cells adapted to toluene and nitrate were used as inoculum (5 %). Cells used for DNA isolation were fed toluene in aliquots of 0.2 mM (final concentration) that were added after toluene depletion. Four aliquots of toluene were added, therefore in total 0.8 mM toluene was consumed in a period of 23 days. Toluene depletion was determined by gas chromatography as described previously (Weelink, Tan *et al.* 2007).

DNA extraction and whole-genome sequencing of strain G5G6^T

Six 80-mL cultures of strain G5G6^T were prepared in 250-mL serum bottles. Cells were harvested using 50-mL Sorvall buckets with screw caps by centrifugation using the SS-34 rotor in a Sorvall RC-6 plus centrifuge (Sorvall, Thermo Fisher Scientific, Waltham, MA, USA). Cell pellets were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and high molecular weight genomic DNA was isolated following the protocol for bacterial genomic DNA isolation using CTAB recommended by the U.S. Department Of Energy, Joint Genome Institute (DOE JGI, Walnut Creek, CA, USA, www.jgi.doe.gov). DNA concentration was measured using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA integrity and quality was determined by loading the genomic DNA (5 µL) on a 1 % (w/v) agarose gel with size and concentration markers according to the instructions of DOE JGI.

Genomic DNA was sent to Baseclear (Baseclear, Leiden, The Netherlands) for paired-end whole genome sequencing (75 bp). The genomic DNA was sequenced to a > 25× depth. Sequence reads were assembled into contigs using the Ray assembler (Boisvert, Laviolette *et al.* 2010). The draft genome was automatically annotated using the RAST tool (Aziz, Bartels *et al.* 2008).

Sequencing of the complete bss*-operon from strain G5G6^T*

In a separate experiment, genomic DNA from strain G5G6^T was extracted using the CTAB extraction method as described above. To generate enough starting material for fosmid library construction genomic DNA of strain G5G6^T was amplified with the GenomiPhi DNA amplification kit (Amersham Biosciences, Piscataway, NJ) according to the supplier's instructions. The amplified DNA was purified using CTAB extraction and was then cloned into fosmids by using the EpiFos fosmid library production kit (Epicentre Technologies) as recommended by the manufacturer. A total of 384 fosmid clones were picked and PCR screened for the *bssA* gene. PCR primers and conditions were described previously (Staats, Braster *et al.* 2011). A single *bssA* positive clone was sequenced using a sub-cloning strategy and spanned gaps were closed via primer walking (Macrogen, Korea). Final fosmid assembly and finishing was performed with Consed (Gordon, Desmarais *et al.* 2001).

Strain G5G6^T gene distribution in the Banisveld aquifer metagenomes

Groundwater samples from three contaminated wells and an uncontaminated control well in the Banisveld aquifer were collected via filtration. Extracted DNA samples were sequenced with Roche 454 GS FLX chemistry (Macrogen, Korea), generating 111-191 Mbp data (425-431 bp average length) per well. Sequences were filtered and assembled using the 454 Newbler assembler with default settings. The genome of strain G5G6^T was compared to Banisveld metagenomes using blast analysis with the tblastX algorithm, allowing all possible hits and using a bit score cut-off of > 80 and an E-value cut-off of $E < 1 \times 10^{-10}$. For each sequence, the blast result with the highest bit score and smallest E-value was selected.

Results and discussion

General characteristics of the strain G5G6^T genome

Genome composition

The 3.42 Mb draft whole genome of strain G5G6^T was assembled into 29 scaffolds with an average length of 118 kb (Table 1). Using automatic annotation 3309 coding sequences were identified in the genome. Among these sequences a single 16S rRNA gene could be identified.

Table 1. General features of the genome of *G. toluolica* strain G5G6^T, *T. aminoaromatica* strain S2^T (Liu, Frostegard *et al.* 2013), *A. aromaticum* strain EbN1 (Rabus, Kube *et al.* 2005) and *G. metallireducens* strain GS-15^T (Aklujkar, Krushkal *et al.* 2009).

Genome feature	*G. toluolica* strain G5G6^T	*T. aminoaromatica* strain S2^T	*A. aromaticum* strain EbN1	*G. metallireducens* strain GS-15^T
Size (bp)	3 424 685	4 252 780	4 727 255; 4 296 230, 207 355, 223 670	4 011 182; 3 997 420, 13 762
Scaffolds	29	NA	3	2
Average size (bp)	118 093	NA	NA	NA
Median size (bp)	88 935	NA	NA	NA
G+C content (mol%)	59.6%	68.6	65.12, 57.63, 63.11	NA
Coding sequences	3 309	3 897	4 133	NA
Coding (%)	74.44	NA	90.9	NA
Average size (bp)	770	NA	945	NA
Median size (bp)	642	NA	NA	NA
Assigned function (%)	70.60	NA	61.94	NA
rDNA operons	1	NA	4	NA
tRNAs	46	NA	58	NA

Sterolibacterium denitrificans strain Chol-1S^T is the closest cultured relative of strain G5G6^T (Weelink, Van Doesburg et al. 2009). This Betaproteobacterium degrades cholesterol (Tarlera and Denner 2003), but does not oxidize toluene anaerobically like G5G6^T (Weelink, Van Doesburg et al. 2009). Although the draft genome sequence of strain Chol-1S^T has been determined, it has not been characterized in detail to date (Dermer and Fuchs 2012). The G+C content of the strain Chol-1S^T DNA is 65.3 mol % (Tarlera and Denner 2003), which is higher than of the strain G5G6^T(59.6%, Table 1). We compared the genome of G5G6^T to genome sequences of other anaerobic toluene-oxidizing bacteria. Anaerobic toluene-oxidizing bacteria are found in other genera of the Betaproteobacteria division, such as *Azoarcus, Aromatoleum* and *Thauera* (Mechichi, Stackebrandt et al. 2002). In contrast to *Georgfuchsia*, they are however facultative anaerobes. The complete genome sequence of *Aromatoleum aromaticum* strain EbN1^T has been determined and described (Rabus, Kube *et al.* 2005) (Table 1). *Thauera aminoaromatica* strain S2^T is the only *Thauera* strain known to use toluene as electron donor (Mechichi, Stackebrandt *et al.* 2002) however its genome has not been described in detail yet (Table 1).

Species from the genus *Geobacter* are abundantly present in the Banisveld landfill (Röling, van Breukelen *et al.* 2001; Staats, Braster *et al.* 2011). Of the isolated *Geobacter* species *G. toluenoxydans* strain TMJ1^T, *G. metallireducens* and *G. grbiciae* have been described to utilize toluene (Kunapuli, Jahn *et al.* 2010). The genome sequence of *G. metallireducens* has been determined (Aklujkar, Krushkal *et al.* 2009), but the general characteristics have not been described in detail (Table1).

Overall, the genome of strain G5G6^T seems to contain less protein coding sequences than the genomes of the other bacteria that can use toluene as electron donor (Table1). This may indicate that strain G5G6^T is metabolically more specialized than of other toluene-degrading microorganisms. Indeed, *T. aminoaromatica* strain S2^T, *A. aromaticum* strain EbN1 and *G. metallireducens* have awider substrate range and also grow on more common

substrates such as acetate (Lovley, Giovannoni *et al.* 1993; Rabus and Widdel 1995; Mechichi, Stackebrandt *et al.* 2002).

Resistance against viral attack

CRISPR/Cas systems, that have been discovered to be a form of bacterial adaptive immunity systems (Brouns, Jore *et al.* 2008) were not present in the genome of strain G5G6^T. The 12 phage-related sequences and seven integrase(-like) genes integrated in its genome show that the strain has been in contact with bacteriophages. Besides CRISPR/Cas systems other bacterial defense mechanisms exist, such as blocking phage attachment, obstructing entry of foreign pathogenic DNA, digestion of non-self DNA, cell death upon infection (Westra, Swarts *et al.* 2012). Although genes encoding bacterial defense mechanisms were not found in the draft genome sequence of strain G5G6^T, its complete genome sequence may contain such genes.

Signal transduction and regulation of transcription

The genome of strain G5G6^T contains several genes involved in signal transduction. Of these genes, eleven are encoding two-component regulators, of which two are two-component histidine kinases. Furthermore, there are 15 other genes encoding histidine kinases, four cAMP-dependent protein kinases, two TonB-dependent receptors, eleven chemotaxis response regulators and 13 that encode other response regulators. For most of the strains relevant for comparison to G5G6^T (Table 1) signal transduction and regulation of transcription has not been well described. The genome of the iron-reducing *G. metallireducens* encodes many signal transduction proteins (Aklujkar, Krushkal *et al.* 2009). For example, there are 83 putative histidine kinase genes, which is considerably more than in the genome of strain G5G6^T and more than 200 putative genes coding for transcriptional regulators. The strain G5G6^T genome contains various types of transcriptional regulators, but much less than the *G. metallireducens* genome. Among these are genes of the LysR (nine genes), MarR (eight), GntR (three), TetR (five), AraC (four) and Crp/Fnr (three) family. Several genes have specific regulatory functions, such as nitrite-sensitive transcriptional repressors (two), NO response proteins (two), specific Nir and Nor expression regulator (one) and nitrogen regulatory proteins (seven). Furthermore, seven genes encode RNA polymerase sigma factor Rpo, one gene codes for RsbR (positive), one RsbS (negative) and one RsbT (negative) regulators of sigma factor B, three genes encode sigma-54-dependent regulators and one gene codes for transcription termination factor Rho, one NusA and one NusB transcription termination protein. *G. metallireducens* also has seven genes that encode sigma factors of RNA polymerase (Aklujkar, Krushkal *et al.* 2009).

Overall, strain G5G6^T seems not to have an extensive array of receptors and regulators. Therefore, the signal transduction system of the strain might be restricted, but it is difficult to be conclusive since it is based on a draft genome sequence.

Stress response and transport

The strain G5G6^T genome contains genes involved in stress response, such as two cold shock, seven heat shock, one osmotic shock and five universal stress protein encoding genes. Furthermore, there are also genes involved in oxidative stress response such as thiol peroxidase (two genes), alkylhydroperoxidase (seven), thioredoxin (five), thioredoxin reductase (two), glutathionine peroxidase (one), thiol peroxidase (two), catalase (one), DyP-type heme peroxidase (one), iron-type superoxide dismutase (three) rubredoxin (one), ruberythrin (two), ferredoxin (eleven) and cytochrome oxidase (see the description of the metabolism of strain G5G6^T). In other relevant strains genes encoding proteins involved in stress response have not been described.

In the genome of strain G5G6^T there are eight genes coding for RND family efflux transporters, two drug efflux pumps, four major facilitator superfamily transporters and two antibiotic (macrolide) resistance genes. Furthermore, there are

17 genes that encode outer membrane efflux pumps in the genome. Thirty-five genes encode ABC transporters. Among these transporters are multidrug and organic solvent efflux proteins and one ABC protein is involved in nitrate, sulfonate and bicarbonate transport. There are also two specific nitrate or nitrite transporter genes, is a formate and nitrite transporter gene and are two genes coding for ammonium transporters. The genome contains genes involved in transport of cofactors such as tungstate (four), zinc (two), molybdenum (three), cobalt, zinc or cadmium (eight), magnesium or cobalt (four), potassium (five), nickel or cobalt (one). Finally, genes coding for transporters of specific organic compounds are in the genome. These are 4-hydroxybenzoate (one), benzoate (one), C4-dicarboxylate or malic acid (three), L-proline, glycine or betaine and ribose transporters. The ribose transporter is the only sugar transporter in the genome and when tested, we observed that strain G5G6^T grew with D-ribose (10 mM) and nitrate (10 mM) as electron acceptor. This finding is remarkable, since the strain was described to only use monoaromatic compounds as electron donors. There are around 250 putative genes encoding transport proteins in the *G. metallireducens* genomes, which is considerably more than in the genome of strain G5G6^T (Aklujkar, Krushkal *et al.* 2009). Transport proteins of other relevant strains have not been described in great detail.

The catabolic metabolism of strain G5G6^T

Strain G5G6^T contains a tricarboxylic acid (TCA) cycle in which a putative bifunctional succinate dehydrogenase-fumarate reductase (peg.1428-1430) converts succinate to fumarate (Figure S1). Different but related enzymes generally perform fumarate reductase and succinate dehydrogenase activities, however bifunctional enzymes capable of performing both activities have been described (Weingarten, Taveirne *et al.* 2009; Wöhlbrand, Jacob *et al.* 2012). The pathogenic bacterium *Campylobacter jejuni* was found to use fumarate reductase in the TCA cycle for conversion of succinate, but it cannot use fumarate as electron donor (Weingarten, Taveirne *et al.* 2009), as was also found for strain G5G6^T (Weelink, Van Doesburg *et al.* 2009). Furthermore, isocitrate lyase (peg.283) and malate synthase (peg.285) likely are involved in a bypass of the TCA cycle by the conversion of isocitrate to malate. This bypass may be used to keep the TCA cycle active when there is too little succinate production, such as when fumarate produced in the TCA cycle is used for activation of toluene degradation.

No pathway for lactate degradation could be found using the whole-genome sequence (Figure S2). Accordingly, it does not grow with lactate (Weelink, Van Doesburg *et al.* 2009). Furthermore, although strain G5G6^T does also not grow with carboxylic acids such as propionate, crotonate, butyrate and acetate, under nitrate-reducing conditions, genes possibly involved in their degradation were identified (Figure S2). *S. denitrificans* strain Chol-1S^T, the closest cultured relative of strain G5G6^T, also does not degrade carboxylic acids with nitrate (Tarlera and Denner 2003).

Genes involved in degradation of sugars are present (Figure S3). Possibly, cells are not able to take up other sugars than ribose, as described earlier.

Anaerobic degradation of aromatic compounds in strain G5G6^T

Anaerobic toluene degradation in strain G5G6^T proceeds via a pathway that includes benzylsuccinate synthase (Bss) (Weelink, Van Doesburg *et al.* 2009) and in the genome of strain G5G6^T we found genes involved in this pathway (Figure S3). The *bss* cluster of strain G5G6^T is organized differently than that of strains EbN1^T and GS-15^T (Figure 1). Unlike the denitrifying *A. aromatoleum* strain EbN1^T, strain G5G6^T, like iron-reducing *G. metallireducens* strain GS-15^T, does not contain *bssGH* in the Bss cluster. Previous research with the toluene-degrading strain *Desulfobacula toluolica* Tol2^T indicated that these genes are specific for denitrifiers (Wöhlbrand, Jacob *et al.* 2012). Our research indicates that these genes are not present in all denitrifiers. Next to *bssGH* also *bssD* is not present in the *bssABCEF* gene cluster of strain G5G6^T. A transposase-like gene is located upstream of *bssF,* indicating that the Bss genes were acquired by horizontal gene transfer.

Strain EbN1^T contains all *bssABCDEFGH* genes and strain GS-15^T has *bssABCD* in the Bss gene cluster. Previous research showed that BssABC are subunits of the benzylsuccinate synthase, BssD is an activase for this enzyme and BssEFGH are proteins of which the function is not yet clear (Kube, Heider *et al.* 2004).

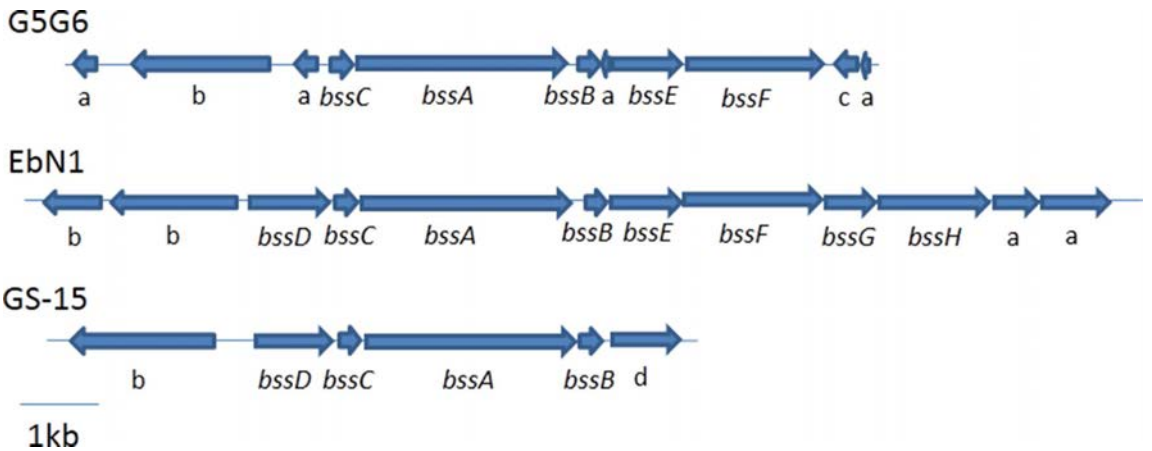


Figure 1. Schematic representation of *Bss*-gene clusters for activation of toluene degradation to benzylsuccinate from *G. toluolica* strain G5G6^T (G5G6), *A. aromaticum* strain EbN1^T (EbN1) and *G. metallireducens* strain GS-15^T (GS-15). Genes indicated with letters are: a) pseudogene, b) transcriptional regulator gene, c) transposase-like gene, d) ATPase gene.

In the draft genome genes encoding Bss are located on a different scaffold, and operon, than genes coding for succinyl-CoA:benzylsuccinate CoA-transferase (BbsEF), 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase (BbsCD) and benzoylsuccinyl-CoA thiolase (BbsAB). Putative benzylsuccinyl-CoA dehydrogenase (BbsG) and phenylitaconyl-CoA hydratase (BbsH) genes are located upstream of BbsF. In strain G5G6^T a pseudogene is present in between the genes encoding BbsE and BbsF, while such pseudogene is absent in the genome of strain EbN1^T and there is a gene coding for BbsJ inbetween *bbsF* and *bbsG* in strain EbN1^T, which is absent in strain G5G6^T (Figure 2). Furthermore, in strain GS-15^T *bbsEFGH* are clustered together and *bbsABCD* located upstream *bbsH* with genes coding for an electron transfer flavoprotein located inbetween. The electron transfer flavoproteins of strain GS-15^T could be involved in conversion of benzylsuccinyl-CoA to phenylitaconyl-CoA (Leuthner and Heider 2000). While we found acyl-CoA dehydrogenases that could be involved in this conversion (peg.2888 and 2890), an electron transfer flavoprotein was not found on the same scaffold as the Bbs genes in strain G5G6^T.

Not all putative genes involved in conversion of benzoyl-CoA to acetyl-CoA that can enter the TCA cycle were found by automatic annotation of the genome of strain G5G6^T (Figure S4), likely because the draft genome is not yet complete. By previous PCR-based detection of the gene encoding 6-ketocyclohex-1-ene-1-carbonyl-CoA hydrolase (Weelink, Van Doesburg *et al.* 2009; Staats, Braster *et al.* 2011), while we did not find it in the draft genome. Further analysis will lead to the identification of this gene in the draft genome of strain G5G6^T (Schaap, personal communication). The benzoyl-coA degrading pathway is well described for facultative anaerobic, but less well for strictly anaerobic bacteria (Peters, Shinoda *et al.* 2007). A gene encoding benzoyl-CoA reductase (Peg.681-684) was found in the draft genome sequence of strain G5G6^T. The reaction catalyzed by this benzoyl-CoA reductase, which is conversion to cyclohexa-1,5-dienoyl-1-carbonyl-CoA, is ATP-dependent. Denitrifying microorganisms, like

strain G5G6^T, were described to have an enzyme that depends on ATP while the enzyme of obligate anaerobes does not (Löffler, Kuntze *et al.* 2011).

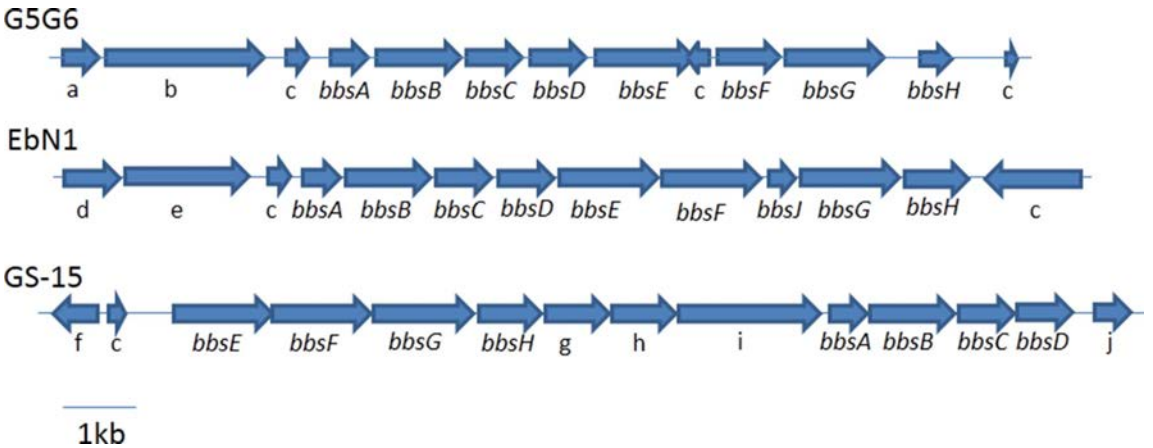


Figure 2. Schematic comparison of *Bbs*-clusters involved in benzylsuccinate degradation to benzoyl-CoA of *G. toluolica* strain G5G6^T (G5G6), *A. aromaticum* strain EbN1^T (EbN1) and *G. metallireducens* strain GS-15^T (GS-15). Genes indicated with letters are: a) *oadA*-like gene, b) -CoA carboxylase gene, c) pseudogene/hypothetical gene, d) transposition helper gene, e) transposase gene, f) TedR family transcriptional regulator coding gene, g) electron transfer flavoprotein subunit beta coding gene, h) electron transfer flavoprotein subunit alpha coding gene, i) electron transfer flavoprotein subunit Cyt B and Fe-S oxidoreductase coding gene, j) cyclic nucleotide-binding protein coding gene.

Ethylbenzene is degraded using a similar mechanism in strains G5G6^T and denitrifying EbN1 (Kühner, Wöhlbrand *et al.* 2005; Weelink, Van Doesburg *et al.* 2009). This mechanism involves ethylbenzene dehydrogenase (EbdABCD), acetophenone carboxylase (Apc1234), benzoylacetate CoA-ligase (bal), 1-phenylethanol dehydrogenase and benzoylacetyl-CoA acetyltransferase. Putative genes encoding these enzymes are present in the genome of strain G5G6^T (Figure S5). In the draft genome sequence of *S. denitrificans* strain Chol-1S^T putative ethylbenzene dehydrogenase beta, gamma and delta coding genes are present (genbank accession numbers JQ293010, JQ293012 and JQ293012). Other putative genes involved in ethylbenzene degradation have not been found and to our knowledge, strain Chol-1S^T has not been described to degrade ethylbenzene. An alternative strategy for ethylbenzene degradation involves fumarate addition as found in the sulfate-reducing marine strain EbS7 (Kniemeyer, Fischer *et al.* 2003), the sulfate-reducing strain PRTOL1 (Beller and Spormann 1997) and the denitrifying bacterium *Thauera aromatica* (Biegert, Fuchs *et al.* 1996). Although the initial steps of the two described mechanisms are different, both involve the benzoyl-CoA pathway eventually.

Other genes in the genome of strain G5G6^T that might be involved in anaerobic degradation of aromatic compounds include a gene coding for benzaldehyde dehydrogenase (peg.1505). This enzyme is involved in anaerobic benzaldehyde degradation. Benzoate-CoA ligase (Peg.1514) is involved in degradation of benzoate. However, the strain was not reported to degrade benzoate (Weelink, Van Doesburg *et al.* 2009). Furthermore, the strain was reported to degrade phenol, *p*-cresol and *m*-cresol anaerobically, but known genes possibly involved in anaerobic degradation of these compounds could not be retrieved from the draft genome sequence.

Aerobic degradation pathways for aromatic compounds in strain G5G6^T

Mono- and dioxygenases are enzymes that convert compounds by integration of oxygen atoms in the chemical structure and that can be involved in aerobic degradation

of aromatic compounds (Di Gennaro, Bargna *et al.* 2011). Oxygenase genes were found in the genome of strain G5G6^T, while this strain was described as a strictly anaerobic bacterium (Table 2). Catechol, the central intermediate of aerobic aromatic compounds, can be converted to 2-hydroxymuconate semialdehyde by catechol 2, 3-dioxygenase. Further aerobic degradation of this compound can proceed via the hydrolytic branch of the *meta*-cleavage pathway of catechol degradation (Harayama, Mermod *et al.* 1987) of which the genes are encoded in the genome (Peg.1386-1392). The genome sequence suggests that phenol and *p*-hydroxybenzoate as well as gentisate, salicylate, catechol and benzoyl-CoA also can be degraded aerobically. Aerobic degradation of toluene by strain G5G6^T was not observed previously (Weelink, Van Doesburg *et al.* 2009). Thus far, no indications were obtained at which environmental conditions strain G5G6^T would be able to grow aerobically or micro-aerophilically.

Table 2. Oxygenase encoding genes in the draft genome sequence of strain G5G6^T.

Oxygenase (geneID)	Conversion catalyzed by oxygenase
Phenol hydroxylase (peg.1397-1402)	phenol to catechol
Gentisate 1,2-dioxygenase (peg.1849, 2918)	2,5-dihydroxybenzoate to maleylpyruvate
Salicylate hydroxylase (peg.2919)	Salicylate to catechol
Catechol 2,3-dioxygenase (peg.1394, 1886)	catechol to 2-hydroxymuconate semialdehyde
Protocatechuate 4,5-dioxygenase (peg.2659, 2660)	protocatechuate to 4-carboxy-2-hydroxymuconate semialdehyde
Toluene 4-monooxygenase (peg.2844-2849)	converts various aromatic compounds
p-Hydroxybenzoate hydroxylase (peg.2665)	p-Hydroxybenzoate to protocatechuate
Benzoyl-CoA oxygenase (peg.1507-1509)	benzoyl-CoA to 3-hydroxybenzoyl-CoA
Taurine dioxygenase (peg.3118)	taurine to aminoacetaldehyde
Anthraniloyl-CoA monooxygenase (peg.3162)	2-aminobenzoyl-CoA to 2-amino-5-oxocyclohex-1-enecarboxyl-CoA
2-nitropropane dioxygenase (peg.1044)	nitroalkanes to carbonyl compounds
2-octaprenyl-6-methoxyphenol hydroxylase (peg.2031)	2-octaprenyl-6-methoxyphenol to 2-octaprenyl-6-methoxy-1,4-benzoquinone
2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase (peg.478, 2195)	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol to 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol
Ubiquinone biosynthesis monooxygenase UbiB (peg.2161)	Ubiquinone biosynthesis
FAD-dependent monooxygenase (peg.1500)	Unknown

Electron acceptor metabolism of strain G5G6^T

A variety of terminal oxidases is capable of transferring electrons to oxygen in aerobic electron transport chains (Morris and Schmidt 2013). Terminal oxidases can be grouped into the haem-copper oxidases and cytochrome *b,d*-type oxidases. Haem-copper oxidases can be further divided into low-affinity oxidases (Class A), high-affinity bacterial-only (Class C) and high-affinity archaeal and bacterial oxidases (Class B). Genes encoding of Class A low-affinity aerobic- (peg.1632-1634) and Class C high-affinity cbb_3-type (peg.561, 562, 564) terminal oxidases were found in the genome sequence of strain G5G6^T. Presence of both low- and high-affinity oxidases indicates that strain G5G6^T is a facultative instead of a strict anaerobe (Morris and Schmidt 2013). However, thus far no indications for aerobic growth have been obtained. The closest cultured relative *S. denitrificans* strain Chol-1S^T is known to respire aerobically, and anaerobically with nitrate (Tarlera and Denner 2003).

Genes involved in nitrate reduction, coding for nitrate (peg.592-594, 2088-2091) and nitric oxide (Peg.731, 732) reductase subunits are present in the strain G5G6^T genome. Two different Nar-type nitrate reductases are encoded in the genome. Of one nitrate reductase, genes (peg.592-594) are close to a scaffold end. These genes code for the beta, gamma and delta chain (NarHJI) of nitrate reductase while the alpha chain (NarG) coding gene is missing. NarGHIJ enzymes reduce nitrate to nitrite in the cytoplasm (Kraft, Strous *et al.* 2011) and toxic nitrite can be excreted using nitrate and nitrite transporters (peg.1818, 2223) or a formate and nitrite (peg.108) transporter that are encoded in the genome. Other nitrate reductase genes (peg. 2088-2099) encode NarG, Y, I and J2 subunits that are homologs of the subunits of NarGHIJ (Moreno-Vivián, Cabello *et al.* 1999). Nitrite reductase and nitrous oxide reductase encoding genes were not found. It is unclear if these genes are missing due to the fact that the genome of strain G5G6^T is not completely gap-close sequenced, or if the bacterium has an atypical denitrification pathway. Alternative nitrate reduction pathways have been suggested and an enzyme that reduces both nitrate and nitrite was found in *G. metallireducens* (Martínez Murillo, Gugliuzza *et al.* 1999; Ettwig, Speth *et al.* 2012). Further research is needed to get a complete picture of the nitrate reduction pathway of strain G5G6^T.

Reduction of iron(III) and manganese(IV) oxides takes place at the cell surface (Richter, Schicklberger *et al.* 2012). A protein similar to metal-reducing protein A, MtrA, a periplasmic decaheme cytochrome *c* protein (Peg.3009) is present in the genome of strain G5G6^T. This protein is involved in iron(III) and manganese(IV) reduction in *Shewanella oneidensis* strain MR-1 (Schuetz, Schicklberger *et al.* 2009). Two decaheme cytochrome *c* MtrF proteins are also present in the G5G6^T genome (Peg.3131, 3133). In *S. oneidensis* MtrF is also involved in reduction of iron(III) and manganese(IV) (Clarke, Edwards *et al.* 2011). Other genes in the genome of strain G5G6^T located near MtrA and MtrF encoding genes are predicted genes and genes encoding general secretion proteins (peg.3029-3031, 3080-3086). General secretion proteins were not described in the iron(III) and manganese(IV) reduction pathway of *S. oneidensis* (Clarke, Edwards *et al.* 2011; Richter, Schicklberger *et al.* 2012). Next to MtrA and MtrF, MtrBCDE and CymA are described to be involved in iron(III) and manganese(IV) reduction in *S. oneidensis* (Richardson, Butt *et al.* 2012). MtrB is a trans-outer membrane β-barrel protein that could form a pore in the membrane. MtrC/OmcA is another decaheme cytochrome *c* protein and MtrDE another porin-cytochrome complex. CymA is a tetraheme *c* cytochrome that transfers electrons from the quinone pool to MtrA. Another microorganism for which the iron(III) and manganese(IV) reduction pathway is relatively well described is *G. metallireducens*. Cytochrome *c*-type proteins are also involved in this strain with OmcB embedded in the outer membrane and acting as Fe(III) reductase. Other outer-membrane proteins involved are OmcE and OmcS. Additional research is needed on electron transfer through the membrane, but the outer membrane proteins are hypothesized to shuttle electrons from type IV pili (Lovley, Ueki *et al.* 2011). In strain G5G6^T MtrA could accept electrons for iron(III) and manganese(IV) reduction. Most likely this protein interacts with one of the two MtrF decaheme cytochrome *c* metal-reducing proteins for further transport of the electrons to the MtrF that can reduce the Iron(III) and manganese(IV). Further genetic or proteomic research needs to be performed to confirm the presence of this iron(III) and manganese(IV) reduction in strain G5G6^T

Analysis of the Banisveld metagenome using the genome of strain G5G6^T

The genome sequence of strain G5G6^T was compared to the metagenomes of Banisveld aquifer samples. All samples contained genes from strain G5G6^T, indicating that the strain is widely distributed and is an important member of the microbial community. Genes from strain G5G6^T were also detected in a clean groundwater sample located outside of the pollution plume. Accordingly, earlier cultivation-independent, PCR amplification based analysis of clean groundwater for functional genes contributing to anaerobic BTEX degradation, showed the presence of sequences closest related to strain G5G6^T (Staats, Braster *et al.* 2011). Among the top fifty hits of the blast of the strain G5G6^T genome against the metagenomes were genes involved in degradation of aromatic compounds, ethanol and carboxylic acids such as propionate. Furthermore, genes of strain G5G6^T involved in respiratory nitrate reduction were present in three of the four metagenomes and in one metagenome cytochrome *c* oxidase genes were present (Table 3).

Concluding remarks

Based on whole genome analysis, the monoaromatic compound-degrading strain *G. toluolica* G5G6^T has a limited number of genes involved in regulation and transport, which will have an effect on its general metabolism. For example, we found only one sugar transporter that can transport ribose and ribose is the only sugar that the strain can use for growth, while other genes suggested the potential for degradation of other sugars.

Our metagenomic analysis and a previous molecular diversity study (Staats, Braster *et al.* 2011) of the aquifer showed that *G. toluolica* is widely distributed. Next to in The Netherlands*, G. toluolica* strains were found in Germany and USA (Pilloni, von Netzer *et al.* 2011; Sun, Sun *et al.* 2013), indicating the importance of *G. toluolica* in these different ecosystems. Whole genome analysis of strain G5G6^T revealed more about its properties, for example about anaerobic toluene degradation and about the intriguing nitrate and iron(III) reduction pathways of this strain. Further research is needed to fully understand these proposed pathways in strain G5G6^T.

Acknowledgements

This research was supported by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053 and under the auspices of the graduate school WIMEK (Wageningen Institute for Environment and Climate Research, which is part of SENSE Research School for Socio-Economic and Natural Sciences of the Environment, www.wimek-new.wur.nl and www.sense.nl. We thank Mark Davids for his suggestions that helped in understanding the metabolism of strain G5G6^T.

Table 3. Overview of genes from the draft genome found in the metagenomes of clean groundwater (clean GW), 6 meters from the pollution plume (6 meters), 21 meters from the pollution plume (21 meters) and 39 meters from the pollution plume (39 meters), + is present, - is absent.

Query	Function	Metabolic role	Clean GW	6 meters	21 meters	39 meters
peg.268	Pyrogallol hydroxytransferase(EC:1.97.1.2)	Aromatic compound metabolism	+	-	-	-
peg.681	Benzoyl-CoA reductase subunit BadD (EC 1.3.99.15)	Aromatic compound metabolism	-	+	-	-
peg.684	Benzoyl-CoA reductase subunit BadG (EC 1.3.99.15)	Aromatic compound metabolism	+	-	-	-
peg.1382	BssE/Nitric oxide reductase activation protein NorQ	Aromatic compound metabolism	+	-	-	-
peg.2666	Positive regulator of phenol hydroxylase	Aromatic compound metabolism	-	+	-	-
peg.1379	BssA/Pyruvate formate-lyase (EC 2.3.1.54) Benzylsuccinate synthase alpha subunit	Aromatic compound metabolism	-	+	-	-
peg.2659	Protocatechuate 4,5-dioxygenase alpha chain (EC 1.13.11.8)	Aromatic compound metabolism	-	-	+	-
peg.2850	Positive regulator of phenol hydroxylase	Aromatic compound metabolism	-	-	-	+
peg.992	Biotin carboxylase of acetyl-CoA carboxylase (EC 6.3.4.14)	Carboxylic acid metabolism	+	-	-	-
peg.3247	3-ketoacyl-CoA thiolase (EC 2.3.1.16) @ Acetyl-CoA acetyltransferase (EC 2.3.1.9)	Carboxylic acid metabolism	+	-	-	-
peg.3261	Enoyl-CoA hydratase (EC 4.2.1.17)	Carboxylic acid metabolism	-	+	-	-
peg.398	NAD(FAD)-utilizing dehydrogenase, sll0175 homolog	Carboxylic acid metabolism	-	+	-	-
peg.3003	[NiFe] hydrogenase metallocenter assembly protein HypE	Carboxylic acid metabolism	-	+	-	-
peg.709	Propionate--CoA ligase (EC 6.2.1.17)	Carboxylic acid metabolism	-	-	+	-
peg.504	Alcohol dehydrogenase (EC 1.1.1.1)	Alcohol metabolism	-	-	-	+
peg.735	Nitric oxide reductase activation protein NorD	Respiratory nitrate reduction	+	+	-	-
peg.592	Respiratory nitrate reductase beta chain (EC 1.7.99.4)	Respiratory nitrate reduction	-	+	-	-
peg.2091	Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	Respiratory nitrate reduction	-	-	+	-
peg.1633	Cytochrome c oxidase polypeptide I Cox (EC 1.9.3.1)	Respiration	-	+	-	-
peg.562	Cytochrome c oxidase subunit CcoO (EC 1.9.3.1)	Respiration	-	+	-	-

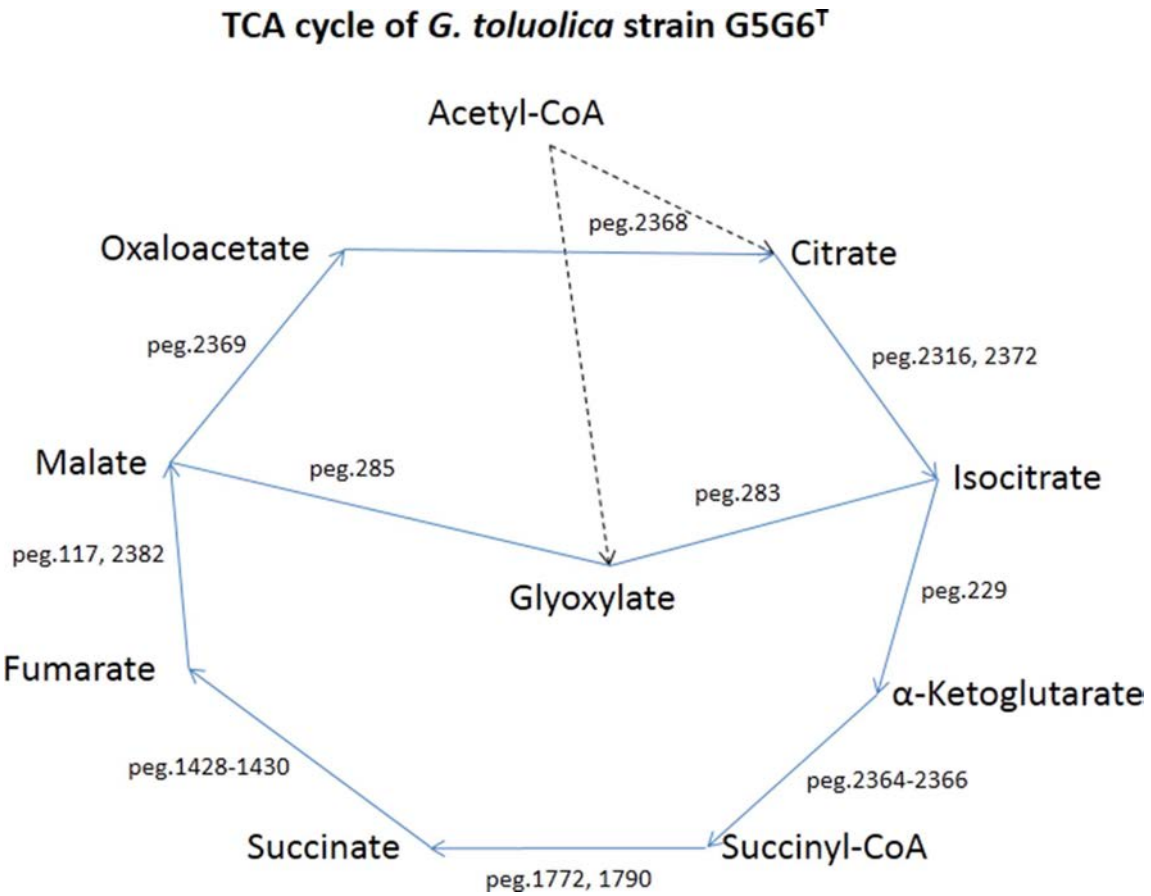


Figure S1. Schematic representation of the tricarboxylic acid (TCA) cycle of *G. toluolica* strain G5G6^T based on the draft genome of the strain. Identifiers of genes coding for the enzymes involved in the TCA cycle are indicated.

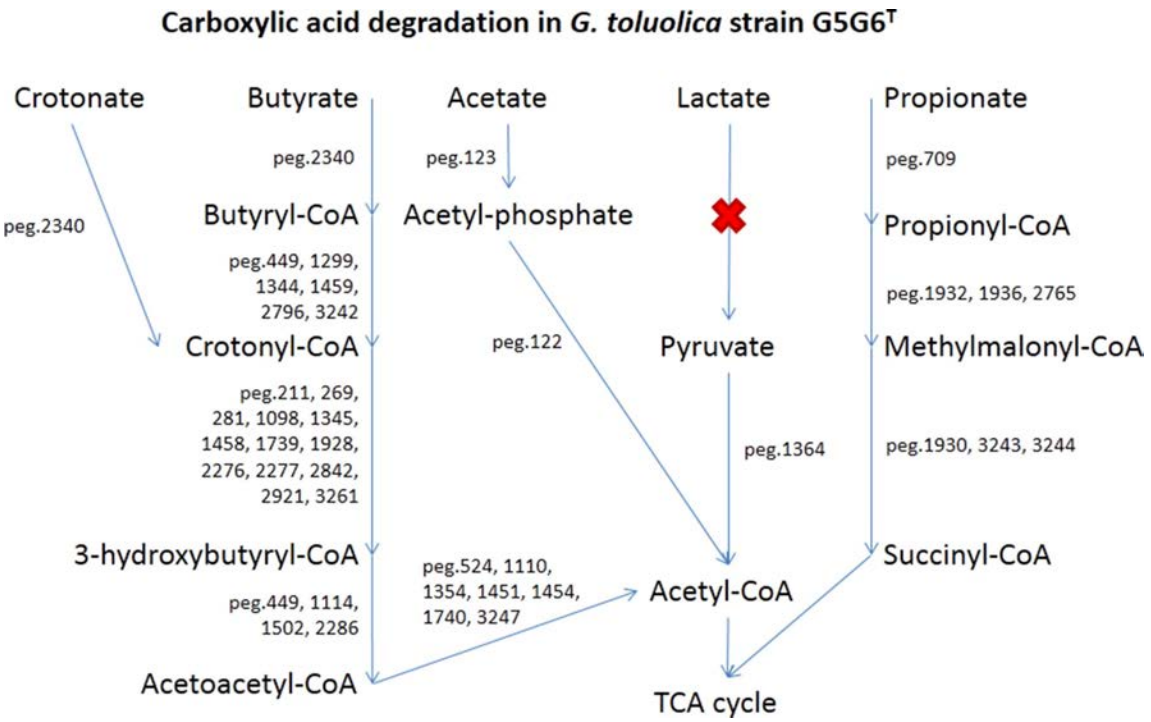


Figure S2. Overview of carboxylic acid degradation pathways in *G. toluolica* strain G5G6^T based on its draft genome. Identifiers of genes coding for the enzymes putatively involved in carboxylic acid degradation are indicated and a cross indicates that a gene involved in the conversion involved was not found.

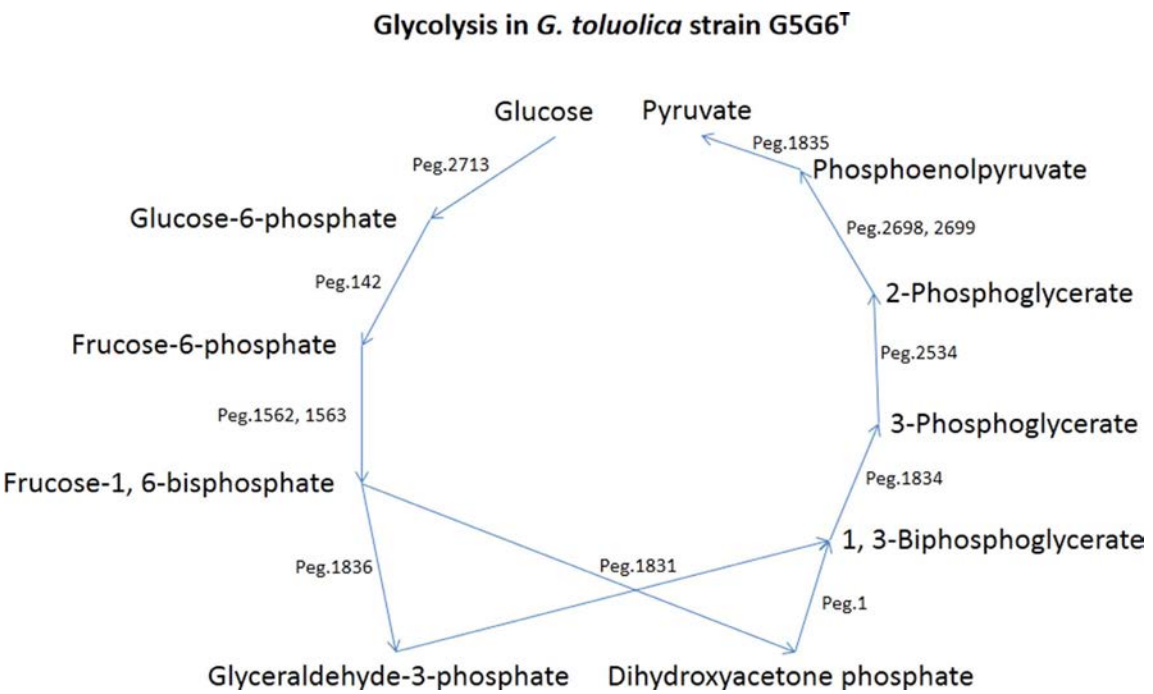


Figure S3. Schematic representation of glycolysis in *G. toluolica* strain G5G6^T based on the draft genome of the strain. Identifiers of genes coding for the enzymes involved are indicated.

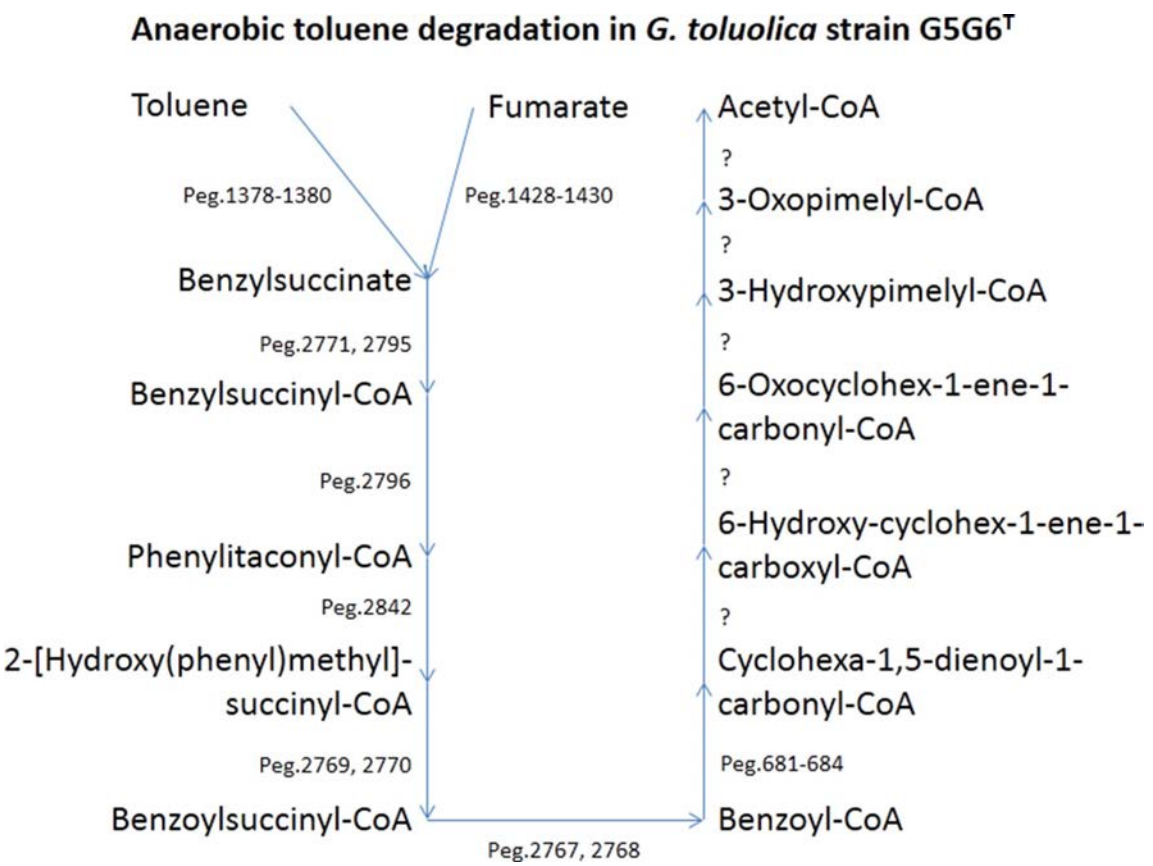


Figure S4. Overview of the anaerobic toluene degradation pathway of *G. toluolica* strain G5G6^T based on its draft genome. Identifiers of genes coding for the enzymes involved are indicated and question marks are used when a gene coding for the enzyme involved in a conversion could not be found in the draft genome.

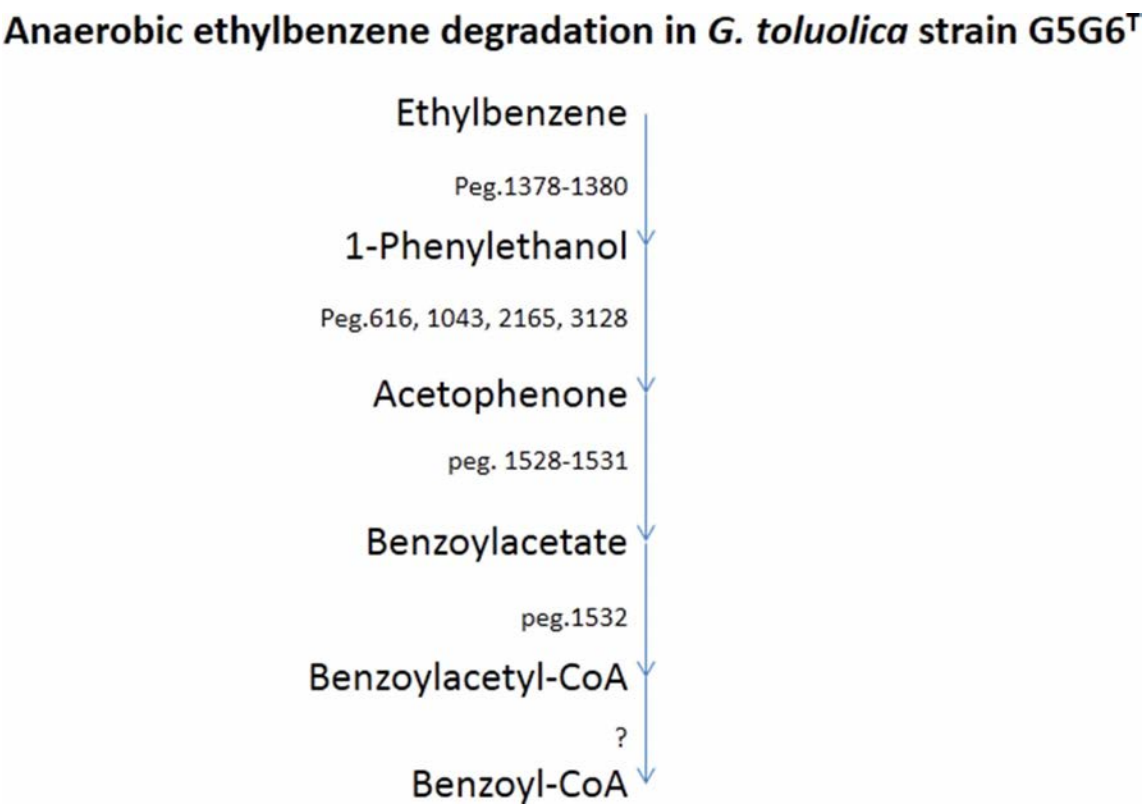


Figure S5. Overview of the anaerobic ethylbenzene degradation pathway of *G. toluolica* strain G5G6^T based on the draft genome of the strain. Identifiers of genes coding for the enzymes involved are indicated and a question mark is used when a gene coding for the enzyme involved in a conversion could not be found in the draft genome.

Chapter 8

Proteome and genome analysis of *Pseudomonas chloritidismutans* AW-1^T that oxidizes *n*-decane with chlorate or oxygen as electron acceptor

Authors and affiliations
Farrakh Mehboob[1(a)#], Margreet J Oosterkamp[1#(b)], Sumaira Farrakh[1], Teun Veuskens[1], Caroline M Plugge[1], Sjef Boeren[2], Willem M de Vos[1], Gosse Schraa[1], Alfons JM Stams[1] and Peter J Schaap[3§]

1 Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands
2 Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
3 Laboratory of Systems and Synthetic Biology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, The Netherlands
(a) Current addres: Pakistan Agricultural Research Council, Ecotoxicology Research Programme, National Institute of Bioremediation, National Agriculture Research Centre, Park Road, Islamabad, Pakistan
(b) Current address: Laboratory of Animal Sciences, Energy Biosciences Institute, Institute of Genomic Biology, 1207 W Gregory Dr, Urbana, IL 61801, USA
§ Corresponding author: Email: Peter.Schaap@wur.nl, Tel.: (+31) 317 485142, Fax: (+31) 317 483829.
# These authors contributed equally.

Keywords
n-Decane degradation, chlorate reduction, nitrate reductase, alkane 1-monooxygenase, bioremediation

Manuscript status
Thesis chapter (new version of previous thesis chapter (Mehboob 2010))

Abstract

Pseudomonas chloritidismutans AW-1^T is a bacterium that oxidizes C7 to C12 *n*-alkanes in the absence of externally supplied oxygen by generating molecular oxygen from chlorate. Analysis of the proteome of *P. chloritidismutans* AW-1^T showed the versatility of this bacterium to adapt to aerobic and anaerobic growth conditions with acetate or *n*-decane as electron donor. The unknown enzymes involved in the alkane oxidation pathway were identified. An alkane monooxygenase was detected in *n*-decane-grown cells, but not in acetate-grown cells. The enzyme was found when grown in the presence of oxygen or chlorate, indicating that under both of these conditions an oxygenase mediated pathway is employed for alkane degradation. Proteomic and biochemical data also showed that both chlorate reductase and chlorite dismutase are constitutively present and abundant under chlorate-reducing conditions, but are less abundant under aerobic conditions. Previously, *P. chloritidismutans* AW-1^T was proposed as the type strain of a novel species closely related to *Pseudomonas stutzeri.* By comparing the genomes of *P. chloritidismutans* AW-1^T and three *P. stutzeri* strains it was confirmed that *P. chloritidismutans* and *P. stutzeri* are phylogenetically distinct species.

Introduction

Pseudomonas chloritidismutans AW-1^T is a Gram-negative, facultative anaerobic bacterium originally isolated from chlorate and bromate polluted wastewater (Wolterink, Jonker et al. 2002). Later studies showed that this bacterium is able to degrade C7 to C12 *n*-alkanes under anaerobic conditions with chlorate as electron acceptor (Mehboob, Junca et al. 2009). Generally, anaerobic biodegradation of *n*-alkanes is slow and it has been reported to occur under nitrate-reducing, iron-reducing, sulfate-reducing and methanogenic conditions (Spormann and Widdel 2000; Wentzel, Ellingsen et al. 2007; Rojo 2009; Gray, Sherry et al. 2010). Information on biochemical mechanisms of anaerobic *n*-alkane degradation is limited. While aerobic bacteria employ oxygenases for initial attack of these hydrocarbons, anaerobes seem to initiate degradation of *n*-alkanes mainly by fumarate addition (Wentzel, Ellingsen et al. 2007; Rojo 2009). Knowledge of alternative capacities for anaerobic degradation of hydrocarbons is important since oil spills in the environment may lead to problematic contamination of groundwater aquifers which are often anoxic or may become anoxic by oxygen consumption by aerobes. More detailed knowledge of the biochemical processes occurring in anaerobic biodegradation of hydrocarbons can be applied to understand the effectiveness of bioremediation efforts and to predict which conditions are optimal for degradation of hydrocarbons and their degradation products.

Currently *P. chloritidismutans* AW-1^T is the only known microorganism that can efficiently degrade medium chain *n*-alkanes (C7-C12) in the absence of oxygen by generating molecular oxygen from chlorate. Generation of molecular oxygen from the supplied electron acceptor represents an alternative mechanism for the fumarate addition mechanism of strict anaerobes (Mehboob, Weelink et al. 2009). Two enzymes are involved in intracellular oxygen formation in *P. chloritidismutans* AW-1^T. Chlorate (ClO_3^-) is first reduced to chlorite (ClO_2^-) by chlorate reductase. Chlorite is then split into chloride (Cl^-) and oxygen (O_2) by chlorite dismutase, as found in other bacteria that reduce chlorate or perchlorate (Rikken, Kroon et al. 1996; Bardiya and Bae 2011). Evidence for the occurrence of intracellular oxygen transfer from chlorite dismutase to the alkane oxygenase was obtained, but a gene involved in alkane oxidation could not be identified using a large set of primers that target a large variety of oxygenase genes (Mehboob, Junca et al. 2009).

To get more insight into the *n*-alkane and chlorate metabolism we sequenced the genome of *P. chloritidismutans* AW-1^T. Further insight into the metabolism of this microorganism was obtained through 'shotgun proteomic analysis' of the expressed portion of the genome under specific growth conditions (Sadygov, Cociorva et al. 2004). Here, we present the results of a study of the *n*-decane metabolism of *P. chloritidismutans* AW-1^T in the absence of oxygen while generating molecular oxygen from chlorate. Results obtained from proteomic analyses of strain AW-1^T were compared with biochemical data of key enzymes. In addition we reevaluated the phylogenetic relationship of *P. chloritidismutans* AW-1^T with *Pseudomonas stutzeri*. Previously, *P. chloritidismutans* AW-1^T was proposed as the type strain of a novel species (Wolterink, Jonker et al. 2002). Subsequently, by sequence analysis of the 16S rRNA genes in combination with sequence analysis of four housekeeping genes Cladera *et al.,* 2006 concluded that *P. chloritidismutans* AW-1^T and *P. stutzeri* are the same species (Cladera, García-Valdés et al. 2006). Here the draft genome is compared with the genomes of three *P. stutzeri* species, confirming that *P. chloritidismutans* AW-1^T and *P. stutzeri* are separate species.

Experimental Procedures

Strains, media and cultivation

P. chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink, Jonker et al. 2002). For experiments with nitrate, it was adapted to nitrate as described (Mehboob, Junca et al. 2009). *P. stutzeri* strain A1501 (GenBank accession number CP000304) was obtained from the Collection of Institute Pasteur (CIP, Paris, France).

P. chloritidismutans strain AW-1^T was grown in medium as described previously (Mehboob, Junca et al. 2009) and cultivated at 30°C in 1-L flasks containing 600 ml of medium. For anaerobic growth experiments the medium was prepared with anoxic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with butyl rubber stoppers and aluminum caps, and the headspace was replaced with N_2 gas (140 kPa). All solutions that were added to the medium were anoxic and autoclaved at 121°C for 20 minutes. Sodium salts of chlorate and nitrate were supplied from a 0.4 M stock solution to a final concentration of 10 mM.

Preparation of cell free extracts

Cell free extracts of strain AW-1^T were prepared under anaerobic conditions as previously described (Mehboob, Junca et al. 2009). Cell free extracts were stored in 12-mL serum vials at -80°C for proteomic analysis and under anaerobic conditions at -20°C for biochemical analysis.

The protein content of the cell free extract fraction was determined using the Bio-Rad protein assay (Bio-rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions with bovine serum albumin as standard.

Enzyme activity measurements

Chlorate reductase, nitrate reductase and nitrite reductase activities were determined spectrophotometrically as described previously (Kengen, Rikken et al. 1999). The substrate-dependent oxidation of reduced methylviologen 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 previously (Wolterink, Jonker et al. 2002). One unit (U) of activity is defined as the amount of enzyme required to convert 1 µMol of chlorite per minute.

Genome sequencing and annotation

The genome *P. chloritidismutans* strain AW-1^T was sequenced to a > 25× depth using Roche 454 pyrosequencing with GS FLX Titanium chemistry. Sequence reads were assembled into contigs using the Newbler assembler which resulted in 5.9 Mbp assembled sequence data. The draft genome was automatically annotated using the RAST tool (Aziz, Bartels et al. 2008). Anib values were calculated with JSpecies (Richter and Rosselló-Móra 2009). Signal peptides were predicted using the SignalP 3.0 and TatP software (Bendtsen, Nielsen et al. 2004; Bendtsen, Nielsen et al. 2005).

Sample preparation for tandem-MS

A comparative analysis of the proteome of cells grown under the 5 different conditions was made. Equal amounts of each sample (250 µg protein) were separated on 12 %-SDS polyacrylamide gels, and gels were stained according to the manufacturer's protocol using Colloidal Coomassie 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 1 h 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. Samples were analysed on LC-MS/MS as previously described (Rooijers, Kolmeder et al. 2011).

Mass spectrometry database searching

The resulting spectra from the MS analysis were submitted to a local implementation in the OMSSA search engine (Geer, Markey et al. 2004). MS/MS spectra were searched against a peptide database derived from a six frame translation of *P. 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. Top hit spectral matches to peptides in the reversed database were used for FDR calculation as described (Elias and Gygi 2007). Unambiguously assigned peptides were used to identify expressed proteins. Spectral counting was subsequently used for comparative quantification (Liu, Sadygov et al. 2004).

Results

Comparison of the* P. chloritidismutans *AW-1^T draft genome with genomes of* P. stutzeri *strains

Whole genome shotgun sequencing of *P. chloritidismutans* AW-1^T resulted in 5.9 Mbp assembled sequence data. Out of this 5.9 Mbp, 4.5 Mbp were assembled in 112 contigs larger than 10 kbp. Overall the GC content of the sequenced genome was 62,8%. The RAST annotation service (Aziz, Bartels et al. 2008) identified some 5500 protein-encoding genes and subsequently could assign putative functions to almost 80% of these proteins.

P. chloritidismutans AW-1^T was proposed as the type strain of a novel species (Wolterink, Jonker et al. 2002). Based on 16S rRNA gene sequence analysis *P. chloritidismutans* AW-1^T is most closely related to *P. stutzeri.* The relationship between *P. chloritidismutans* AW-1^T and *P. stutzeri* has been studied using a multilocus phylogeny approach with a limited set of four 'housekeeping genes'. This study suggested that they are the same species (Cladera, García-Valdés et al. 2006). However, a comparison of all *P. chloritidismutans* AW-1^T proteins with the protein complement of the most closely related fully sequenced genome of *P. stutzeri* A1501 (Yan, Yang et al. 2008) by 'reciprocal best hits' revealed that only 55% of the *P. chloritidismutans* AW-1^T proteins show over 50% identity in more than 90% of the sequence length of the set of proteins of *P. stutzeri* A1501. Furthermore, 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, which all are present in *P. stutzeri* A1501, are missing. On the other hand, *P. stutzeri* A1501 lacks the genes coding for the four subunits of chlorate reductase, chlorite dismutase and for alkane 1-monooxygenase (see below). Further analysis confirmed that *P. stutzeri* A1501 is unable to grow on *n*-decane and also cannot reduce chlorate or perchlorate. Moreover the genomes of the three fully sequenced *P. stutzeri* strains, A1501, ATCC17588 and DSM4302 (Yu, Yuan et al. 2011) code for 4128, 4217 and 4302 proteins respectively, which in all cases is more than 1000 protein-coding genes less than the number of protein coding genes encoded by the genome of *P. chloritidismutans* AW-1^T. Pairwise average nucleotide identity (ANI) blastn values (Goris, Konstantinidis *et al.* 2007), showed a clear-cut difference between ANIb values of *P. chloritidismutans* AW-1^T and the three *P. stutzeri* strains (86%) with ANIb values of 97% within this clique. Average nucleotide identity values based on MUMmer (ANIm) were very similar to these ANIb values.

Analysis of the genes coding for chlorate reduction by* P. chloritidismutans *AW-1

P. chloritidismutans AW-1^T is a bacterium which is able to use chlorate as a source of oxygen and as electron acceptor. Chlorate is reduced to chlorite by chlorate reductase and in a separate reaction chlorite is converted to chloride by chlorite dismutase while producing molecular oxygen, both of these enzymes of strain AW-1^T have been characterized previously (Wolterink, Schiltz et al. 2003; Mehboob, Wolterink et al. 2009). Since the chlorate reductase encoding genes are relatively poorly characterized, the RAST service was not able to detect the genes encoding the four subunits of chlorate reductase directly. However sequences, which were misinterpreted by RAST to encode 'respiratory nitrate reductase alpha beta and gamma chain' were matching the N-terminal protein sequences of the alpha, beta and gamma subunits of chlorate reductase obtained from the previously purified chlorate reductase (Wolterink, Schiltz et al. 2003). The relative position of these N-terminal sequences, which were obtained from the mature alpha, beta and gamma subunits, suggested the presence of a signal peptide for secretion for the alpha and gamma subunit, but not for the beta subunit. This was confirmed by SignalP3 (Bendtsen, Nielsen et al. 2004) and TatP analyses (Bendtsen, Nielsen et al. 2005) (Fig 1). The alpha subunit preprotein has a conserved twin-arginine motif located between the N-region and the hydrophobic region of the signal sequence and thus is secreted by the TAT pathway. In contrast the Sec secretion pathway is involved in secretion of the the gamma subunit (Fig. 1). The beta subunit and delta subunit were predicted to have no signal peptide for secretion.

The arrangement of the *P. chloritidismutans* AW-1^T chlorate reductase encoding genes is the same as for *Ideonella dechloratans* and *Alicycliphilus denitrificans* chlorate reductase encoding genes, i.e. *clrABDC* (Thorell, Stenklo et al. 2003; Oosterkamp, Veuskens et al. 2011; Oosterkamp, Veuskens et al. 2013). A transposase was found 6-kb downstream of the chlorate reductase genes, which points towards a lateral transfer of the *clrABDC* gene cluster. Like what has been observed for most genomic islands, also the GC content (55% GC) of the 12-kb region from the *clrABDC* gene-cluster up until the transposase gene is markedly different from the average GC content of the *P. chloritidismutans* AW-1^T genome.

A Blastp analysis (Altschul, Madden et al. 1997) shows that the molybdopterin containing alpha subunit of chlorate reductase (ClrA) is 70% identical to the alpha subunit of dimethylsulfide dehydrogenase of *Citreicella* sp. SE45 and 44% identical with the ClrA of *I. dechloratans*. Similarly, the Fe-S containing 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 *I. dechloratans*. The gamma subunit of chlorate reductase (ClrC) has 54% identity with a hypothetical protein of *S. stellata* E-37 and only 34% identity

with the ClrC of *I. dechloratans*. The delta subunit of chlorate reductase (ClrD) has 46% identity with protein DdhD of *Citreicella* sp. SE45 and 40% with ClrD of *I. dechloratans*. In *I. dechloratans* the ClrD is not part of the mature enzyme, but is a cytoplasmic chaperone required for the assembly of the enzyme (Thorell, Stenklo et al. 2003; Bender, Shang et al. 2005).

```
>ClrA (PseChl_4948)
MGMWKLKRRDFLKGLSVTGAGVMLSGNVWGLNRLEPVGETLASEYPYRDWEDLYRNEWTW
                                NmLEPVGETLASEYPYxk

>ClrB (PseChl_4949)
MTVKRQLSMVLDLNKCIGCQTCTAACKLMWTNRNGREFMYWNNVETMPGKGYPRDYQNMG
 TVKxQLSMVLDLNKeIGgqTxTAA

>ClrC (PseChl_4951)
MKLGHFMKGSLTLALVLASAGALAERSEQNPNILEIKPGDTVKVSTIPDTIFLRSQNDPD
                        ExSEQNPNILEIKPGDTVKVxT
```

Fig. 1. Identification of chlorate reductase encoding genes in *P. chloritidismutans* AW-1^T. Alignment of the predicted ClrA, ClrB and ClrC protein sequences with N-terminal sequences of the alpha, beta and gamma subunits of the purified enzyme (accession numbers P83448-50). Underlined: Putative signal peptide predicted by TatP (ClrA) and SignalP3 (ClrC). The conserved twin-arginine motif (regular expression: RR.[FGAVML][LITMVF]) located between the N region and the hydrophobic region of the signal sequence is indicated in bold.

Shotgun proteomic analysis of P. chloritidismutans *AW-1^T under five different growth conditions*

Shotgun tandem mass spectrometry has proven to be an effective high-throughput approach for protein identification and quantification (Sadygov, Cociorva et al. 2004). For effective large-scale protein identification a complete proteome is a prerequisite. Most contig ends of the draft genome of *P. chloritidismutans* AW-1^T consist of sequence repeats hampering further assembly with the present data. However, with 25 to 30x sequence coverage of the present contigs it is likely that the vast majority of protein-encoding genes was identified.

To study the differential expression of proteins involved in chlorate and nitrate reduction and in *n*-decane utilization three anaerobic growth conditions were selected and two aerobic growth conditions were used as control. For each condition approx. 20,000 to 25,000 MS/MS spectra were obtained from trypsin-digested protein mixtures of cell free extracts. Overall, out of 118,362 spectra obtained 45,581 peptides were identified over the five conditions with a peptide spectrum match (PSM) efficiency of 38% and a false discovery rate (FDR) of <5% (Table 1). Expression data were obtained from 2133 proteins. Differential protein expression was measured with spectral counting where the number of spectra matched to peptides from a protein is used as a proxy for relative protein abundance (Liu, Sadygov et al. 2004). To study differential protein expression a subset of 1151 proteins was selected which, over the five growth conditions applied, have accumulated at least five PSM.

Table 1. Number of MS spectra obtained, peptide spectrum matches and proteins detected from trypsin digested extracts of *P. chloritidismutans* AW-1^T grown under five different conditions.

	Acetate + Oxygen	Acetate + Nitrate	Acetate + Chlorate	Decane + Oxygen	Decane + Chlorate	Total
Spectra	25214	19580	21988	25228	26352	118362
Number of PSM*	8737	7742	8627	9459	10953	45518
Number of proteins detected	1298	1071	1311	1273	1442	2133

*PSM: peptide spectrum match

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

Compared with growth of strain AW-1^T in acetate and oxygen the expression of 65 proteins was at least three-fold increased during growth in acetate and chlorate. Similarly the expression of 49 proteins was increased at least three-fold in strain AW-1^T cells grown with *n*-decane and chlorate in comparison with cells grown in *n*-decane in the presence of oxygen (Supplementary file 1). Expression of seven proteins was high in chlorate-grown cells irrespective of the carbon source. Three of these proteins are directly involved in chlorate reduction, being chlorite dismutase and the alpha and gamma chain of chlorate reductase. Enzyme activities of chlorate reductase, and chlorite dismutase were also elevated in chlorate-grown cells (Table 2). The other four proteins that were increased in expression in chlorate-grown cells were outer membrane protein OprC, involved in Fe/heme transport, cytochrome c oxidase *cbb3*-type diheme CcoP subunit, 4a-hydroxytetrahydrobiopterin dehydratase putatively involved in phenylalanine degradation and enoyl-CoA hydratase (Table 2).

During anaerobic growth with acetate and nitrate versus aerobic growth with acetate, 96 proteins are at least three-fold higher in expression (Supplementary file 1). When nitrate is added to anoxic cells of *P. stutzeri* nitrate is reduced to nitrite (Körner and Zumft 1989). In the subsequent denitrification pathway, NO is produced from nitrite by the enzyme Nir (nitrite reductase) which in *Pseudomonas* species is carried out by *cd*1 NiR with haem as cofactor. The membrane-bound enzyme NOR (nitric oxide reductase) is responsible for NO conversion into N_2O. Both enzyme systems are present in *P. chloritidismutans* AW-1^T and both enzyme systems are strongly induced in acetate- and nitrate-grown cells (Table 2).

In *P. stutzeri*, *P. aeruginosa* and *Pseudomonas fluorescens* the respiratory nitrate reductase (Nar) is used for nitrate reduction. Nar is encoded by the narGHJI operon and under the control of NarXL, a nitrate responsive two-component system. The genes for the nitrate sensor, *narX*, and the response regulator, *narL*, are located immediately upstream of the *nar* operon in *P. stutzeri* (Härtig, Schiek et al. 1999), *P. aeruginosa* (Schreiber, Krieger et al. 2007), and *P. fluorescens* (Philippot, Mirleau et al. 2001). The *narGHJI* operon and upstream *narXL* nitrate sensory system are absent in the draft genome of *P. chloritidismutans* AW-1^T, however the *napDABC* operon is present and at least the NapA catalytic subunit is shown to be expressed in nitrate-grown cells (Table 2). Since purified chlorate reductase from *P. chloritidismutans* AW-1^T cannot use nitrate as electron acceptor (Wolterink, Schiltz et al. 2003) this periplasmic Nap nitrate reductase is most probably responsible for anaerobic nitrate reduction in acetate- and nitrate-grown cells in *P. chloritidismutans* AW-1^T. Nitrate and nitrite reductase activities under these different growth conditions are also presented in Table 2.

Table 2. Number of PSM detected and enzyme activity of key enzymes in *P. chloritidismutans* AW-1^T grown under five different growth conditions.

	Protein (number)	Acetate + Oxygen	Acetate + Nitrate	Acetate + Chlorate	Decane + Oxygen	Decane + Chlorate
Number of PSM	Chlorate reductase - alpha subunit (PseChl_4948)	15	47	95	48	158
	Chlorate reductase - beta subunit (PseChl_4949)	10	13	31	18	50
	Chlorate reductase - gamma subunit (PseChl_4951)	4	0	17	2	12
	Chlorate reductase - delta subunit (PseChl_4950)	1	2	11	2	4
	Chlorate reductase	30	62	154	70	224
Activity (U/mg)	Chlorate reductase	1.7 ± 0.1	10.6 ± 0.9	21.3 ± 0.1	0.47 ± 0.04	46.3 ± 3.2
Number of PSM	Chlorite dismutase (PseChl_5209)	25	24	100	5	121
Activity (U/mg)	Chlorite dismutase (PseChl_5209)	0.15 ± 0.01	7.9 ± 1.5	6.8 ± 1.2	0.25 ± 0.0	7.4 ± 2.1
Number of PSM	Nitrate reductase - catalytic subunit (PseChl_3101)	2	36	17	20	29
Activity (U/mg)	Nitrate reductase	bdl	0.73 ± 0.10	bdl	bdl	bdl
Number of PSM	Cytochrome cd_1 nitrite reductase (PseChl_4607)	2	195	6	1	0
Activity (U/mg)	Nitrite reductase	bdl	0.23 ± 0.42	0.0021 ± 0.00046	n.d	n.d

PSM Peptide Spectrum Match; n.d: not determined; bdl: below detection limit

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

A total of 97 proteins showed at least three-fold more expression in *n*-decane- and chlorate- versus acetate- and chlorate-grown cells. Out of these proteins, at least 17 proteins were only detected in *n*-decane-grown cells (Supplementary file 1). When comparing oxygen-grown *n*-decane and oxygen-grown acetate cells, a total of 124 proteins were three-fold more expressed in *n*-decane, 11 proteins were exclusively detected in *n*-decane-grown cells.

Alkane hydroxylation is the key step in *n*-alkane degradation. In *P. chloritidismutans* AW-1^T alkane 1- monooxygenase is an integral-membrane protein that is exclusively detected in cell-free extracts of *n*-decane-grown cells. Like all other members of this superfamily, there are eight conserved histidines within three His boxes and a HYG motif that is highly conserved in AlkB hydroxylases. These histidine residues are reported to be catalytically essential and

proposed to be the ligands for the iron atoms (Fig. 2).Table 3 shows the number of PSMs found for alkane 1-monooxygenase and other proteins and enzymes possibly involved in transport and further oxidation of *n*-decane. The terminal oxidation of *n*-alkanes by alkane 1-monooxygenase generates primary fatty alcohols, which are further oxidized to aldehydes and fatty acid by alcohol and aldehyde dehydrogenases. Table 3 lists some alcohol and aldehyde dehydrogenases that are exclusively detected in *n*-decane-grown cells.

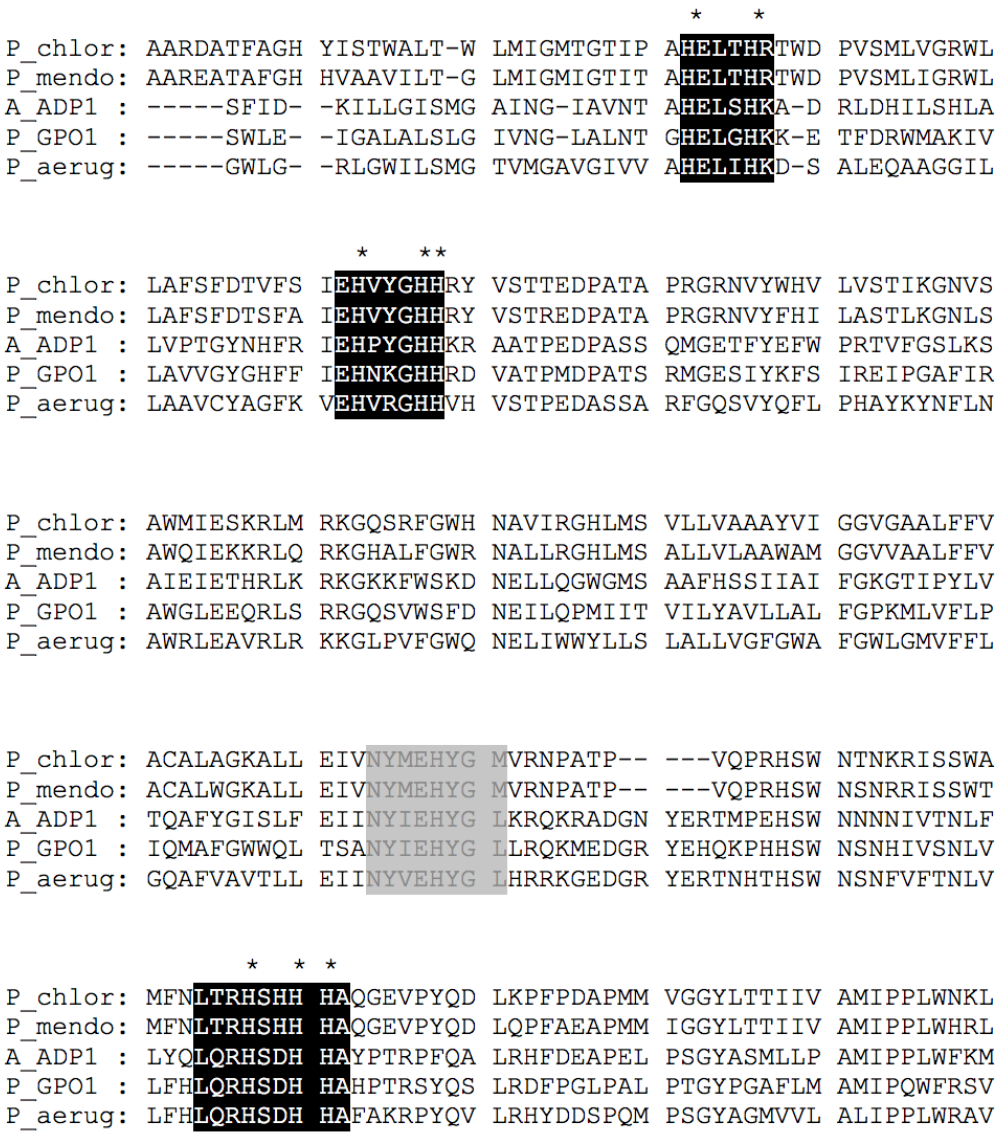


Fig. 2. Alignment of alkane-1-monooxygenases. AlkB hydroxylases have eight conserved and catalytically essential histidines (*) within three His boxes (Shaded). Abbreviations: P_chlor, *Pseudomonas chloritidismutans* alkane-1-monooxygenase; *P_mendo*, *P. mendocina* ymp (YP001188029); A_ADP1, *Acinetobacter* ADP-1 AlkM (AJ002316); P_GPO1, *P. putida* GPo1 Alk B (AJ245436); P_aerug, *P. aeruginosa* Alk B1 (NP251264).

Table 3. Differential expression of proteins involved in *n*-decane degradation.

	Peptide Spectrum Matches under the indicated growth conditions					
Protein	**Ace/Oxy**	**Ace/Nit**	**Ace/Chl**	**Dec/Oxy**	**Dec/Chl**	**Annotation**
PseChl_613	0	0	0	5	5	Short-chain dehydrogenase, Reductase
PseChl_629	0	0	0	1	5	Gamma-glutamyltranspeptidase (EC_2.3.2.2)
PseChl_775	0	0	0	2	5	Homogentisate 1,2-dioxygenase (EC 1.13.11.5)
PseChl_1132	0	0	0	28	15	Esterase_EstA
PseChl_3353	10	18	15	103	135	Enoyl-CoA_hydratase (EC_4.2.1.17), Fatty acid metabolism
PseChl_3354	4	5	6	30	25	3-ketoacyl-CoA thiolase (EC 2.3.1.16)
PseChl_3626	0	0	0	2	5	3-hydroxyacyl-CoA dehydrogenase
PseChl_3904	2	1	4	48	23	Long-chain fatty acid transport protein
PseChl_4162	2	0	3	57	1	Outer membrane porin oprD superfamily
PseChl_4421	0	0	0	7	5	Lipase precursor (EC_3.1.1.3)
PseChl_4798	0	0	0	26	27	Acyl-CoA dehydrogenase-like
PseChl_4799	0	0	0	12	24	3-ketoacyl-CoA thiolase (EC 2.3.1.16)
PseChl_4800	0	0	0	7	37	AMP-dependent synthetase and ligase
PseChl_4802	0	0	0	0	5	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100), Fatty Acid Biosynthesis FASII
PseChl_4803	0	0	0	9	19	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100), Fatty_Acid_Biosynthesis_FASII
PseChl_5007	0	0	0	9	12	Periplasmic aromatic aldehyde oxidoreductase, Molybdenum binding subunit YagR
PseChl_5170	0	0	0	13	13	Aldehyde dehydrogenase (EC 1.2.1.3)
PseChl_5172	0	0	0	2	6	Oxidoreductase, GMC family
PseChl_5262	0	0	0	13	12	Alkane-1 monooxygenase_(EC 1.14.15.3)
PseChl_5274	0	0	0	33	24	Choline dehydrogenase (EC 1.1.99.1)

Abbreviations: Ace/Oxy; acetate/oxygen, Ace/Nit; acetate/nitrate, Ace/Chl; acetate/chlorate, Dec/Oxy; *n*-decane/oxygen, Dec/Chl; n-decane/chlorate

In Gram-negative bacteria, the lipopolysaccharide layer in the outer membrane (OM) is an efficient barrier for hydrophobic molecules, such as *n*-alkanes, long-chain fatty acids and aromatic hydrocarbons destined for biodegradation (Nikaido 2003). Transport of these molecules across the OM requires an OM transport protein of the OMPP1/FadL/TodX family. The *P. chloritidismutans* AW-1^T homolog of this OM transport protein, PseChl_3904 is strongly induced in *n*-decane-grown cells.

Among the enzymes of strain AW-1^T likely involved in β-oxidation, one out of two acyl CoA dehydrogenases, AMP-dependent synthase and ligase, two out of three enoyl CoA hydratases and L-3 hydroxyacyl dehydrogenase were found exclusively in *n*-decane-grown cells. β-ketothiolase was also found to be more expressed in *n*-decane-grown cells.

Finally, based on proteome analysis, proteins PseChl_3353 to 3354 and PseChl_4798 to PseChl_4803 are putatively involved in fatty acid metabolism.

Discussion

The chlorate reduction pathway of strain AW-1^T

P. chloritidismutans AW-1^T is a Gram-negative, facultative anaerobic, chlorate-reducing bacterium originally isolated with acetate and chlorate (Wolterink, Jonker et al. 2002). Strain AW-1^T is a versatile organism that can also degrade C7 to C12 *n*-alkanes in the presence of oxygen or under anaerobic conditions with chlorate as electron acceptor (Mehboob, Junca et al. 2009). The ability to use chlorate as an electron acceptor is a characteristic that distinguishes *P. chloritidismutans* AW-1^T from other *Pseudomonas* species. Up to date 24 *Pseudomonas* genome sequences can be retrieved from the NCBI sequence repository (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/) and on average these genomes code for some 5300 proteins. The draft genome of *P. chloritidismutans* AW-1^T is no exception to that and harbors some 5500 protein encoding genes which is considerable more than the average of 4215 proteins encoded by the three sequenced genomes of the most closely related *P. stutzeri*. None of the other sequenced *Pseudomonas* genomes and plasmids encode the two key enzymes involved in chlorate reduction, chlorate reductase and chlorite dismutase, encoded by the *clrABDC* and *cld* genes, respectively. Thus this physiological trait differentiates *P. chloritidismutans* AW-1^T from other sequenced *Pseudomonas* species. An alignment of the protein sequences of the alpha, beta and gamma subunits of chlorate reductase of *P. chloritidismutans* with the N-terminal end of the alpha, beta and gamma subunits of the purified mature enzyme not only confirmed the identity of the Clr encoding genes, but also suggests that two secretion pathways are used in parallel to export the ClrABC enzyme to the periplasmic space. ClrB most likely first forms a complex with ClrA since there is no evidence for a signal-peptide for ClrB. Like in *I. dechloratans* the ClrD protein may function as a cytoplasmic chaperone required for formation of this complex (Thorell, Stenklo et al. 2003; Bender, Shang et al. 2005). Subsequently the Tat-pathway is used to export the ClrAB complex while the ClrC subunit uses the Sec pathway.

Table 2 shows that the enzymes directly involved in chlorate reduction i.e. chlorate reductase and chlorite dismutase are constitutive. An increased abundance of all the subunits of chlorate reductase and of chlorite dismutase, and a concomitant increase in activity of both enzymes was observed during growth with chlorate. These results are in agreement with previous findings. Although the chlorate reductase of *P. chloritidismutans* AW-1^T appears to be oxygen sensitive (Wolterink, Schiltz et al. 2003), the bacterium can simultaneously reduce chlorate and oxygen when oxygen is added to a chlorate-reducing culture (Wolterink, Jonker et al. 2002). Similarly, the chlorate reductase of *Pseudomonas* sp. PDA is reported to be constitutive (Steinberg, Trimble et al. 2005).

Some other proteins essential for an efficient chlorate reduction also have an increased abundance. Since chlorite dismutase is a heme-containing protein an increase in the formation of heme is expected. Evidence for that is an increased abundance of the oxygen-independent coproporphyrinogen III oxidase (PseChl_5183) involved in heme formation. We also found evidence for expression of PseChl_4604. Protein sequence PseChl_4604 is a mature soluble 8.6 kDa periplasmic cytochrome preceded by a predicted N-terminal signal peptide. This cytochrome shares the motif KLVGPxxKDVAAK with a soluble 6 kDa periplasmic *c* cytochrome involved in periplasmic chlorate reduction in *I. dechloratans* (Bäcklund, Bohlin et al. 2009).

Enzymes of strain AW-1^T involved in n-*alkane degradation*

P. chloritidismutans AW-1^T is known to degrade *n*-alkanes via an oxygenase mediated pathway (Mehboob, Junca et al. 2009). From Table 3 it is evident that an alkane 1-oxygenase (PseChl_5262) is exclusively present when the bacterium is grown with *n*-decane. The sequence of alkane 1-monooxygenase from strain AW-1^T is 76% identical with the putative alkane 1-monooxygenase of *P. mendocina* ymp and 39% identical with the alkane 1-monoxygenase of *P. aeruginosa* PAO1 (Fig. 2).

Rubredoxin (Rd) is a redox-active protein. One of its functions is storing and transferring electrons to alkane 1-monooxygenase from Rd reductase. In *P. chloritidismutans* AW-1^T Rd and Rd reductase are fused into a single open reading frame (PseChl_651). We were unable to find an alcohol oxygenase in the proteome as we postulated before (Mehboob, Junca et al. 2009). Instead, we found a number of different alcohol dehydrogenases that are mainly present during growth on *n*-decane.

Apart from these proteins, which are directly involved in alkane degradation some other proteins putatively involved in alkane degradation are also abundant. PseChl_4162 is a porin of the oprD superfamily and strongly induced upon growth with *n*-decane but under aerobic conditions only. PseChl_3904 appears to be a long chain fatty acid outer membrane transporter and is strongly induced in *n*-decane-grown cells both under aerobic and chlorate-reducing conditions.

Also taking into account localization of chlorate reductase, differential expression of subunits of chlorate reductase, chlorite dismutase, ubiquinol-cytochrome-*c*-reductase, cytochrome *cbb*$_3$ oxidase Rd and presence and expression of an 8.6 kDa cytochrome in genome, we propose that *n*-decane degradation with chlorate as electron acceptor proceeds in *P. chloritidismutans* AW-1^T as presented in Fig. 3.

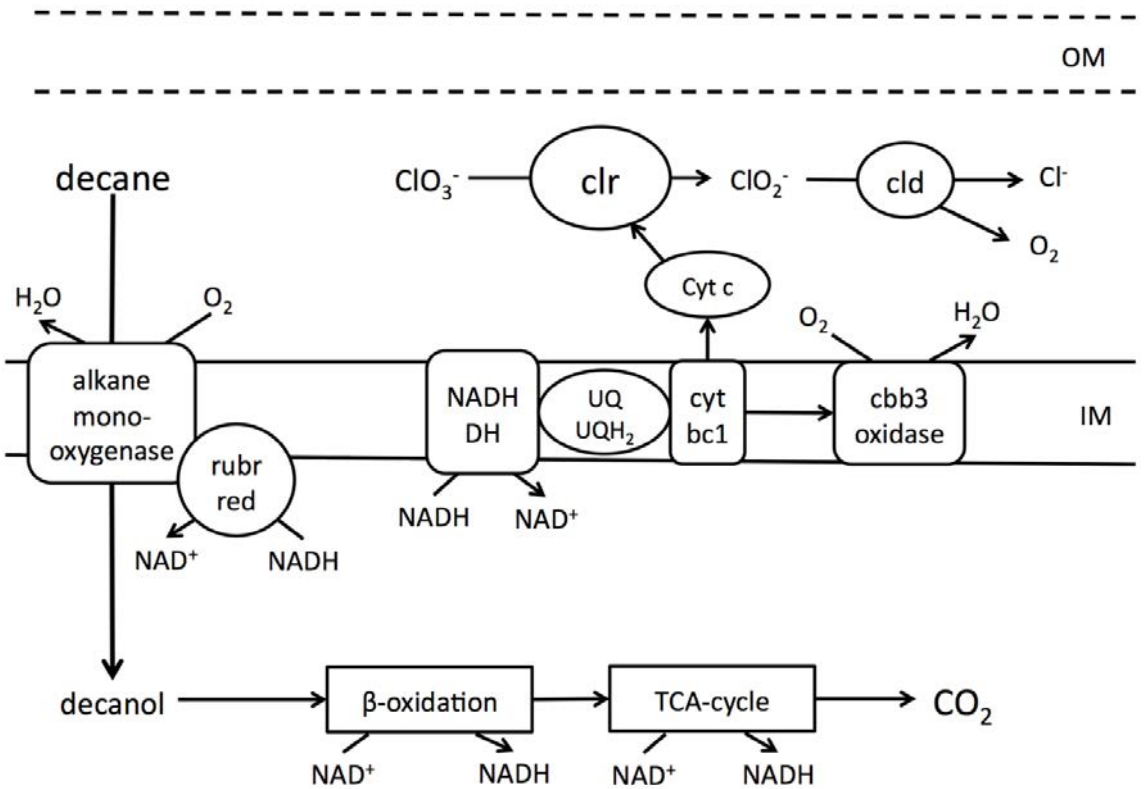


Fig. 3. Proposed model of anaerobic *n*-decane degradation. After reduction of chlorate by chlorate reductase the resulting chlorite is decomposed into chloride ions and molecular oxygen. Under anaerobic conditions the produced oxygen can be used by alkane-1-monooxygenase to oxidize decane to decanol. Abbreviations: OM, outer membrane; IM, Inner membrane; clr, chlorate reductase; cld, chlorite dismutase; Cyt C, 8.6 kDa periplasmic cytochrome; cbb$_3$ oxidase; cbb$_3$-type cytochrome c oxidase; UQ/UQH$_2$ ubiquinone/ubihydroxyquinone; rubr/red rubredoxin and rubredoxin reductase, which in *P. chloritidismutans* AW-1^T are fused into a single protein.

Nitrate reductase of strain AW-1^T

P. chloritidismutans AW-1^T is most closely related to *P. stutzeri.* Up to date three *P. stutzeri* genomes have been sequenced and all encode the well-studied Nar operon involved in anaerobic nitrogen reduction. *P. chloritidismutans* AW-1^T,which has previously been described as a non-denitrifying species, lacks this operon completely. Nevertheless, we and others (Cladera, García-Valdés et al. 2006) have shown that the isolation of variants that are able to grow anaerobically in the presence of nitrate are readily obtained, suggesting that the Nap periplasmic nitrate reductase can be used as a substitute under anaerobic conditions. Notably, the Nar nitrate reductase can also reduce chlorate (Bell, Richardson et al. 1990). Due to the toxic effects of chlorite accumulation in the cytoplasm, a bacterial capability to generate molecular oxygen from chlorate may be not compatible with the presence of a cytoplasm-oriented, chlorate-reducing Nar nitrate reductase. The Nap nitrate reductase, in contrast, is not cytoplasm-oriented and does not reduce chlorate.

Concluding remark

This is the first report about the proteogenomics of a bacterium 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. This study confirms previous findings showing that an oxygenase-mediated pathway is employed by *P. chloritidismutans* during growth on *n*-alkanes and chlorate. It further shows that there are two separate pathways for growth on chlorate and nitrate and demonstrated that chlorate reductase and chlorite dismutase activity is increased when grown with chlorate and decreased when grown with oxygen.

Acknowledgements

We thank the Technology Foundation STW, Applied Science Division of the Netherlands Science Foundation NWO, Chemical Sciences (CW) of NWO, the technology program Economy Ecology Technology and the Higher Education Commission of Pakistan (HEC), the Teaching Grant of Wageningen University for financial support of our research and the Spinoza Prize Award for providing funds for genome sequencing. We also thank Dr. Willem van Berkel for useful suggestions about proteomics.

Chapter 9

General discussion

Author and affiliations
Margreet J. Oosterkamp[1(a)]
1 Wageningen University, Agrotechnology and food sciences group, Laboratory of microbiology, Dreijenplein 10, 6703HB, Wageningen, The Netherlands
(a) Current address: Laboratory of Animal Sciences, Energy Biosciences Institute, Institute of Genomic Biology, 1207 W Gregory Dr, Urbana, IL 61801, USA

Keywords
Chlorate, nitrate, anaerobic degradation, aromatic compound, aliphatic compound, *Alicycliphilus denitrificans*, *Pseudomonas stutzeri*, *Georgfuchsia toluolica*, *Pseudomonas chloritidismutans*, biochemistry, physiology, genome, proteome

Manuscript status
Thesis chapter

Abstract

The environmental conditions and the microorganisms present are central factors that determine the intrinsic remediation potential of contaminated soil sites. For *in situ* bioremediation, appropriate conditions for the microorganisms often need to be created. In this research project four bacterial strains that degrade hydrocarbons with nitrate and/or chlorate as electron acceptor were studied. *Alicycliphilus denitrificans* strain BC was studied with the focus on biochemical pathways involved in nitrate and chlorate reduction and the benzene, toluene and acetone degradation pathway (chapters 2 to 5). Studies using the benzene-degrading *Pseudomonas stutzeri* strain BN gave insight in aerobic and anaerobic pathways for aromatic compound degradation (chapter 6). The properties of *Georgfuchsia toluolica* strain G5G6^T that degrades toluene with nitrate were studied and insight in the prevalence of the strain at the polluted site that is its natural habitat, was obtained (chapter 7). Finally, *Pseudomonas chloritidismutans* strain AW-1^T was studied focusing on *n*-decane degradation with chlorate as electron acceptor (chapter 8). Field studies at contaminated soil sites are necessary to show the application and bioremediation potential of these strains more clearly. Further research on chlorate and nitrate reduction will increase the understanding of factors involved in regulation of these respiratory pathways in bacteria that degrade hydrocarbons.

Introduction

Naturally occurring or introduced microorganisms can be used to clean-up anaerobic polluted soil sites. Reduction of chlorate, perchlorate and nitrate can be coupled to degradation of recalcitrant organic pollutants, such as benzene, toluene, ethylbenzene, xylene isomers (BTEX) and *n*-alkanes. Aerobic degradation of these compounds is mediated by oxygenases. These enzymes incorporate oxygen atoms into their substrate, which activates degradation of the compound (Butler and Mason 1996; Eltis and Bolin 1996; Gibson and Parales 2000; Leahy, Batchelor *et al.* 2003; Vaillancourt, Bolin *et al.* 2006). Anaerobic degradation of aromatic compounds is a much slower process that can be initiated by hydroxylation, methylation or carboxylation of the compounds, for example (Vogt, Kleinsteuber *et al.* 2011). Bacteria that couple chlorate or perchlorate reduction to pollutant degradation are less well studied than nitrate-reducing pollutant-degrading bacteria.

Recent advances in genomics and proteomics have opened more possibilities to investigate pollutant-degrading microorganisms (Zhao and Poh 2008). Four nitrate- and/or chlorate-reducing microorganisms that are employed for bioremediation of polluted anaerobic soil sites were studied using different techniques (Figure 1).

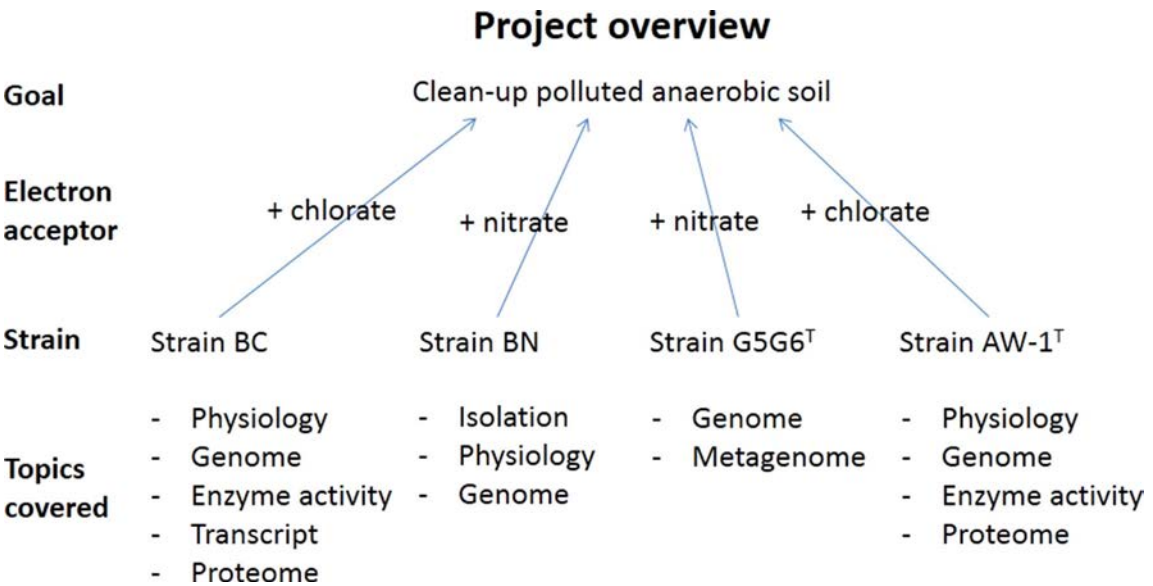


Figure 1. Overview of the project results described in this thesis. *A. denitrificans* strain BC (Strain BC), *P. stutzeri* strain BN (Strain BN), *G. toluolica* strain G5G6^T (Strain G5G6^T) and *P. chloritidismutans* strain AW-1^T (Strain AW-1^T) were studied as shown (Topics covered). These strains can be used for the clean-up of polluted anaerobic soil sites using the electron acceptor that is indicated.

Bioremediation with chlorate, nitrate and oxygen as electron acceptor

Naturally, organisms that can deal with or degrade pollutants are selected by the environment. Therefore, human intervention is not always needed and can be limited to monitoring and controlling the polluted site. If the remediation capacity of the intrinsic soil community is poor and the pollutant is harmful, human intervention is necessary. There are various techniques that can be used to clean-up soil. Soil is excavated and treated elsewhere

(*ex situ* methods) or treated on site (*in situ* methods). In *in situ* bioremediation there is no standard and reliable method, but a variety of methods can be applied. Often a combination of methods is used to treat contaminated soil sites (Cao, Nagarajan *et al.* 2009). Microorganisms capable of degrading soil pollutants may be stimulated or introduced in the soil to increase the bioremediation capacity of the soil. Appropriate conditions for these microorganisms to degrade pollutants often need to be created. For example, by introducing compounds necessary for survival and functioning of the microorganisms. Soil pollutants can be used as electron donor, electron acceptor or both. Electron acceptors are for example, oxygen, nitrate or chlorate. Electrons can be derived from electron donors such as aromatic hydrocarbons or other organic compounds.

Aerobic microorganisms use oxygen as electron acceptor. Pseudomonadaceae, Mycobacteriaceae, Coccaceae, Spirillaceae, Bacteriaceae and Bacillaceae family members for example are known to degrade aromatic compounds aerobically (Evans 1963). These microorganisms are in general inhabitants of aerobic soil and provide their bioremediation capacity directly to the soil. In soil sites that are more closed systems, due to high water saturation that limits air flow, these and other aerobic microorganisms use all oxygen available, creating anaerobic conditions. In anaerobic conditions soil pollutants often persist, because anaerobic biodegradation is generally slower.

Anaerobic soils can be aerated using compressed air to introduce oxygen. However, usually in anaerobic soil sites water saturation is high and solubility of oxygen in water is low (solubility 3.5 x 10^{-4} g per 100 g water of oxygen from air at 1 atm at 10°C). Therefore aeration of anaerobic soils is difficult to accomplish. Chlorate (solubility 8.2 g per 100 g water at 1 atm at 10°C) and nitrate (solubility 9.5 g per 100 g water of at 1 atm at 10°C) can be easily solubilized in water as sodium salts and therefore are easier to introduce in anaerobic soil sites. Skepticism towards the use of chlorate for bioremediation rose because of the toxic chlorite that is produced as an intermediate compound. However, the more accepted alternative nitrate also is converted to a toxic intermediate, nitrite. The reason that nitrate may be more accepted is because denitrification, including the detoxification of nitrite, is a more general characteristic performed by a broad spectrum of microorganisms indigenous to soil. Given the fact that in conjunction with chlorate (a variety of) chlorate-reducing microorganisms that convert the toxic intermediate chlorite can be introduced and that chlorate- and perchlorate-reducing microorganisms are also present in soil (Nozawa-Inoue, Scow *et al.* 2005), chlorate should be more generally accepted as electron acceptor in bioremediation of anaerobic polluted soil sites.

Bacterial chlorate and nitrate reduction pathways

Most isolated bacteria that reduce chlorate are Proteobacteria of the *Dechloromonas* and *Azospira* genera while nitrate reduction cannot be described as a characteristic of a particular taxon (chapter 1). The enzymes chlorate reductase and chlorite dismutase are involved in the two step conversion of chlorate to chlorite and of chlorite to chloride and dioxygen, respectively (Nilsson, Rova *et al.* 2012). In the chlorate-reducing *Ideonella dechloratans* the electron transfer pathway consists of two steps (Bäcklund, Bohlin *et al.* 2009) (Figure 2). In the reduction of chlorate to chlorite two electrons are consumed, these electrons are derived from a cytochrome bc_1 complex and electron transfer is mediated by a soluble cytochrome *c*. The cytochrome bc_1 complex is re-oxidized by ubiquinone which, in turn, is re-oxidised by an ubihydroquinone dehydrogenase. The soluble cytochrome *c* is also involved in electron transfer to a cytochrome cbb_3 oxidase complex and the involvement of a larger cytochrome *c* complex in this electron transfer was also proposed. The cytochrome cbb_3 oxidase functions in the reduction of oxygen to water. This oxygen is produced in the conversion of chlorite to chloride and oxygen (Bäcklund, Bohlin *et al.* 2009).

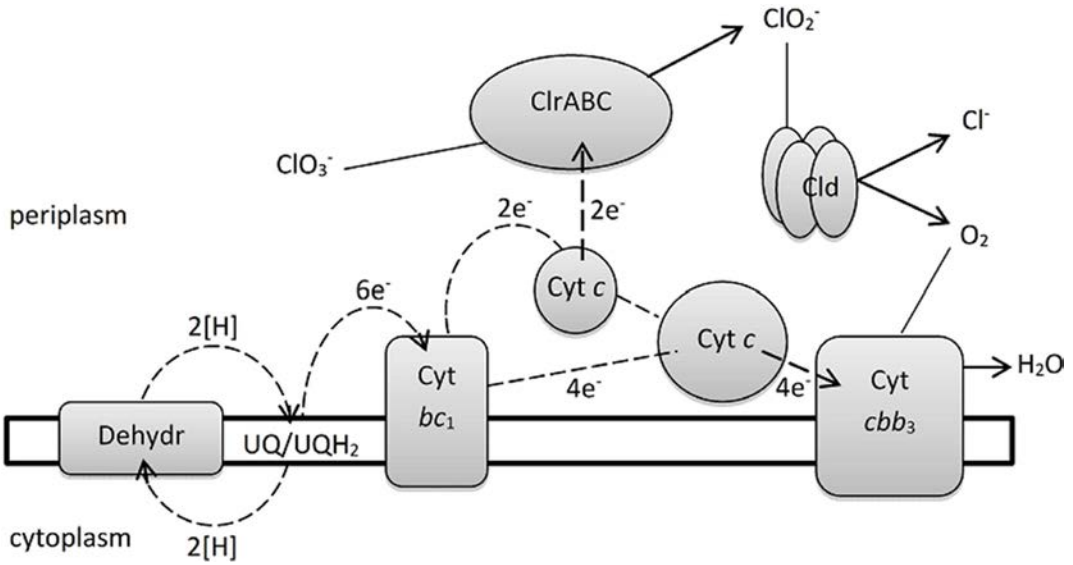


Figure 2. Proposed chlorate reduction and electron transfer pathway in *I. dechloratans* (Bäcklund, Bohlin *et al.* 2009). Chlorate (ClO_3^-) is reduced to chlorite (ClO_2^-), this conversion is mediated by chlorate reductase (clr). Chlorite dismutase (cld) is involved in conversion of chlorite (ClO_2^-) to chloride (Cl^-) and oxygen (O_2). Oxygen can be converted to water by a terminal cytochrome oxidase (cyt cbb_3). Electrons (e^-) are derived from an electron donor and transferred via an ubiquinone/ubihydroquinone complex (UQ/UQH_2) that is oxidized by an ubihydroquinone dehydratase (Dehydr) that delivers protons ([H]). A cytochrome bc_1 complex and soluble cytochrome *c* proteins are involved in further transfer of the electrons.

The oxygen produced by chlorate reduction, may be used as electron acceptor or to activate degradation of recalcitrant compounds by using oxygenases. A similar mechanism might be employed in atypical nitrate reduction. Nitrate reductase converts nitrate to nitrite and nitrite reductase mediates NO production from nitrite. A putative NO dismutase might convert NO to dinitrogen and dioxygen gas. Although an NO dismutase has not been isolated and characterized yet, there is evidence that oxygen can be produced from NO (Ettwig, Butler *et al.* 2010; Wu, Ettwig *et al.* 2011; Zedelius, Rabus *et al.* 2011; Ettwig, Speth *et al.* 2012). This is not a general mechanism for denitrifying microorganisms and in general anaerobic degradation pathways for recalcitrant compounds are used by these microorganisms (Vogt, Kleinsteuber *et al.* 2011). Bacterial reduction of nitrate may proceed via a periplasmic nitrate reductase, Nap, that takes up electrons from membrane-associated subunits of the enzyme (Figure 3) or via a membrane-associated nitrate reductase, Nar. There are two Nar enzyme systems known (Figure 4). Nar can reduce nitrate in the cytoplasm as well as in the periplasm, depending on the system. Nap poorly uses chlorate as substrate (Bell, Richardson *et al.* 1990), where Nar also can reduce chlorate. In (per)chlorate-reducing microorganisms this might aid chlorate reduction, while in other microorganisms toxic levels of chlorite are produced.

Respiratory nitrate reduction leads to the production of dinitrogen gas, where assimilatory nitrate reduction results in ammonia production. This is a less common pathway of nitrate reduction compared to denitrification to dinitrogen gas. Microorganisms known to produce ammonia are strains of the *Alcaligenes, Pseudomonas, Bradyrhizobium* and *Blastobacter* families (Liu, Tiquia *et al.* 2003). Ammonia can also be produced from dinitrogen gas fixation. Microorganisms of the *Rhizobia* and *Frankia* families are among the known nitrogen fixing organisms (Benson, Brooks *et al.* 2011; Terpolilli, Hood *et al.* 2012). Finally, ammonia-oxidizing bacteria and archaea can form nitrite and nitrate from ammonia in the nitrification process (Junier, Molina *et al.* 2010).

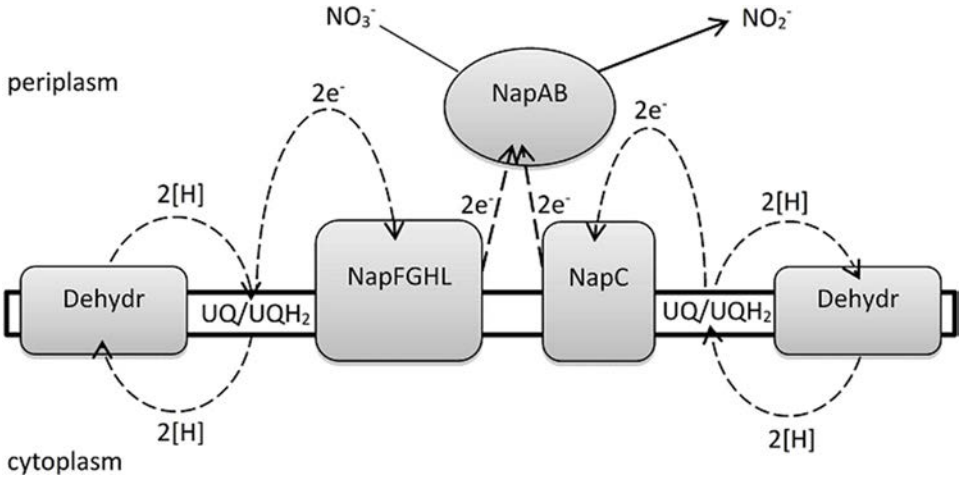


Figure 3. Nitrate reduction by the periplasmic nitrate reductase Nap. Figure adapted from (Kraft, Strous *et al.* 2011). Nitrate (NO_3^-) is reduced to nitrite (NO_2^-), this conversion is mediated by the periplasmic complex (NapAB). Electrons (e^-) are derived from an electron donor and transferred via an ubiquinone/ubihydroquinone complex (UQ/UQH_2) that is oxidized by an ubihydroquinone dehydratase (Dehydr) that delivers protons ([H]). Further transfer of electrons can go via membrane-associated NapFGHL or NapC subunits, depending on the enzyme system.

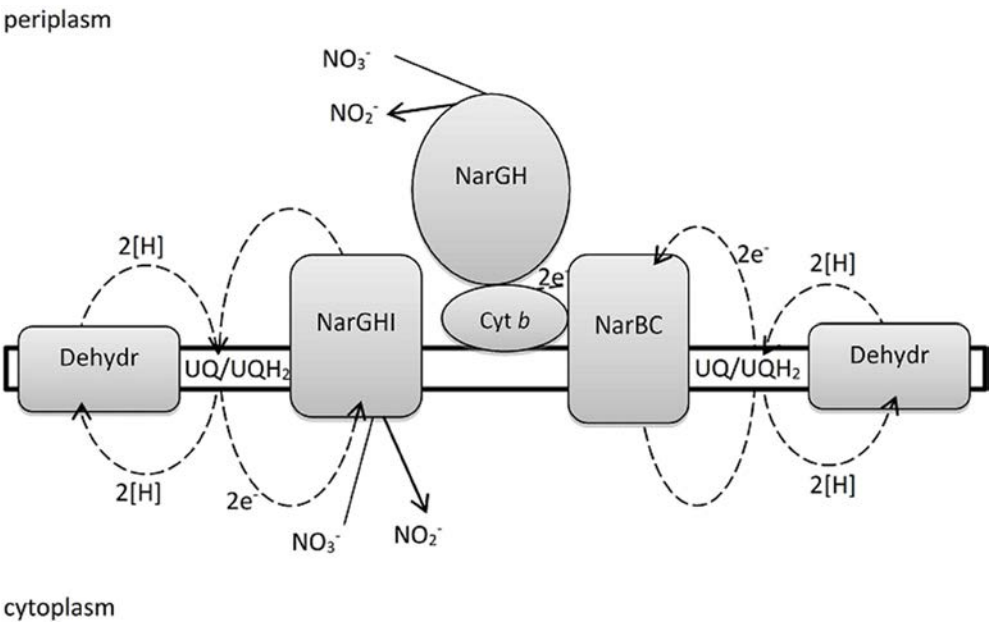


Figure 4. Nitrate reduction by the respiratory nitrate reductase Nar. Figure adapted from (Kraft, Strous *et al.* 2011) and right Nar system from (Martinez-Espinosa, Dridge *et al.* 2007). Nitrate (NO_3^-) is reduced to nitrite (NO_2^-), this conversion is mediated either by the membrane-associated NarGHI, that reduces nitrate on the cytoplasmatic side, or by the periplasmic NarGH. Periplasmic NarGH receives electrons (e^-) from the membrane-associated NarBC complex, which is mediated by a cytochrome b (Cyt *b*). Electrons are derived from an electron donor and transferred via an ubiquinone/ubihydroquinone complex (UQ/UQH_2) that is oxidized by an ubihydroquinone dehydratase (Dehydr) that delivers protons ([H]).

Genomes of *Alicycliphilus denitrificans* strains BC and K601^T

The nitrate-reducing *A. denitrificans* strain BC is highly related to the type strain *A. denitrificans* strain K601^T, but there are marked physiological differences. While strain BC is also capable of chlorate reduction, strain K601^T cannot reduce chlorate. On the other hand, strain K601^T can degrade cyclohexanol with nitrate or oxygen as electron acceptor and strain BC cannot degrade cyclohexanol (Mechichi, Stackebrandt *et al.* 2003; Weelink, Tan *et al.* 2008). The whole-genome sequences of strains BC and K601^T were studied (chapter 2). The strains have identical 16S rDNA gene sequences and the G+C content of the genomes is also similar, 67.8 and 67.9 mol% for strains K601^T and BC, respectively. Experimental and *in silico* DNA-DNA hybridization showed that the strains belong to the same species. Strains BC and K601^T are highly related to *Acidovorax avenae* strain C1 (99.9% 16S rRNA similarity). More detailed genome analysis confirmed that strains BC is more closely related to *Alicycliphilus* than to *Acidovorax*. Bidirectional Blast analysis of the proteomes showed that 857 proteins encoded by the genome of strain K601^T are not present in strain BC. Similarly the genome of strain BC encodes 721 proteins that are not present in strain K601^T. Chlorate reductase and chlorite dismutase genes are located on a plasmid of strain BC. Furthermore, Nar-type nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase genes involved in respiratory nitrate reduction are present on the chromosomes of strains BC and K601^T. Enzymes involved in cyclohexanol degradation are only encoded by the chromosome of strain K601^T. Overall, the genome sequences of *A. denitrificans* strains BC and K601^T give insight in the properties of the strains and, more specifically, in chlorate reduction by strain BC, cyclohexanol degradation by strain K601^T and nitrate reduction and aerobic aromatic compound degradation by strains BC and K601^T.

Chlorate and nitrate reduction by *Alicycliphilus denitrificans* strain BC

Detailed physiological experiments showed that *A. denitrificans* strain BC cells adapted to chlorate cannot easily grow with nitrate as electron acceptor and vice versa (chapter 3). Furthermore, aerobically grown cells are difficult to adapt to growth with nitrate or chlorate as electron acceptor, whereas cells adapted to growth with nitrate or chlorate grow aerobically easily. Proteins involved in nitrate reduction were more abundant in nitrate-adapted cells. Although specific activity of chlorate reductase was higher in cells adapted to nitrate, chlorate reductase and chlorite dismutase activities were higher in chlorate-adapted cells. An insert encoding an integrase and an IS3/IS911-family transposase is present in *narG* of chlorate-adapted cells (chapter 4). Chlorate-adapted cells with putative deletions in *narG* were also found and the transposon was not observed in nitrate-adapted cells. We proposed that presence of the insert in *narG* is depending on chlorate and that it can cause a deletion in that gene. Further research needs to reveal how the insert is activated to integrate in *narG* depending on the presence of chlorate.

Acetone biodegradation by *Alicycliphilus denitrificans* strain BC

Strain BC grows with acetone and nitrate, but not with oxygen or chlorate as electron acceptor. Acetone carboxylase of *A. denitrificans* strain K601^T was described previously (Dullius, Chen *et al.* 2011). The enzyme is found in many Proteobacteria and especially in Beta-proteobacteria, to which *A. denitrificans* also belongs, but the complete pathway is not well understood. In strain BC, an acetone carboxylase that is homologous to the enzyme from strain K601^T, an AMP-dependent synthase and ligase and an acetyl-CoA acetyltransferase are involved in nitrate-dependent acetone degradation (chapter 5). Accordingly, acetone is converted to acetoacetate, acetoacetate to acetoacetyl-CoA and acetoacetyl-CoA to acetyl-CoA. With the available genome sequence the acetone metabolism of *A. denitrificans* can be studied further into depth.

Benzene degradation coupled to denitrification in *Pseudomonas stutzeri* strain BN

P. stutzeri strain BN was isolated from a chemostat that was degrading benzene coupled to nitrate reduction (chapter 6). This strain degrades aromatic compounds with nitrate and oxygen as electron acceptor. Aerobic degradation of aromatic compound by strain BN is mediated by oxygenases, which incorporate molecular oxygen in the aromatic ring. Aerobic aromatic hydrocarbon degradation is better understood than anaerobic degradation (Weelink, van Eekert *et al.* 2010; Vogt, Kleinsteuber *et al.* 2011). Carboxylation or hydroxylation are possible mechanisms for activation of anaerobic aromatic acid degradation in strain BN. A putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase may be involved in carboxylation, since in an iron-reducing enrichment culture, subunits of benzene carboxylase were related to this type of enzyme (Abu Laban, Selesi *et al.* 2010). A putative benzyl-CoA ligase that converts benzoates to benzoyl-CoA is also present in the genome of strain BN. Hydroxylation would require the use of oxygen atoms from water, a reaction that is not thermodynamically favorable. Genes encoding phenol hydroxylase, a carboxy-lyase, thioesterase and reductase can be involved in formation of benzoyl-CoA if aromatic hydrocarbons are degraded using hydroxylation. Proteomic approaches will give more information about the anaerobic aromatic hydrocarbon degradation route in strain BN.

Genome of *Georgfuchsia toluolica* strain G5G6^T and comparison to Banisveld metagenome

G. toluolica strain G5G6^T degrades toluene coupled to nitrate, iron(III) and manganese(IV) and this strain was isolated from an anaerobic aquifer polluted with monoaromatic compounds (Weelink, Van Doesburg *et al.* 2009). The genome sequence of strain G5G6^T gave more information about its metabolic potential (chapter 7). Next to a high affinity cbb_3-type terminal oxidase, an aerobic-type oxidase is encoded in the genome. Furthermore, oxygenases involved in aerobic degradation of aromatic compounds are present in the genome. Remarkably, the strain has not been observed to grow aerobically or microaerobically. The genome sequence of the strain also encodes pathways for anaerobic degradation of aromatic compounds such as toluene, ethylbenzene, benzaldehyde and benzoate. The *bss* and *bbs* clusters of genes involved in anaerobic toluene degradation are present. Furthermore, typical nitrite reductase genes are not present. Likely, the genome of strain G5G6^T encodes an atypical denitrification pathway, such as in the iron(III)-reducing *Geobacter metallireducens*. In this microorganism a novel enzyme that reduces both nitrate and nitrite was described (Martínez Murillo, Gugliuzza *et al.* 1999). Comparison of the genome of strain G5G6^T to metagenome data of the Banisveld aquifer from which the strain was isolated, indicated that the strain is an important member of the intrinsic community involved in the clean-up of the site. Other studies also indicated that the strain is an important bacterium in the aquifer (Staats, Braster *et al.* 2011).

***n*-Decane degradation of *Pseudomonas chloritidismutans* strain AW-1^T**

Strain AW-1^T degrades a large number of aromatic compounds, including catechol, benzoate and *n*-decane with oxygen and chlorate as electron acceptor (Mehboob, Junca *et al.* 2009). More detailed proteomic experiments showed that in aerobic conditions an alkane-1-monooxygenase was involved in the initial activation of *n*-decane followed by further degradation in a general *beta*-oxidation pathway of fatty acids (chapter 8). Since these enzymes are also abundant with chlorate as electron acceptor, oxygen produced from chlorate reduction is likely introduced in *n*-decane. Chlorate and nitrate reduction are separate pathways in this strain. The respiratory periplasmic nitrate reductase Nap of strain AW-1^T, unlike membrane-associated Nar nitrate reductases, does not convert chlorate to chlorite. This indicates that nitrate reductase does not interfere with chlorate reduction in this strain. Therefore, it is an interesting target for further studies on the degradation of recalcitrant compounds using chlorate as electron acceptor.

Future perspectives

Implications for application of chlorate and nitrate as electron acceptor

The chlorate- and nitrate-reducing *A. denitrificans* strain BC and *P. chloritidismutans* strain AW-1^T degrade different pollutants with chlorate than with nitrate as electron acceptor and are therefore useful for bioremediation with chlorate as electron acceptor. The nitrate-reducing *P. stutzeri* strain BN and *G. toluolica* strain G5G6^T can degrade pollutants with nitrate, but not with chlorate. For application of such microorganisms for bioremediation of polluted anaerobic soils, there is no easy method to select appropriate strains. Further research should therefore be aimed at identification and isolation of appropriate strains and also at the development of novel methods to screen biodegradation capacity of intrinsic and introduced microorganisms. One of the options for such a screening method could be the development of a box that can be introduced below ground and that captures the anaerobic soil in multiple compartments. In these compartments microenvironments can be created by adding strips on the walls of the compartments that introduce a suitable electron acceptor, if not already present, and different microbiological strains (including blank compartments). Degradation of pollutants in these microenvironments can be compared to the blanks, which will reveal the bioremediation capacity of the microorganisms used. Practical, low-cost and easy selection methods for appropriate strains will enhance application of such strains for bioremediation of polluted anaerobic soil sites.

Electron acceptor metabolism of chlorate- and nitrate-reducing microorganisms

P. chloritidismutans strain AW-1^T and *A. denitrificans* strain BC contain chlorate reductase and chlorite dismutase, the enzymes involved in chlorate reduction. Similarity of chlorite dismutase (63%) and the alpha (45%), beta (58%), gamma (36%) and delta (40%) subunits of chlorate reductase of strains AW-1^T and BC is low. However, the function of the enzymes is similar in the strains (Figure 2). For adaptation of the strains to chlorate reduction, it is necessary to take nitrate reductase into account. In strain AW-1^T the periplasmic Nap enzyme is present (Figure 3). This nitrate reductase poorly reduces chlorate and, although it is unlikely to occur, if it produces chlorite, the toxic compound is produced in the periplasmic space, where the chlorite dismutase of strain AW-1^T is located and can detoxify chlorite. Strain BC, on the other hand, contains the membrane-associated NarGHI complex, which is causing a metabolic challenge if chlorate is present. This nitrate reductase can also reduce chlorate and produces chlorite at the cytoplasmic side of the cell membrane (Figure 4). Since there is no possibility for the periplasmic chlorite dismutase to reach the chlorite, this compound cannot be detoxified if NarGHI reduces chlorate in strain BC. Based on the experiments described in this thesis, there are three nitrate reductase genotypes. In cells adapted to nitrate and oxygen the intact 3795 bp *narG* is present. Chlorate-reducing cells can contain *narG* as intact gene, with a 1324 bp insert or with a deletion (Chapter 4). Since no *narG* transcript with insert or deletion was found (Chapter 3), the insert and deletion likely inactivate the gene. When nitrate-adapted cells are transferred to chlorate for the first time, the culture likely becomes heterogeneous, because the presence of chlorate directs integration of a genetic element in *narG* of part of the total amount of strain BC cells. After several transfers, the cells are fully adapted to chlorate and *narG* is inactivated in all cells. Theoretically, a minor amount of nitrate-adapted cells may contain the gene with insert or deleted gene and transfer of these cells to chlorate may have stimulated selection of these cells specifically, because a non-functional Nar is advantageous if chlorate is present. This possibility cannot be excluded, however, so far these *narG* genotypes were not found in nitrate-adapted cells and more evidence for the existence of these genotypes in nitrate-adapted cells could not be obtained in this study. Therefore, our hypothesis is that chlorate triggers integration of a genetic element in *narG*. Furthermore, deletions of this gene might be caused when the insert jumps out of the gene. Further studies to test this hypothesis are needed. These studies would require a set-up with the use of advanced genetic techniques such as fluorescent or radio-active nucleotide labeling (van der Oost, personal communication).

Acknowledgements

This research was supported by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053 and under the auspices of the graduate school WIMEK (Wageningen Institute for Environment and Climate Research, which is part of SENSE Research School for Socio-Economic and Natural Sciences of the Environment, www.wimek-new.wur.nl and www.sense.nl. I thank Prof. J. van der Oost for his contribution regarding the insert of *narG* that was found in *A. denitrificans* strain BC.

Thesis summary

Introduction

Denitrification pathways have been more extensively studied than perchlorate and chlorate reduction pathways. Denitrification is used as a mechanism for anaerobic bioremediation of petroleum hydrocarbons. Bioremediation with perchlorate and chlorate reduction however, is not commonly applied yet and it seems controversial as perchlorate and chlorate are considered as soil pollutants as well. Oxygen is an intermediate of perchlorate and chlorate reduction and this may enhance bioremediation, as aerobic degradation of petroleum hydrocarbons is easier to accomplish. In a similar fashion oxygen can be formed in a recently discovered atypical nitrate reduction pathway. Perchlorate, chlorate and nitrate are more soluble than oxygen and easy to introduce in anaerobic soil sites via the groundwater. Perchlorate and chlorate can be harmful for living organisms, but perchlorate- and chlorate-reducing microorganisms are abundantly present in soil to convert these compounds to harmless chloride. Most of the known perchlorate- and chlorate-reducing microorganisms are Proteobacteria, while nitrate reduction is present in a wide range of microorganisms and is not a characteristic of a particular clade. The enzymes involved in perchlorate and chlorate reduction are perchlorate reductase, chlorate reductase and chlorite dismutase. Both perchlorate- and chlorate-reducing bacteria possess chlorite dismutase. This enzyme is essential for these bacteria, since it detoxifies chlorite. Bacteria that reduce perchlorate also reduce chlorate. Reduction of both compounds may be mediated by one enzyme, perchlorate reductase. Chlorate-reducing microorganisms contain a distinct chlorate reductase. Perchlorate and chlorate reductases are highly related to nitrate reductases of the NarGHIJ-type. The interaction of nitrate reduction and perchlorate and chlorate reduction is not well studied.

More background information that can be used to get a better understanding of the research described in this thesis is given in Chapter 1. The aim of this study was to further explore the metabolic pathways in bacteria that degrade aromatic hydrocarbons and *n*-alkanes with chlorate or nitrate as electron acceptor.

Genome and physiology of *Alicycliphilus denitrificans* strains BC and K601^T

A. denitrificans strain BC couples degradation of benzene and a variety of other aromatic hydrocarbons to chlorate reduction. Strain BC degrades these compounds using aerobic degradation pathways in which oxygenases incorporate molecular oxygen in the aromatic ring to initiate degradation. When degradation is coupled to chlorate reduction, chlorite must be converted to chloride and oxygen for the pathway to function. Strain BC is highly related to *A. denitrificans* strain K601^T. The genomes and physiology of these strains were studied (Chapter 2). Strains BC and K601^T use nitrate, nitrite and oxygen as electron acceptors. Unlike strain BC, strain K601^T does not reduce chlorate. Accordingly, strain BC contains a plasmid encoding chlorate reductase and chlorite dismutase, which is not present in strain K601^T. Strains BC and K601^T cannot reduce perchlorate. Genes encoding nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase are located on the chromosomes of strains BC and K601^T. Therefore, these strains contain the complete denitrification pathway. Furthermore, the genomes of strains BC and K601^T encode low-oxygen affinity cytochrome *c* and cytochrome *o* ubiquinol oxidases, that can be used under aerobic conditions. Cytochrome *bd* ubiquinol and cbb_3-type cytochrome *c* oxidases are high-oxygen affinity cytochrome oxidases of strains BC and K601^T. These different types of cytochrome oxidases allow the strains to thrive with both high and low oxygen availability.

Strains BC and K601^T have a broad electron donor range. Besides a variety of aromatic hydrocarbons, they use carboxylic acids and amino acids. Accordingly, genes involved in the degradation of these compounds have been identified in the genomes of strains BC and K601^T. Strain K601^T degrades cyclohexanol with nitrate and oxygen as electron acceptor, but strain BC cannot degrade this alcohol. Enzymes involved in cyclohexanol degradation are encoded on the chromosome of strain K601^T, but not of strain BC.

Electron acceptor metabolism of strain BC

Strain BC cells grown with acetate as electron donor have difficulties to change their electron acceptor metabolism. Cells adapted to nitrate have a prolonged lag-phase when transferred to chlorate and vice versa. Furthermore, cells adapted to oxygen do not grow easily with nitrate or chlorate as electron acceptor. The specific chlorate reductase activity in nitrate-adapted cells was higher than in chlorate-adapted cells, but the chlorate reductase protein and mRNA transcript were more abundant in chlorate-adapted cells than in cells adapted to nitrate. Nitrate reductase protein is more abundant in nitrate-adapted cells than in cells adapted to chlorate. The nitrate reductase of the Nar-type can reduce chlorate and thus interferes with the specific chlorate reductase (Chapter 3). Further analysis showed that there is a chlorate-depending regulation of the gene encoding the catalytic subunit of nitrate reductase, NarG. A regulation mechanism involving integration of a transposon (containing an IS3/IS911 family transposase and an integrase) in the *narG* locus of strain BC when chlorate is present is proposed (Chapter 4). This integration seems to lead to a deletion in *narG*. Oxygen-adapted cells possess nitrate reductase, but not nitrite reductase, chlorate reductase and chlorite dismutase. Therefore, toxic nitrite may accumulate when switched from oxygen to nitrate and chlorite may accumulate when switched from oxygen to chlorate (Chapter 3). Overall, this research showed that cells did not always grow easily when switching from one electron acceptor to another and that regulation of enzymes involved in electron acceptor metabolism in strain BC is rather complex.

Anaerobic acetone degradation pathway in strain BC

A. denitrificans strains BC and K601^T degrade acetone with nitrate as electron acceptor, but not with chlorate or oxygen as electron acceptor. Under anaerobic conditions, acetone can be converted to acetoacetate, acetoacetyl-CoA and acetyl-CoA, respectively. Acetyl-CoA can enter the tricarboxylic acid cycle. This anaerobic acetone degradation pathway is proposed in anaerobic photosynthetic acetone degradation and in acetone degradation using nitrate or sulfate as electron acceptors. It is unclear which enzymes are involved in this degradation pathway, besides acetone carboxylase. Our proteome studies using strain BC gave more insight in the anaerobic acetone degradation pathway. Also in strain BC, the enzyme initiating the degradation of acetone is acetone carboxylase, which catalyzes the ATP-dependent conversion of acetone to acetoacetate. Based on proteome data from strain BC, an AMP-dependent synthetase and ligase is proposed to be involved in the conversion of acetoacetate to acetoacetyl-CoA and the enzyme involved in the conversion of acetoacetyl-CoA to acetyl-CoA is proposed to be acetyl-CoA acetyltransferase (Chapter 5).

The benzene-degrading *Pseudomonas stutzeri* strain BN

P. stutzeri strain BN is a mesophilic bacterium that degrades aromatic hydrocarbons with nitrate and oxygen as electron acceptors. The pathway for aerobic benzene degradation involves oxygenases that incorporate oxygen into the benzene-ring, such as phenol hydroxylase and benzoate 1, 2-dioxygenase. Analysis of the draft genome sequence suggests that carboxylation to benzoates and/or hydroxylation to phenols is the most likely mechanism for benzene degradation with nitrate as electron acceptor. A putative NO dismutase, that produces oxygen from NO, is not present in the genome of strain BN, indicating that aerobic degradation pathways for aromatic hydrocarbons cannot be used with nitrate as electron acceptor (Chapter 6).

Georgfuchsia toluolica strain G5G6^T genome and gene distribution in metagenomes of its habitat

G. toluolica strain G5G6^T degrades toluene with nitrate, iron(III) and manganese(IV). This bacterium was isolated from a polluted aquifer and seems to play an important role in biodegradation of toluene at this polluted site. Genes encoding high and low-oxygen affinity cytochrome *c* oxidases and oxygenases were present in the genome, indicating that the strain should be able to grow aerobically, but aerobic growth of the strain has not been observed yet and no indications how to achieve that were obtained thus far. Typical nitrite reductase and nitrous oxide reductase encoding genes were not found in the genome. Therefore, atypical enzymes are likely involved in the denitrification pathway of strain G5G6^T. The anaerobic toluene degradation pathway of strain G5G6^T was proposed to involve benzylsuccinate synthase. Genes encoding this enzyme and other enzymes of the anaerobic toluene degradation pathway (genes of the Bss and Bbs clusters) were present in the genome. Genes involved in anaerobic ethylbenzene and benzoate degradation were also present in the strain G5G6^T genome. The strain was previously described not to be able to degrade benzoate, but it was described to degrade phenol, *p*-cresol and *m*-cresol under anaerobic conditions. However, genes involved in anaerobic degradation of these compounds could not be identified from the genome sequence yet. Strain G5G6^T gene distribution in metagenomes from wells of the polluted aquifer where the strain was isolated from, indicated that the strain is widely distributed. Its genes are also present in clean groundwater outside of the pollution plume at the site. Genes highly similar to genes of strain G5G6^T involved in degradation of aromatic compounds and carboxylic acids and genes of the respiratory nitrate reduction pathway and high- and low-oxygen affinity cytochrome *c* oxidases are present at the aquifer (Chapter 7).

n-Alkane degradation pathway of *Pseudomonas chloritidismutans* strain AW-1^T and genome comparison of *P. chloritidismutans* to *P. stutzeri* strains

P. chloritidismutans strain AW-1^T degrades C7- to C12-alkanes with chlorate and oxygen as electron acceptor. Genome comparison showed that the genome of strain AW-1^T is significantly different from genomes of *P. stutzeri* strains. The strain has distinct physiological characteristics, such as the ability to use chlorate as electron acceptor and *n*-alkanes as electron donor and is lacking properties characteristic for *P. stutzeri* strains, such as nitrogen fixation (Chapter 8). In strain AW-1^T degradation of *n*-decane is initiated by an alkane 1-monooxygenase with both chlorate and oxygen as electron acceptor. This enzyme mediates production of decanol. Other enzymes involved in *n*-decane degradation include alcohol and aldehyde dehydrogenases that allow conversion of the alcohol to aldehydes and fatty acid. Enzymes of the Beta-oxidation pathway and tricarboxylic acid cycle are involved in further degradation of these compounds. Since the Nap-type nitrate reductase of strain AW-1^T poorly reduces chlorate, it is proposed that nitrate reductase does not interfere with chlorate respiration in this strain (Chapter 8).

Future perspectives

There are various techniques that can be used to clean-up soil and often a combination of techniques is applied. To enhance biodegradation of petroleum hydrocarbons, microorganisms and electron acceptors can be introduced. Like nitrate, chlorate is an electron acceptor that is easy to introduce in anaerobic soil sites. Oxygen produced from chlorate reduction allows aerobic degradation of recalcitrant compounds, which can enhance bioremediation. Further research should aim at isolation and characterization of appropriate microorganisms. This is important for unraveling the metabolism and properties of such strains and for obtaining appropriate microorganisms that can be applied at specific soil conditions. In addition, research is needed for the development of novel methods that screen the

biodegradation capacity of the intrinsic and introduced microorganisms, which improves selection of appropriate microorganisms.

Strains BC and AW-1^T can be used for bioremediation using chlorate as electron acceptor and strains BN and G5G6^T for bioremediation with nitrate. It is proposed that Nap-type nitrate reductase of strain AW-1^T does not interfere with chlorate respiration and that Nar-type nitrate reductase of strain BC interferes with chlorate respiration. A novel mechanism of chlorate-dependent Nar regulation was proposed to occur and involves integration of a transposon in the gene coding for the catalytic subunit of Nar (Chapter 9). Further studies need to confirm this novel mechanism. Furthermore, the genome of strain G5G6^T was proposed to contain genes encoding an atypical denitrification pathway. Further studies need to show which enzymes are involved in nitrite reduction and nitrous oxide reduction in this strain. Also the possible aerobic or micro-aerophilic metabolism of the strain requires further research.

Samenvatting proefschift

Inleiding

Denitrificatieroutes zijn uitgebreider onderzocht dan perchloraat- en chloraatreductieroutes. Denitrificatie wordt gebruikt als mechanisme voor anaerobe bioremediatie van de koolwaterstoffen in petroleum. Echter, bioremediatie met perchloraat- en chloraatreductie wordt nog niet veel toegepast en deze toepassing lijkt controversieel, omdat perchloraat en chloraat ook als bodemvervuilende stoffen worden beschouwd. Zuurstof is een intermediair van perchloraat- en chloraatreductie en deze stof kan bioremediatie versnellen, omdat aerobe afbraak van de koolwaterstoffen in petroleum eenvoudig te bewerkstelligen is. In een onlangs ontdekte atypische enzymatische route van nitraatreductie wordt zuurstof op soortgelijke manier gevormd. Perchloraat, chloraat en nitraat zijn beter oplosbaar dan zuurstof en kunnen via het grondwater makkelijker geïntroduceerd worden in anaerobe delen van de bodem. Perchloraat en chloraat kunnen schadelijk zijn voor levende organismen, maar perchloraat en chloraat reducerende microorganismen die deze stoffen kunnen omzetten in het onschadelijke chloride zijn overvloedig aanwezig in de bodem. De meeste perchloraat- en chloraatreducerende microorganismen zijn Proteobacteriën, terwijl nitraatreductie uitgevoerd kan worden door een breed scala aan microorganismen en niet kenmerkend is voor een bepaalde klasse. De enzymen die betrokken zijn bij perchloraat en chloraatreductie zijn perchloraatreductase, chloraatreductase en chloriet dismutase. Zowel perchloraat- als chloraatreducerende bacteriën hebben chloriet dismutase. Dit enzym is essentieel voor deze bacteriën, omdat het chloriet onschadelijk maakt. Bacteriën die perchloraat reduceren kunnen ook chloraat reduceren en de reductie van beide stoffen kan versneld worden door één enzym, perchloraatreductase. Chloraatreducerende microorganismen bevatten chloraatreductase dat alleen chloraat reduceert. Perchloraat- en chloraatreductases zijn sterk verwant aan NarGHIJ-type nitraatreductases. De interactie tussen nitraatreductie en perchloraat- en chloraatreductie is niet goed onderzocht.

Meer achtergrondinformatie die gebruikt kan worden om het onderzoek dat beschreven is in dit proefschrift beter te begrijpen is beschreven in Hoofdstuk 1. Het doel van deze studie was het verder onderzoeken van metabole omzettingsroutes in bacteriën die aromatische koolwaterstoffen en *n*-alkanen kunnen afbreken met chloraat of nitraat als electronacceptor.

Genomen en fysiologie van *Alicycliphilus denitrificans* stammen BC en K601^T

A. denitrificans stam BC koppelt de afbraak van benzeen en een scala aan andere aromatische koolwaterstoffen aan chloraatreductie. Stam BC breekt deze stoffen af met aerobe omzettingsroutes waarbij oxygenases moleculair zuurstof in de aromatische ring opnemen, wat afbraak initiëert. Als afbraak gekoppeld wordt aan chloraatreductie, moet chloriet omgezet worden naar chloride en zuurstof om de omzettingsroute te laten functioneren. Stam BC is sterk verwant met *A. denitrificans* stam K601^T. De genomen en fysiologie van deze stammen zijn bestudeerd (Hoofdstuk 2). Stammen BC en K601^T gebruiken nitraat, nitriet en zuurstof als electronacceptoren. Anders dan stam BC, kan stam K601^T geen chloraat reduceren. In overeenstemming hiermee, heeft stam BC een plasmide dat chloraatreductase en chloriet dismutase codeert en dat niet aanwezig is in stam K601^T. De stammen BC en K601^T kunnen geen perchloraat reduceren. Genen die coderen voor nitraatreductase, nitrietreductase, NO reductase en N_2O reductase bevinden zich op het chromosoom van de stammen BC en K601^T. Dus bevatten de stammen de complete nitraatreductieroute. Verder bevatten de genomen van de stammen BC and K601^T cytochroom *c* en cytochroom *o* ubiquinol oxidases met een lage affiniteit voor zuurstof, die gebruikt kunnen worden onder aerobe condities. Cytochroom *bd* ubiquinol en *cbb*$_3$-type cytochroom *c* oxidases zijn cytochroom oxidases van de stammen BC en K601^T met een hoge affiniteit voor zuurstof. Deze verschillende types cytochroom oxidases zorgen ervoor dat de stammen bij zowel hoge als lage beschikbaarheid van zuurstof kunnen leven.

De stammen BC en K601^T kunnen een breed scala aan electrondonoren afbreken. Naast een verscheidenheid aan aromatische koolwaterstoffen kunnen ze carbonzuren en aminozuren gebruiken. In overeenstemming hiermee, zijn de genen die betrokken zijn bij de afbraak van deze stoffen gevonden in de genomen van de stammen BC en K601^T. Stam K601^T breekt cyclohexanol af met nitraat en zuurstof als electronacceptor, maar stam BC kan deze alcoholverbinding niet afbreken. Enzymen die betrokken zijn bij cyclohexanolafbraak zijn gecodeerd op het chromosoom van stam K601^T, maar niet op die van stam BC.

Electronacceptormetabolisme van stam BC

Cellen van stam BC die opgegroeid zijn met acetaat als electrondonor hebben moeite met het veranderen van hun electronacceptormetabolisme. Cellen die aangepast zijn aan het gebruik van nitraat hebben een verlengde fase van vertraagde groei als ze omgezet worden naar chloraat en vice versa. Verder groeien cellen die aangepast zijn aan zuurstof niet makkelijk met nitraat of chloraat als electronacceptor. De specifieke chloraatreductase activiteit in cellen aangepast aan nitraat was hoger dan in cellen aangepast aan chloraat, maar het eiwit en mRNA transcript van chloraatreductase kwamen vaker voor in cellen aangepast aan chloraat dan in cellen aangepast aan nitraat. Het nitraatreductase eiwit is meer aanwezig in cellen aangepast aan nitraat dan in cellen aangepast aan chloraat. Het Nar-type nitraat reductase kan chloraat reduceren en dus interfereren met de specifieke chloraatreductase (Hoofdstuk 3). Verdere analyse liet zien dat er chloraatafhankelijke regulatie is van het gen dat codeert voor het katalytische subunit van nitraatreductase, NarG. Een regulatoir meganisme waar integratie van een transposon (dat een transposase van de IS3/IS911 familie en een integrase bevat) onderdeel van uitmaakt, in het *narG* locus van stam BC als chloraat aanwezig is, wordt voorgesteld (Hoofdstuk 4). Deze integratie lijkt te leiden tot een deletie in *narG*. Cellen aangepast aan zuurstof hebben nitraatreductase, maar geen nitrietreductase, chloraatreductase en chlorietdismutase. Daardoor zou het toxische nitriet kunnen ophopen als er van zuurstof naar nitraat gewisseld wordt en chloriet zou kunnen op hopen als er gewisseld wordt van zuurstof naar chloraat (Hoofdstuk 3). Over het algemeen liet dit onderzoek zien dat cellen niet altijd makkelijk groeien als er van de ene naar de andere electronacceptor gewisseld werd en dat de regulatie van enzymen die betrokken zijn bij electronacceptormetabolisme in stam BC erg complex is.

Anaerobe acetonafbraakroute in stam BC

A. denitrificans stammen BC en K601^T breken aceton af met nitraat als electonacceptor, maar niet met chloraat of zuurstof als electronacceptor. Onder anaerobe condities kan aceton af worden gebroken naar acetoacetaat, acetoacetyl-CoA en acetyl-CoA, respectievelijk. Acetyl-CoA kan de citroenzuurcyclus in. Deze anaerobe acetonafbraakroute wordt voorgesteld voor anaerobe acetonafbraak met fotosynthese en voor acetonafbraak met nitraat of sulfaat als electronacceptor. Het is onduidelijk welke enzymen naast acetoncarboxylase betrokken zijn bij deze afbraakroute. Onze proteoomstudies met stam BC gaven meer inzicht in de anaerobe acetonafbraakroute. Ook in stam BC is het enzym dat acetonafbraak initieert acetoncarboxylase, welke de ATP-afhankelijke afbraak van aceton naar acetoacetaat katalyseert. Gebaseerd op proteoomdata van stam BC, wordt voorgesteld dat een AMP-afhankelijk synthetase betrokken is bij de omzetting van acetoacetaat naar acetoacetyl-CoA en wordt voorgesteld dat het enzym betrokken bij de omzetting van acetoacetyl-CoA naar acetyl-CoA een acetyl-CoA acetyltransferase is (Hoofdstuk 5).

De benzeenafbrekende *Pseudomonas stutzeri* stam BN

P. stutzeri stam BN is een mesofiele bacterie die aromatische koolwaterstoffen kan afbreken met nitraat en zuurstof als electronacceptor. De aerobe benzeenafbraakroute bevat oxygenases die zuurstof laten opnemen in de benzeenring, zoals fenolhydroxylase en

benzoaat 1, 2-dioxygenase. Analyse van de DNA volgorde van het kladgenoom wekt de suggestie dat carboxylatie naar benzoaten en/of hydroxylatie naar fenolen het meest waarschijnlijke mechanisme van benzeendegradatie is met nitraat als electronacceptor. Een mogelijk NO dismutase dat zuurstof produceert uit NO is niet aanwezig op het genoom van stam BN, dat duidt aan dat aerobe afbraakroutes van aromatische koolwaterstoffen niet gebruikt kunnen worden met nitraat als electronacceptor (Hoofdstuk 6).

Het genoom van *Georgfuchsia toluolica* stam G5G6^T en gendistributie in metagenomen van zijn habitat

G. toluolica stam G5G6^T breekt tolueen af met nitraat, ijzer(III) en mangaan(IV). Deze bacterie werd geïsoleerd uit een vervuilde waterbron en lijkt een belangrijke rol te spelen bij de biodegradatie van tolueen op deze vervuilde locatie. Genen die cytochroom *c* oxydases met hoge en lage affiniteit voor zuurstof en die oxygenases coderen, waren aanwezig in het genoom, dit geeft aan dat de stam eigenlijk aeroob zou moeten kunnen groeien, maar aerobe groei van de stam is nog niet geobserveerd en er zijn nog geen mogelijkheden gevonden om dat mogelijk te maken. Typische nitriet reductase en N_2O reductase coderende genen werden niet gevonden in het genoom. Daarom zijn er mogelijk atypische enzymen betrokken bij de denitrificatieroute van stam G5G6^T. Betrokkenheid van benzylsuccinaat synthase werd voorgesteld bij de anaerobe tolueenafbraakroute van stam G5G6^T. Genen die coderen voor dit enzym en voor andere enzymen van de anaerobe tolueenafbraakroute (genen van de Bss en Bbs clusters) waren aanwezig in het genoom. Genen die betrokken zijn bij anaerobe ethylbenzeen- en benzoaatafbraak waren ook aanwezig in het genoom van stam G5G6^T. Van de stam werd beschreven dat deze geen benzoaat kan afbreken, maar dat fenol, *p*-cresol en *m*-cresol wel afgebroken konden worden onder anaerobe condities. Echter, genen betrokken bij de anaerobe degradatie van deze stoffen konden nog niet worden geïdentificeerd in de DNA volgorde van het genoom. De gendistributie van stam G5G6^T in metagenomen van de boorgaten in de vervuilde waterput waaruit de stam geïsoleerd werd, toonde aan dat de stam wijd verspreid is. De genen van de stam waren ook aanwezig in schoon grondwater buiten de vervuilde pluim op de locatie. Genen die sterk lijken op genen van stam G5G6^T en die betrokken zijn bij de afbraak van aromatische stoffen en carbonzuren en genen van de respiratoire nitraatreductieroute en cytochroom *c* oxidases met hoge en lage affiniteit voor zuurstof zijn aanwezig in de waterbron (Hoofdstuk 7).

***n*-Alkaanafbraakroute van *Pseudomonas chloritidismutans* stam AW-1^T en vergelijking van de genomen van *P. chloritidismutans* en *P. stutzeri* stammen**

P. chloritidismutans stam AW-1^T breekt C7- tot C12-alkanen af met chloraat en zuurstof als electronacceptor. Door vergelijking werd duidelijk dat het genoom van stam AW-1^T significant verschilt van de genomen van *P. stutzeri* stammen. De stam heeft onderscheidende fysiologische kenmerken, zoals de mogelijkheid om chloraat als electronacceptor te gebruiken en *n*-alkanen als electrondonor en het mist eigenschappen die kenmerkend zijn voor *P. stutzeri* stammen, zoals stikstoffixatie (Hoofdstuk 8). In stam AW-1^T wordt de afbraak van *n*-decaan geïnitieerd door alkaan 1-monooxygenase zowel bij chloraat als bij zuurstof als electronacceptor. Dit enzym draagt zorg voor de productie van decanol. Andere enzymen betrokken bij *n*-decaan degradatie zijn onder andere alcohol en aldehyde dehydrogenases die de omzetting van de alcoholverbinding naar aldehydes en vetzuren mogelijk maken. Enzymen van de Beta-oxidatieroute en citroenzuurcyclus zijn betrokken bij de verdere afbraak van deze stoffen. Aangezien het Nap-type nitraatreductase van stam AW-1^T chloraat maar moeizaam reduceert, wordt voorgesteld dat nitraatreductase niet interfereert met chloraatreductie in deze stam (Hoofdstuk 8).

Toekomstperspectieven

Er zijn verschillende technieken die gebruikt kunnen worden bij het schoonmaken van de bodem en vaak wordt er een combinatie van technieken toegepast. Om biodegradatie van petroleum koolwaterstoffen te bevorderen, kunnen microorganismen en electronacceptors geïntroduceerd worden. Net zoals nitraat, is chloraat een electronacceptor die eenvoudig te introduceren is in anaerobe bodemdelen. De zuurstof die geproduceerd wordt bij de reductie van chloraat maakt het mogelijk om recalcitrante stoffen aeroob af te breken, wat bioremediatie kan bevorderen. Verder onderzoek zou zich moeten richten op de isolatie en karakterisatie van geschikte microorganismen. Dit is belangrijk voor het ontrafelen van het metabolisme en de eigenschappen van zulke stammen en voor het verkrijgen van microorganismen die toegepast kunnen worden bij specifieke bodemcondities. Ook is onderzoek nodig naar nieuwe methodes om de biodegradatiecapaciteit van de intrinsieke en geïntroduceerde microorganismen te testen, wat de selectie van geschikte microorganismen bevordert.

De stammen BC en AW-1^T kunnen gebruikt worden voor bioremediatie met chloraat als electronacceptor en stammen BN en G5G6^T voor bioremediatie met nitraat. Er wordt voorgesteld dat Nap-type nitraatreductase van stam AW-1^T niet interfereert met chloraatrespiratie en dat Nar-type nitraatreductase van stam BC wel interfereert met chloraatrespiratie. Het bestaan van een nieuw mechanisme van chloraat-afhankelijke regulatie van Nar werd voorgesteld en dit houdt in dat er een transposon integreert in het gen dat codeert voor het katalytische subunit van Nar (Hoofdstuk 9). Verdere studies zijn nodig om het bestaan van dit nieuwe mechanisme te bevestigen. Ook werd voorgesteld dat het genoom van stam G5G6^T genen bevat die voor een atypische denitrificatieroute coderen. Vervolgstudies moeten aantonen welke enzymen bij nitrietreductie en N_2O reductie betrokken zijn in deze stam. Ook moet het mogelijk aerobe of micro-aerofiele metabolisme van de stam nog verder onderzocht worden.

References

Abu Laban, N., D. Selesi*, et al.* (2009). Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria. FEMS Microbiol Ecol **68**(3): 300-311.
Abu Laban, N., D. Selesi*, et al.* (2010). Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environ Microbiol **12**(10): 2783-2796.
Achenbach, L. A., U. Michaelidou*, et al.* (2001). *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int J Syst Evol Microbiol **51**(2): 527-533.
Aklujkar, M , J. Krushkal*, et al.* (2009). The genome sequence of *Geobacter metallireducens*: features of metabolism, physiology and regulation common and dissimilar to *Geobacter sulfurreducens*. BMC Microbiol **9**: 109.
Allen, M. S., K. T. Welch*, et al.* (2004). Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium *Thauera sp*. MZ1T. Environ Microbiol **6**(8): 780-790.
Altenschmidt, U. and G. Fuchs (1991). Anaerobic degradation of toluene in denitrifying *Pseudomonas sp.*: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. Arch Microbiol **156**(2): 152-158.
Altschul, S. F., T. L. Madden*, et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**(17): 3389-3402.
Andrade, S. L. A., C. D. Brondino*, et al.* (2000). Aldehyde oxidoreductase activity in *Desulfovibrio alaskensis* NCIMB 13491. FEBS J **267**(7): 2054-2061.
Aslander, A. (1928). Experiments on the eradication of Canada Thistle *Cirsium arvense* with chlorates and other herbicides. J Agric Res **36**: 915-935.
Aziz, C. E. and P. B. Hatzinger (2008). Perchlorate sources, source identification and analytical methods. *In situ* bioremediation of perchlorate in ground water. H. F. Stroo and C. H. Ward. New York, Springer**:** 55-77.
Aziz, R., D. Bartels*, et al.* (2008). The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics **9**(1): 75.
Aziz, R. K., M. Breitbart*, et al.* (2010). Transposases are the most abundant, most ubiquitous genes in nature. Nucl Acids Res **38**(13): 4207-4217.
Bäcklund, A. S., J. Bohlin*, et al.* (2009). Periplasmic *c* cytochromes and chlorate reduction in *Ideonella dechloratans*. Appl Environ Microbiol **75**(8): 2439-2445.
Bäcklund, A. S. and T. Nilsson (2011). Purification and characterization of a soluble cytochrome *c* capable of delivering electrons to chlorate reductase in *Ideonella dechloratans*. FEMS Microbiol Lett **321**(2): 115-120.
Balasubramanian, P., L. Philip*, et al.* (2011). Biodegradation of chlorinated and non-chlorinated VOCs from pharmaceutical industries. Appl Biochem Biotechnol **163**(4): 497-518.
Balk, M., F. Mehboob*, et al.* (2010). (Per)chlorate reduction by an acetogenic bacterium, *Sporomusa sp.*, isolated from an underground gas storage. Appl Microbiol Biotechnol **88**(2): 595-603.
Balk, M., T. van Gelder*, et al.* (2008). (Per)chlorate reduction by the thermophilic bacterium *Moorella perchloratireducens* sp. nov., isolated from underground gas storage. Appl Environ Microbiol **74**(2): 403-409.
Ball, H. A., H. A. Johnson*, et al.* (1996). Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol **178**(19): 5755-5761.
Bardiya, N. and J.-H. Bae (2011). Dissimilatory perchlorate reduction: A review. Microbiol Res **166**(4): 237-254.
Basu, S., N. A. Azarova*, et al.* (2008). Nitrite reductase activity of cytochrome *c*. J Biol Chem **283**(47): 32590-32597.
Bedzyk, L., T. Wang*, et al.* (1999). The periplasmic nitrate reductase in *Pseudomonas sp.* strain G-179 catalyzes the first step of denitrification. J Bacteriol **181**(9): 2802-2806.

Beilen, J. B. v., E. G. Funhoff*, et al.* (2006). Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading Eubacteria lacking integral membrane alkane hydroxylases. Appl Env Microbiol **72**(1): 59-65.
Beilen, J. v. and E. Funhoff (2007). Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol **74**(1): 13-21.
Belancio, V. P., D. J. Hedges*, et al.* (2008). Mammalian non-LTR retrotransposons: For better or worse, in sickness and in health. Genome Res **18**(3): 343-358.
Bell, L. C., D. J. Richardson*, et al.* (1990). Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: the periplasmic enzyme catalyzes the first step in aerobic denitrification. FEBS Lett **265**(1-2): 85-87.
Beller, H. R. and A. M. Spormann (1997). Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J Bacteriol **179**(3): 670-676.
Bender, K. S., M. R. Rice*, et al.* (2004). Metabolic primers for detection of (per)chlorate-reducing bacteria in the environment and phylogenetic analysis of *cld* gene sequences. Appl Environ Microbiol **70**(9): 5651-5658.
Bender, K. S., C. Shang*, et al.* (2005). Identification, characterization, and classification of genes encoding perchlorate reductase. J Bacteriol **187**(15): 5090-5096.
Bendtsen, J. D., H. Nielsen*, et al.* (2004). Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340**(4): 783-795.
Bendtsen, J. D., H. Nielsen*, et al.* (2005). Prediction of twin-arginine signal peptides. BMC Bioinformatics **6**(1): 167.
Bennett, S. (2004). Solexa Ltd. Pharmacogenomics J **5**(4): 433-438.
Benson, D. A., I. Karsch-Mizrachi*, et al.* (2008). GenBank. Nucl Acids Res **36**(suppl_1): D25-30.
Benson, D. R., J. M. Brooks*, et al.* (2011). The biology of *Frankia sp*. strains in the post-genome era. Mol Plant Microbe Interact **24**(11): 1310-1316.
Berks, B. C. (1996). A common export pathway for proteins binding complex redox cofactors? Mol Microbiol **22**(3): 393-404.
Bertero, M. G., R. A. Rothery*, et al.* (2003). Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. Nat Struct Mol Biol **10**(9): 681-687.
Biegert, T., G. Fuchs*, et al.* (1996). Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur J Biochem **238**(3): 661-668.
Birks, S. J. and D. J. Kelly (1997). Assay and properties of acetone carboxylase, a novel enzyme involved in acetone-dependent growth and CO_2 fixation in *Rhodobacter capsulatus* and other photosynthetic and denitrifying bacteria. Microbiology **143**(3): 755-766.
Bohlin, J., A. S. Bäcklund*, et al.* (2010). Characterization of a cytochrome *c* gene located at the gene cluster for chlorate respiration in *Ideonella dechloratans*. Microbiol Res **165**(6): 450-457.
Boisvert, S., F. Laviolette*, et al.* (2010). Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. J Comp Biol **17**(11): 1519-1533.
Boll, M., G. Fuchs*, et al.* (2002). Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr Opin Chem Biol **6**(5): 604-611.
Bollagh, J.-M. and N. M. Henniger (1978). Effects of nitrite toxicity on soil bacteria under aerobic and anaerobic conditions. Soil Biol Biochem **10**(5): 377-381.
Bonnet-Smits, E. M., L. A. Robertson*, et al.* (1988). Carbon dioxide fixation as the initial step in the metabolism of acetone by *Thiosphaera pantotropha*. J Gen Microbiol **134**(8): 2281-2289.
Brouns, S. J., M. M. Jore*, et al.* (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. Science **321**(5891): 960-964.
Brunori, M., A. Giuffrè*, et al.* (2005). Cytochrome *c* oxidase, ligands and electrons. J Inorg Biochem **99**(1): 324-336.
Brzezinski, P. and R. Gennis (2008). Cytochrome *c* oxidase: exciting progress and remaining mysteries. J Bioenerg Biomembr **40**(5): 521-531.

Bustin, S. A., V. Benes*, et al.* (2009). The MIQE guidelines: Minimum Information for publication of Quantitative real-time PCR Experiments. Clin Chem **55**(4): 611-622.
Butler, C. S. and J. R. Mason (1996). Structure-function analysis of the bacterial aromatic ring-hydroxylating dioxygenases. Adv Microb Physiol. R. K. Poole, Academic Press. **38:** 47-84.
Cafaro, V., V. Izzo*, et al.* (2004). Phenol hydroxylase and toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes. Appl Environ Microbiol **70**(4): 2211-2219.
Caldwell, M. E. and J. M. Suflita (2000). Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. Environ Sci Technol **34**(7): 1216-1220.
Callaghan, A. V., B. Wawrik*, et al.* (2008). Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. Biochem Biophys Res Comm **366**(1): 142-148.
Cao, B., K. Nagarajan*, et al.* (2009). Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl Microbiol Biotechnol **85**(2): 207-228.
Carmona, M., M. T. Zamarro*, et al.* (2009). Anaerobic catabolism of aromatic compounds: a genetic and genomic view. Microbiol Mol Biol Rev **73**(1): 71-133.
Carter, J. P., Y. H. Hsiao*, et al.* (1995). Soil and sediment bacteria capable of aerobic nitrate respiration. Appl Environ Microbiol **61**: 2852-2858.
Casacuberta, J. M. and N. Santiago (2003). Plant LTR-retrotransposons and MITEs: control of transposition and impact on the evolution of plant genes and genomes. Gene **311**: 1-11.
Chakraborty, R. and J. D. Coates (2004). Anaerobic degradation of monoaromatic hydrocarbons. Appl Microbiol Biotechnol **64**(4): 437-446.
Chakraborty, R. and J. D. Coates (2005). Hydroxylation and carboxylation--two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain RCB. Appl Environ Microbiol **71**(9): 5427-5432.
Chakraborty, R., S. M. O'Connor*, et al.* (2005). Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. Appl Environ Microbiol **71**(12): 8649-8655.
Chaudhuri, S. K., S. M. O'Connor*, et al.* (2002). Environmental factors that control microbial perchlorate reduction. Appl Environ Microbiol **68**(9): 4425-4430.
Chen, W., H. Liu*, et al.* (2011). Effects of nitrite and toxic *Microcistis aeruginosa* PCC7806 on the growth of freshwater rotifer *Brachionus calyciflorus*. Bull Environ Contam Toxicol **86**(3): 263-267.
Cladera, A., E. García-Valdés*, et al.* (2006). Genotype versus phenotype in the circumscription of bacterial species: the case of *Pseudomonas stutzeri* and *Pseudomonas chloritidismutans*. Arch Microbiol **184**(6): 353-361.
Clarke, T. A., M. J. Edwards*, et al.* (2011). Structure of a bacterial cell surface decaheme electron conduit. Proc Nat Acad Sciences **108**(23): 9384-9389.
Coates, J. D. and L. A. Achenbach (2004). Microbial perchlorate reduction: rocket-fuelled metabolism. Nat Rev Micro **2**(7): 569-580.
Coates, J. D., R. A. Bruce*, et al.* (1999). Hydrocarbon bioremediative potential of (per)chlorate-reducing bacteria. Bioremed J **3**(4): 323-334.
Coates, J. D., R. Chakraborty*, et al.* (2001). Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature **411**(6841): 1039-1043.
Coates, J. D., R. Chakraborty*, et al.* (2002). Anaerobic benzene biodegradation—a new era. Res Microbiol **153**(10): 621-628.
Coates, J. D., U. Michaelidou*, et al.* (1999). Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl Env Microbiol **65**(12): 5234-5241.
Cole, J. A. and C. M. Brown (1980). Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. FEMS Microbiol Lett **7**(2): 65-72.
Couri, D., M. S. Abdel-Rahman*, et al.* (1982). Toxicological effects of chlorine dioxide, chlorite and chlorate. Environ Health Perspect **46**: 13-17.

Cox, J. R., N. Neuhauser*, et al.* (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res **10**(4): 1794-1805.
Dangel, W., A. Tschech*, et al.* (1988). Anaerobic metabolism of cyclohexanol by denitrifying bacteria. Arch Microbiol **150**(4): 358-362.
Dangel, W., A. Tschech*, et al.* (1989). Enzyme reactions involved in anaerobic cyclohexanol metabolism by a denitrifying *Pseudomonas* species. Arch Microbiol **152**: 273-279.
Dasgupta, P. K., P. K. Martinelango*, et al.* (2005). The origin of naturally occurring perchlorate: the role of atmospheric processes. Environ Sci Technol **39**(6): 1569-1575.
Davidova, I. A., L. M. Gieg*, et al.* (2007). Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment. ISME J **1**(5): 436-442.
De Geus, D. C., E. A. J. Thomassen*, et al.* (2009). Crystal structure of chlorite dismutase, a detoxifying enzyme producing molecular oxygen. J Mol Biol **387**(1): 192-206.
De Groot, G. N. and A. H. Stouthamer (1969). Regulation of reductase formation in *Proteus mirabilis*. I. Formation of reductases and enzymes of the formic hydrogen lyase complex in the wild type and in chlorate resistant mutants. Arch Microbiol **66**: 220-233.
De Long, S. K., K. A. Kinney*, et al.* (2010). qPCR assays to quantify genes and gene expression associated with microbial perchlorate reduction. J Microbiol Methods **83**(2): 270-274.
Dermer, J. and G. Fuchs (2012). Molybdoenzyme that catalyzes the anaerobic hydroxylation of a tertiary carbon atom in the side chain of cholesterol. J Biol Chem **287**(44): 36905-36916.
Di Gennaro, P., A. Bargna*, et al.* (2011). Microbial enzymes for aromatic compound hydroxylation. Appl Microbiol Biotechnol **90**(6): 1817-1827.
Dojka, M. A., P. Hugenholtz*, et al.* (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl Environ Microbiol **64**(10).
Dou, J., A. Ding*, et al.* (2010). Anaerobic benzene biodegradation by a pure bacterial culture of *Bacillus cereus* under nitrate reducing conditions. J Environ Sci **22**(5): 709-715.
Dou, J., X. Liu*, et al.* (2008). Anaerobic BTEX biodegradation linked to nitrate and sulfate reduction. J Hazard Mater **151**(2–3): 720-729.
Dudley, M., A. Salamone*, et al.* (2008). Kinetics of a chlorate-accumulating, perchlorate-reducing bacterium. Water Res **42**(10–11): 2403-2410.
Dullius, C. H., C.-Y. Chen*, et al.* (2011). Nitrate-dependent degradation of acetone by *Alicycliphilus* and *Paracoccus* strains and comparison of acetone carboxylase enzymes. Appl Environ Microbiol **77**(19): 6821-6825.
Duval-Valentin, G. and M. Chandler (2011). Cotranslational control of DNA transposition: A window of opportunity. Mol Cell **44**(6): 989-996.
Ebihara, A., A. Okamoto*, et al.* (2005). Structure-based functional identification of a novel heme-binding protein from *Thermus thermophilus* HB8. J Struct Funct Gen **6**(1): 21-32.
Ehrenreich, P., A. Behrends*, et al.* (2000). Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol **173**(1): 58-64.
Elias, J. E. and S. P. Gygi (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Meth **4**(3): 207-214.
Eltis, L. D. and J. T. Bolin (1996). Evolutionary relationships among extradiol dioxygenases. J Bacteriol **178**(20): 5930-5937.
Emanuel, B. S. and T. H. Shaikh (2001). Segmental duplications: an 'expanding' role in genomic instability and disease. Nat Rev Genet **2**(10): 791-800.
Ensign, S. A., F. J. Small*, et al.* (1998). New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. Arch Microbiol **169**(3): 179-187.
Ettwig, K. F., M. K. Butler*, et al.* (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464**(7288): 543-548.
Ettwig, K. F., D. R. Speth*, et al.* (2012). Bacterial oxygen production in the dark. Front Microbiol **3**: 273.

Evans, W. C. (1963). The microbiological degradation of aromatic compounds. J gen microbiol **32**: 177-184.
Ewing, B., L. Hillier*, et al.* (1998). Base-calling of automated sequencer traces using Phred. I. accuracy assessment. Genome Res **8**(3): 175-185.
Ewing, B. and P. Green (1998). Base-calling of automated sequencer traces using Phred. II. error probabilities. Genome Res **8**(3): 186-194.
Feretti, D., I. Zerbini*, et al.* (2008). Evaluation of chlorite and chlorate genotoxicity using plant bioassays and in vitro DNA damage tests. Water Res **42**(15): 4075-4082.
Ferroni, F. M., M. G. Rivas*, et al.* (2011). Nitrate reduction associated with respiration in *Sinorhizobium meliloti* 2011 is performed by a membrane-bound molybdoenzyme. Biometals **24**: 891-902.
Feschotte, C. and E. J. Pritham (2007). DNA transposons and the evolution of Eukaryotic genomes. Ann Rev Gen **41**(1): 331-368.
Frazer, A. C., P. W. Coschigano*, et al.* (1995). Toluene metabolism under anaerobic conditions: a review. Anaerobe **1**(6): 293-303.
Frunzke, K., B. Heiss*, et al.* (1993). Molybdopterin guanine dinucleotide is the organic moiety of the molybdenum cofactor in respiratory nitrate reductase from *Pseudomonas stutzeri*. FEMS Microbiol Lett **113**(2): 241-245.
Frunzke, K., P. Hoffmüller*, et al.* (1991). Specific usage of molybdopterin dinucleotides by molybdoenzymes in *Pseudomonas carboxydoflava*: CO dehydrogenase harbors molybdopterin cytosine dinucleotide and nitrate reductase molybdopterin guanine dinucleotide. BioFactors **3**: 134-135.
Fuchs, G. (2008). Anaerobic metabolism of aromatic compounds. Ann NY Acad Sci **1125**(1): 82-99.
Gama-Castro, S., C. Núñez*, et al.* (2001). *Azotobacter vinelandii* aldehyde dehydrogenase regulated by c-54: role in alcohol catabolism and encystment. J Bacteriol **183**(21): 6169-6174.
Geer, L. Y., S. P. Markey*, et al.* (2004). Open mass spectrometry search algorithm. J Prot Res **3**(5): 958-964.
George, H. A., J. L. Johnson*, et al.* (1983). Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. Appl Environ Microbiol **45**(3): 1160-1163.
Gescher, J., W. Ismail*, et al.* (2006). Aerobic benzoyl-Coenzyme A (CoA) catabolic pathway in *Azoarcus evansii*: conversion of ring cleavage product by 3,4-dehydroadipyl-CoA semialdehyde dehydrogenase. J Bacteriol **188**(8): 2919-2927.
Gibson, D. T. and R. E. Parales (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr Opin Biotechnol **11**(3): 236-243.
Ginkel, C. G. v., C. M. Plugge*, et al.* (1995). Reduction of chlorate with various energy substrates and inocula under anaerobic conditions. Chemosphere **31**(10): 4057-4066.
Ginkel, C. G. v., G. B. Rikken*, et al.* (1996). Purification and characterization of chlorite dismutase: a novel oxygen-generating enzyme. Arch Microbiol **166**(5): 321-326.
Goblirsch, B. R., B. R. Streit*, et al.* (2009). Crystallization and preliminary X-ray diffraction of chlorite dismutase from *Dechloromonas aromatica* RCB. Acta Chrystallogr Sect F Struct Biol Cryst Commun **65**: 818-821.
Goksøyr, J. (1951). On the effect of chlorate upon the nitrate reduction of plants I. Experiments with *Aspergillus oryzae*. Physiol Plant **4**: 498-513.
Goksøyr, J. (1952). On the effect of chlorate upon nitrate reduction of plants II. The effect upon the nitrate-reducing system in *Escherichia coli*. Physiol Plant **5**: 228-240.
Gonzalez, J. M., F. Mayer*, et al.* (1997). *Sagittula stellata* gen. nov., sp. nov., a lignin-transforming bacterium from a coastal environment. Int J Syst Bacteriol **47**(3): 773-780.
Gordon, D., C. Abajian*, et al.* (1998). Consed: a graphical tool for sequence finishing. Genome Res **8**(3): 195-202.
Gordon, D., C. Desmarais*, et al.* (2001). Automated Finishing with Autofinish. Genome Res **11**(4): 614-625.

Goris, J., K. T. Konstantinidis*, et al.* (2007). DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol **57**(1): 81-91.
Gray, N. D., A. Sherry*, et al.* (2010). Chapter 5 - Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation, and energy recovery. Advances in applied microbiology. S. S. Allen I. Laskin and M. G. Geoffrey, Academic Press. **72:** 137-161.
Griffiths-Jones, S., A. Bateman*, et al.* (2003). Rfam: an RNA family database. Nucleic Acids Res **31**(1): 439-441.
Grundmann, O., A. Behrends*, et al.* (2008). Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. Environ Microbiol **10**(2): 376-385.
Gullick, R. Q., M. W. Lechvallier*, et al.* (2001). Occurrence of (per)chlorate in drinking water sources. J Am Water Works Assoc **93**: 66-77.
Hackenthal, E. (1965). Die reduktion von perchlorat durch bakterien--II: die identität der nitratreduktase und des perchlorat reduzierenden enzyms aus *B. cereus*. Biochem Pharmacol **14**(9): 1313-1324.
Hacker, J. and J. B. Kaper (2000). Pathogenicity islands and the evolution of microbes. Ann Rev Microbiol **54**(1): 641-679.
Hagedoorn, P. L., D. C. De Geus*, et al.* (2002). Spectroscopic characterization and ligand-binding properties of chlorite dismutase from the chlorate respiring bacterial strain GR-1. Eur J Biochem **269**(19): 4905-4911.
Han, C. S. and P. Chain (2006). Finishing repeat regions automatically with Dupfinisher. Proceedings of the 2006 international conference on bioinformatics & computational biology. H. R. Arabnia and H. Valafar, CSREA Press**:** 141-146.
Harayama, S., N. Mermod*, et al.* (1987). Roles of the divergent branches of the meta-cleavage pathway in the degradation of benzoate and substituted benzoates. J Bacteriol **169**(2): 558-564.
Härtig, E., U. Schiek*, et al.* (1999). Nitrate and nitrite control of respiratory nitrate reduction in denitrifying *Pseudomonas stutzeri* by a two-component regulatory system homologous to NarXL of *Escherichia coli*. J Bacteriol **181**(12): 3658-3665.
Harwood, C. S., G. Burchhardt*, et al.* (1998). Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol Rev **22**(5): 439-458.
Harwood, C. S. and R. E. Parales (1996). The β-ketoadipate pathway and the biology of self-identity. Ann Rev Microbiol **50**(1): 553-590.
Head, I. M., D. M. Jones*, et al.* (2006). Marine microorganisms make a meal of oil. Nat Rev Micro **4**(3): 173-182.
Hecht, M. H., S. P. Kounaves*, et al.* (2009). Detection of perchlorate and the soluble chemistry of Martian soil at the Phoenix Lander site. Science **325**(5936): 64-67.
Heider, J. (2007). Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr Opin Chem Biol **11**(2): 188-194.
Heider, J. and G. Fuchs (1997). Anaerobic metabolism of aromatic compounds. Eur J Biochem **243**(3): 577-596.
Heider, J., A. M. Spormann*, et al.* (1998). Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev **22**(5): 459-473.
Heilig, H. G. H. J., E. G. Zoetendal*, et al.* (2002). Molecular diversity of *Lactobacillus spp*. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol **68**(1): 114-123.
Hellberg Lindqvist, M., N. Johansson*, et al.* (2012). Expression of chlorite dismutase and chlorate reductase in the presence of oxygen and/or chlorate as the terminal electron acceptor in *Ideonella dechloratans*. Appl Environ Microbiol **78**(12): 4380-4385.
Herman, D. C. and W. T. Frankenberger (1999). Bacterial reduction of perchlorate and nitrate in water. J Environ Qual **28**(3): 1018-1024.

Holmes, D. E., C. Risso*, et al.* (2011). Anaerobic oxidation of benzene by the hyperthermophilic archaeon *Ferroglobus placidus*. Appl Environ Microbiol **77**(17): 5926-5933.
Hyatt, D., G.-L. Chen*, et al.* (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics **11**(1): 119.
Iwai, S., F. Kurisu*, et al.* (2007). Development of a 60-mer oligonucleotide microarray on the basis of benzene monooxygenase gene diversity. Appl Microbiol Biotech **75**(4): 929-939.
Iwai, S., F. Kurisu*, et al.* (2010). Characterization of monooxygenase gene diversity in benzene-amended soils. Lett Appl Microbiol **50**(2): 138-145.
Janssen, P. H. and B. Schink (1995). 14CO_2 exchange with acetoacetate catalyzed by dialyzed cell-free extracts of the bacterial strain BunN grown with acetone and nitrate. Eur J Biochem **228**(3): 677-682.
Jin, J., A. J. Straathof*, et al.* (2011). Purification, characterization, and cloning of a bifunctional molybdoenzyme with hydratase and alcohol dehydrogenase activity. Appl Microbiol Biotechnol **89**(6): 1831-1840.
Jobst, B., K. Schühle*, et al.* (2010). ATP-dependent carboxylation of acetophenone by a novel type of carboxylase. J Bacteriol **192**(5): 1387-1394.
Jogl, G. and L. Tong (2004). Crystal structure of yeast acetyl-Coenzyme A synthetase in complex with AMP. Biochem **43**(6): 1425-1431.
Jones, C. M., B. Stres*, et al.* (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Mol Biol Evol **25**(9): 1955-1966.
Jormakka, M., D. Richardson*, et al.* (2004). Architecture of NarGH reveals a structural classification of Mo-bisMGD enzymes. Structure **12**(1): 95-104.
Junier, P., V. Molina*, et al.* (2010). Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. Appl Microbiol Biotechnol **85**(3): 425-440.
Kalapos, M. P. (2003). On the mammalian acetone metabolism: from chemistry to clinical implications. Biochim Biophys Acta **1621**(2): 122-139.
Kalyuzhnaya, M. G., P. De Marco*, et al.* (2006). *Methyloversatilis universalis* gen. nov., sp. nov., a novel taxon within the Betaproteobacteria represented by three methylotrophic isolates. Int J Syst Evol Microbiol **56**(11): 2517-2522.
Kang, N., W. A. Jackson*, et al.* (2008). Perchlorate production by ozone oxidation of chloride in aqueous and dry systems. Sci Total Environ **405**(1-3): 301-309.
Karlsson, J. and T. Nilsson (2005). The C subunit of *Ideonella dechloratans* chlorate reductase: expression, purification, refolding, and heme reconstitution. Protein Expr Purif **41**(2): 306-312.
Kasai, Y., Y. Takahata*, et al.* (2006). RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Appl Environ Microbiol **72**(5): 3586-3592.
Kazazian Jr, H. H. and J. L. Goodier (2002). LINE drive: Retrotransposition and genome instability. Cell **110**(3): 277-280.
Kengen, S. W. M., G. B. Rikken*, et al.* (1999). Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1. J Bacteriol **181**(21): 6706-6711.
Kidwell, M. G. and D. R. Lisch (2001). Perspective: Transposable elements, parasitic DNA, and genome evolution. Evolution **55**(1): 1-24.
Kittichotirat, W., N. M. Good*, et al.* (2011). Genome sequence of *Methyloversatilis universalis* FAM5^T, a methylotrophic representative of the order Rhodocyclales. J Bacteriol **193**(17): 4541-4542.
Kivisaar, M. (2009). Degradation of nitroaromatic compounds: a model to study evolution of metabolic pathways. Mol Microbiol **74**(4): 777-781.
Kniemeyer, O., T. Fischer*, et al.* (2003). Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol **69**(2): 760-768.

Korenkov, V., V. Romanenko*, et al.* (1976). Process for purification of industrial waste waters from perchlorates and chlorate. US patent number 3943055.
Körner, H., H. J. Sofia*, et al.* (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. FEMS Microbiol Rev **27**(5): 559-592.
Körner, H. and W. G. Zumft (1989). Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl Environ Microbiol **55**(7): 1670-1676.
Korolev, K. S., J. B. Xavier*, et al.* (2011). A quantitative test of population genetics using spatiogenetic patterns in bacterial colonies. Am Nat **178**(4): 538-552.
Kostan, J., B. Sjöblom*, et al.* (2010). Structural and functional characterisation of the chlorite dismutase from the nitrite-oxidizing bacterium "*Candidatus Nitrospira defluvii*": identification of a catalytically important amino acid residue. J Struct Biol **172**(3): 331-342.
Kotani, T., H. Yurimoto*, et al.* (2007). Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia sp.* strain TY-5. J Bacteriol **189**(3): 886-893.
Kounaves, S. P., S. T. Stroble*, et al.* (2010). Discovery of natural perchlorate in the antarctic dry valleys and its global implications. Environ Sci Technol **44**(7): 2360-2364.
Kraft, B., M. Strous*, et al.* (2011). Microbial nitrate respiration – Genes, enzymes and environmental distribution. J Biotechnol **155**(1): 104-117.
Krieger, C. J., H. R. Beller*, et al.* (1999). Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus sp.* strain T. J Bacteriol **181**(20): 6403-6410.
Krogh, A., B. Larsson*, et al.* (2001). Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. J Mol Biol **305**(3): 567-580.
Kube, M., J. Heider*, et al.* (2004). Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. Arch Microbiol **181**(3): 182-194.
Kučera, I. (2006). Interference of chlorate and chlorite with nitrate reduction in resting cells of *Paracoccus denitrificans*. Microbiology **152**(12): 3529-3534.
Kühner, S., L. Wöhlbrand*, et al.* (2005). Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J Bacteriol **187**(4): 1493-1503.
Kunapuli, U., C. Griebler*, et al.* (2008). Identification of intermediates formed during anaerobic benzene degradation by an iron-reducing enrichment culture. Environ Microbiol **10**(7): 1703-1712.
Kunapuli, U., M. K. Jahn*, et al.* (2010). *Desulfitobacterium aromaticivorans* sp. nov. and *Geobacter toluenoxydans* sp. nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic compounds. Int J Syst Evol Microbiol **60**(3): 686-695.
Lagesen, K., P. Hallin*, et al.* (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res **35**(9): 3100-3108.
Lajoie, C. A., A. C. Layton*, et al.* (2000). Zoogleal clusters and sludge dewatering potential in an industrial activated-sludge wastewater treatment plant. Water Environ Res **72**(1): 56-64.
Lalucat, J., A. Bennasar*, et al.* (2006). Biology of *Pseudomonas stutzeri*. Microbiol Mol Biol Rev **70**(2): 510-547.
Leahy, J. G., P. J. Batchelor*, et al.* (2003). Evolution of the soluble diiron monooxygenases. FEMS Microbiol Rev **27**(4): 449-479.
Leahy, J. G. and R. R. Colwell (1990). Microbial degradation of hydrocarbons in the environment. Microbiol Rev **54**(3): 305-315.
Lee, A. Q., B. R. Streit*, et al.* (2008). Mechanism of and exquisite selectivity for O-O bond formation by the heme-dependent chlorite dismutase. Proc Nat Acad Sci USA **105**(41): 15654-15659.
Leuthner, B. and J. Heider (2000). Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of β oxidation of the intermediate benzylsuccinate. J Bact **182**(2): 272-277.

Leuthner, B., C. Leutwein*, et al.* (1998). Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol **28**(3): 615-628.
Leutwein, C. and J. Heider (1999). Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and ß-oxidation of the first intermediate, (R)-(+)-benzylsuccinate. Microbiology **145**(11): 3265-3271.
Liebensteiner, M. G., M. W. H. Pinkse*, et al.* (2013). Archaeal (per)chlorate reduction at high temperature: an interplay of biotic and abiotic reactions. Science **340**(6128): 85-87.
Liu, B., A. Frostegard*, et al.* (2013). Draft genome sequences of five strains in the Genus *Thauera*. Genome Announc **1**(1): DOI: 10.1128/genomeA.00052-00012.
Liu, H., R. G. Sadygov*, et al.* (2004). A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem **76**(14): 4193-4201.
Liu, X. and J. A. DeMoss (1997). Characterization of NarJ, a system-specific chaperone required for nitrate reductase biogenesis in *Escherichia coli*. J Biol Chem **272**(39): 24266-24271.
Liu, X., S. M. Tiquia*, et al.* (2003). Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off the Pacific coast of Mexico. Appl Environ Microbiol **69**(6): 3549-3560.
Löffler, C., K. Kuntze*, et al.* (2011). Occurence, genes and expression of the W/Se-containing class II benzoyl-coenzyme A reductases in anaerobic bacteria. Environ Microbiol **13**(3): 696-709.
Logan, B. E. (1998). A review of chlorate- and perchlorate-respiring microorganisms. Bioremed J **2**(2): 69-79.
Logan, B. E. and J. Wu (2002). Enhanced toluene degradation under chlorate-reducing conditions by bioaugmentation of sand columns with chlorate- and toluene-degrading enrichments. Bioremed J **6**(2): 87-95.
Lovley, D. R., S. J. Giovannoni*, et al.* (1993). *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch Microbiol **159**(4): 336-344.
Lovley, D. R., T. Ueki*, et al.* (2011). *Geobacter*: the microbe electric's physiology, ecology, and practical applications. Adv Microb Physiol **59**: 1-100.
Lowe, T. M. and S. R. Eddy (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res **25**(5): 955-964.
Lu, J., S. Boeren*, et al.* (2011). Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins. J Proteomics **75**(1): 34-43.
Maixner, F., M. Wagner*, et al.* (2008). Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium '*Candidatus Nitrospira defluvii*'. Environ Microbiol **10**(11): 3043-3056.
Makkar, N. S. and L. E. Casida (1987). *Cupriavidus necator* gen. nov., sp. nov.; a nonobligate bacterial predator of bacteria in soil. Int J Syst Bacteriol **37**(4): 323-326.
Malmqvist, A., T. Welander*, et al.* (1991). Anaerobic growth of microorganisms with chlorate as electron acceptor. Appl Environ Microbiol **57**(8): 2229-2232.
Malmqvist, A., T. Welander*, et al.* (1994). *Ideonella dechloratans*, gen. nov., sp. nov., a new bacterium capable of growing anaerobically with chlorate as an electron acceptor. Syst Appl Microbiol **12**: 58-64.
Margulies, M., M. Egholm*, et al.* (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature **437**(7057): 376-380.
Martinez-Espinosa, R. M., E. J. Dridge*, et al.* (2007). Look on the positive side! The orientation, identification and bioenergetics of 'Archaeal' membrane-bound nitrate reductases. FEMS Microbiol Lett **276**(2): 129-139.
Martínez Murillo, F., T. Gugliuzza*, et al.* (1999). A heme-C-containing enzyme complex that exhibits nitrate and nitrite reductase activity from the dissimilatory iron-reducing bacterium *Geobacter metallireducens*. Arch Microbiol **172**(5): 313-320.

Mattie, D. R., J. Strawson*, et al.* (2006). Perchlorate toxicity and risk assessment. Perchlorate: environmental occurrence, interactions and treatment. B. Gu and J. D. Coates. New York, NY, USA, Springer Science+Business Media, Inc.**:** 169-196.
McClintock, B. (1950). The origin and behaviour of mutable loci in maize. Proc Natl Acad Sci USA **36**(6): 344-355.
Mechichi, T, E . Stackebrandt*, et al.* (2003). *Alicycliphilus denitrificans* gen. nov., sp. nov., a cyclohexanol-degrading, nitrate-reducing β-proteobacterium. Int J Syst Evol Microbiol **53**(1): 147-152.
Mechichi, T., E. Stackebrandt*, et al.* (2002). Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. Arch Microbiol **178**(1): 26-35.
Mehboob, F. (2010). Anaerobic microbial degradation of organic pollutants with chlorate as electron acceptor. PhD thesis, Laboratory of Microbiology, Wageningen.
Mehboob, F., H. Junca*, et al.* (2009). Growth of *Pseudomonas chloritidismutans* AW-1 on *n*-alkanes with chlorate as electron acceptor. Appl Microbiol Biotechnol **83**(4): 739-747.
Mehboob, F., S. A. Weelink*, et al.* (2009). Microbial degradation of aliphatic and aromatic hydrocarbons with (per)chlorate as electron acceptor. Handbook of hydrocarbon and lipid microbiology K. N. Timmis. Berlin / Heidelberg, Springer-Verlag**:** 935-945.
Mehboob, F., A. F. Wolterink*, et al.* (2009). Purification and characterization of a chlorite dismutase from *Pseudomonas chloritidismutans*. FEMS Microbiol Lett **293**(1): 115-121.
Melnyk, R. A., A. Engelbrektson*, et al.* (2011). A comparative analysis of the genomes of four perchlorate reducers reveals a genomic island with novel regulatory and metabolic genes. Appl Environ Microbiol **77**: 7401-7404.
Miller, J. P. and B. E. Logan (2000). Sustained perchlorate degradation in an autotrophic, gas-phase, packed-bed bioreactor. Environ Sci Technol **34**: 3018-3022.
Mlynek, G., B. Sjöblom*, et al.* (2011). Unexpected diversity of chlorite dismutases: a catalytically efficient dimeric enzyme from *Nitrobacter winogradskyi*. J Bacteriol **193**(10): 2408-2417.
Moreno-Vivián, C., P. Cabello*, et al.* (1999). Prokaryote nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J Bacteriol **181**: 6573-6584.
Morikawa, M. (2010). Dioxygen activation responsible for oxidation of aliphatic and aromatic hydrocarbon compounds: current state and variants. Appl Microbiol Biotechnol **87**(5): 1595-1603.
Morozkina, E. V. and R. A. Zvyagilskaya (2007). Nitrate reductases: structure, functions, and effect of stress factors. Biochem **72**: 1151-1160.
Morris, R. L. and T. M. Schmidt (2013). Shallow breathing: bacterial life at low O_2. Nat Rev Microbiol **11**(3): 205-212.
Moura, J. J. G., C. D. Brondino*, et al.* (2004). Mo and W bis-MGD enzymes: nitrate reductases and formate dehydrogenases. J Biol Inorg Chem **9**(7): 791-799.
Mouttaki, H., J. Johannes*, et al.* (2012). Identification of naphthalene carboxylase as a prototype for the anaerobic activation of non-substituted aromatic hydrocarbons. Environ Microbiol **14**(10): 2770-2774.
Mukherjee, A. and N. Bordoloi (2012). Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium. Environ Sci Pollut Res Int **19**(8): 3380-3388.
Musat, F. and F. Widdel (2008). Anaerobic degradation of benzene by a marine sulfate-reducing enrichment culture, and cell hybridization of the dominant phylotype. Environ Microbiol **10**(1): 10-19.
Muyzer, G., E. C. de Waal*, et al.* (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59**(3): 695-700.

Nagata, Y., R. Ohtomo*, et al.* (1994). Cloning and sequencing of a 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase gene involved in the degradation of gamma-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J Bacteriol **176**(11): 3117-3125.
Nakagawa, H. and N. Yamashita (1986). Chlorate reducing activity of spinach nitrate reductase. Agric Biol Chem **50**(7): 1893-1894.
Neidle, E. L., C. Hartnett*, et al.* (1991). Nucleotide sequences of the *Acinetobacter calcoaceticus benABC* genes for benzoate 1,2-dioxygenase reveal evolutionary relationships among multicomponent oxygenases. J Bacteriol **173**(17): 5385-5395.
Nepomnyashchaya, Y. N., G. B. Slobodkina*, et al.* (2011). *Moorella humiferrea* sp. nov., a novel thermophilic anaerobic, bacterium capable of growth via electron shuttling between humic acid and Fe(III). Int J Syst Evol Microbiol **62**(3): 613-617.
Newman, L. M. and L. P. Wackett (1995). Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry **34**(43): 14066-14076.
Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev **67**(4): 593-656.
Nilsson, T., M. Rova*, et al.* (2012). Microbial metabolism of oxochlorates: a bioenergetic perspective. Biochim Biophys Acta doi:10.1016/j.bbabio.2012.06.010.
NIST-MML (2007). IUPAC-NIST solubility database, version 1.0, IUPAC-NIST solubility dataseries.
Norris, D. B. and P. W. Trudgill (1971). The metabolism of cyclohexanol by *Nocardia globerula* CL1. Biochem J **121**(3): 363-370.
Nozawa-Inoue, M., K. M. Scow*, et al.* (2005). Reduction of perchlorate and nitrate by microbial communities in vadose soil. Appl Environ Microbiol **71**(7): 3928-3934.
O'Reilly, C. and E. Colleran (2005). Toxicity of nitrite toward mesophilic and thermophilic sulfate-reducing, methanogenic and syntrophic populations in anaerobic sludge. J Ind Microbiol Biotechnol **32**(2): 46-52.
Okeke, B. C. and J. W. T. Frankenberger (2003). Molecular analysis of a perchlorate reductase from a perchlorate-respiring bacterium Perc1ace. Microbiol Res **158**(4): 337-344.
Oltmann, L. F., W. N. M. Reijnders*, et al.* (1976). Characterization of purified nitrate reductase A and chlorate reductase C from *Proteus mirabilis*. Arch Microbiol **111**(1): 25-35.
Oosterkamp, M. J., S. Boeren*, et al.* (2013). Metabolic response of *Alicycliphilus denitrificans* strain BC toward electron acceptor variation. Prot J: doi: 10.1002/pmic.201200571.
Oosterkamp, M. J., F. Mehboob*, et al.* (2011). Nitrate and (per)chlorate reduction pathways in (per)chlorate-reducing bacteria. Biochem Soc Trans **39**(1): 230-235.
Oosterkamp, M. J., T. Veuskens*, et al.* (2011). Genome sequences of *Alicycliphilus denitrificans* strains BC and K601^T. J Bacteriol **193**(18): 5028-5029.
Oosterkamp, M. J., T. Veuskens*, et al.* (2013). Genome analysis and physiological comparison of *Alicycliphilus denitrificans* strains BC and K601^T. PLoS One **8**(6): e66971.
Orris, G. J., G. J. Harvey*, et al.* (2003). Preliminary analyses for perchlorate in selected natural materials and their derivative products. Open-File Report 03-314.
Ostertag, E. M. and H. H. Kazazian Jr (2001). Biology of Mammalian L1 retrotransposons. Ann Rev Gen **35**(1): 501-538.
Pati, A., N. N. Ivanova*, et al.* (2010). GenePRIMP: a gene prediction improvement pipeline for prokaryotic genomes. Nat Methods **7**(6): 455-457.
Peng, G., H. Wang*, et al.* (2006). *Azospirillum melinis* sp. nov., a group of diazotrophs isolated from tropical molasses grass. Int J Syst Evol Microbiol **56**(6): 1263-1271.
Peng, K., J. W. M. van Lent*, et al.* (2012). Characterization of novel components of the baculovirus *per os* infectivity factor complex. J Virol **86**(9): 4981-4988.
Pérez-Pantoja, D., R. Donoso*, et al.* (2012). Genomic analysis of the potential for aromatic compounds biodegradation in *Burkholderiales*. Environ Microbiol **14**(5): 1091-1117.
Pérez-Pantoja, D., B. Gonzáles*, et al.* (2010). Aerobic degradation of aromatic hydrocarbons. Handbook of Hydrocarbon and Lipid Microbiology. K. N. Timmis. Berlin, Germany, Springer-Verlag Berlin Heidelberg**:** 799-837.

Peters, F., Y. Shinoda*, et al.* (2007). Cyclohexa-1,5-diene-1-carbonyl-coenzyme A (CoA) hydratases of *Geobacter metallireducens* and *Syntrophus aciditrophicus*: evidence for a common benzoyl-CoA degradation pathway in facultative and strict anaerobes. J Bacteriol **189**(3): 1055-1060.
Philippot, L., P. Mirleau*, et al.* (2001). Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the *nar, nir, nor* and *nos* genes. Biochim Biophys Acta **1517**(3): 436-440.
Pilloni, G., F. von Netzer*, et al.* (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. FEMS Microbiol Ecol **78**(1):165-175.
Platen, H. and B. Schink (1989). Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J Gen Microbiol **135**(4): 883-891.
Platen, H. and B. Schink (1990). Enzymes involved in anaerobic degradation of acetone by a denitrifying bacterium. Biodegradation **1**(4): 243-251.
Platen, H., A. Temmes*, et al.* (1990). Anaerobic degradation of acetone by *Desulfococcus biacutus* spec. nov. Arch Microbiol **154**(4): 355-361.
Polard, P., L. Seroude*, et al.* (1994). One-ended insertion of IS911. J Bacteriol **176**(4): 1192-1196.
Quastel, J. H., M. Stephenson*, et al.* (1925). Some reactions of resting bacteria in relation to anaerobic growth. Biochem J **19**(2): 304-317.
Rabus, R., M. Kube*, et al.* (2005). The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol **183**(1): 27-36.
Rabus, R. and F. Widdel (1995). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol **163**(2): 96-103.
Rabus, R., H. Wilkes*, et al.* (2001). Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: evidence for (1-Methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. J Bacteriol **183**(5): 1707-1715.
Reinhold-Hurek, B., T. Hurek*, et al.* (1993). *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca (L.) Kunth*), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int J Syst Bacteriol **43**(3): 574-584.
Richardson, D. J., B. C. Berks*, et al.* (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell Mol Life Sci **58**(2): 165-178.
Richardson, D. J., J. N. Butt*, et al.* (2012). The 'porin-cytochrome' model for microbe-to-mineral electron transfer. Mol Microbiol **85**(2): 201-212.
Richter, K., M. Schicklberger*, et al.* (2012). Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. Appl Environ Microbiol **78**(4): 913-921.
Richter, M. and R. Rosselló-Móra (2009). Shifting the genomic gold standard for the prokaryotic species definition. Proc Nat Acad Sci **106**(45): 19126-19131.
Rikken, G. B., A. G. M. Kroon*, et al.* (1996). Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Appl Microbiol Biotechnol **45**(3): 420-426.
Rojo, F. (2009). Degradation of alkanes by bacteria. Environ Microbiol **11**(10): 2477-2490.
Röling, W. F. M., B. M. van Breukelen*, et al.* (2001). Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. Appl Environ Microbiol **67**(10): 4619-4629.
Rooijers, K., C. Kolmeder*, et al.* (2011). An iterative workflow for mining the human intestinal metaproteome. BMC Genomics **12**(1): 6.
Rosier, C., N. Leys*, et al.* (2012). Purification and characterization of the acetone carboxylase of *Cupriavidus metallidurans* strain CH34. Appl Environ Microbiol **78**(12): 4516-4518.
Rosselló-Mora, R. A., J. Lalucat*, et al.* (1994). Biochemical and chemotaxonomic characterization of *Pseudomonas stutzeri* genomovars. J Appl Microbiol **76**(3): 226-233.
Rothery, R. A., A. Magalon*, et al.* (1998). The molybdenum cofactor of *Escherichia coli* nitrate reductase A (NarGHI). J Biol Chem **273**(13): 7462-7469.

Rousseau, P., C. Loot, *et al.* (2007). Control of IS911 target selection: how OrfA may ensure IS dispersion. Mol Microbiol **63**(6): 1701-1709.
Rousseau, P., C. Tardin, *et al.* (2010). A model for the molecular organisation of the IS911 transpososome. Mob DNA **1**(1): 16.
Rupakula, A., T. Kruse, *et al.* (2013). Evaluation of the restricted metabolism of the obligate organohalide respiring bacterium *Dehalobacter restrictus* - lessons from a tiered functional genomics approach. Philos Trans R Soc Lond B Biol Sci **368**(1616): 20120325.
Rusmana, I. and D. B. Nedwell (2004). Use of chlorate as a selective inhibitor to distinguish membrane-bound nitrate reductase (Nar) and periplasmic nitrate reductase (Nap) of dissimilative nitrate reducing bacteria in sediment. FEMS Microbiol Ecol **48**(3): 379-386.
Sacks, L. E. and H. A. Barker (1949). The influence of oxygen on nitrate and nitrite reduction. J Bacteriol **58**(1): 11-22.
Sadygov, R. G., D. Cociorva, *et al.* (2004). Large-scale database searching using tandem mass spectra: Looking up the answer in the back of the book. Nat Meth **1**(3): 195-202.
Salinero, K., K. Keller, *et al.* (2009). Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. BMC Genomics **10**(1): 351.
Sanguinetti, C. J., E. Dias Neto, *et al.* (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques **17**(5): 914-921.
Schreiber, K., R. Krieger, *et al.* (2007). The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. J Bacteriol **189**(11): 4310-4314.
Schühle, K. and J. Heider (2012). Acetone and butanone metabolism of the denitrifying bacterium *"Aromatoleum aromaticum*" demonstrates novel biochemical properties of an ATP-dependent aliphatic ketone carboxylase. J Bacteriol **194**(1): 131-141.
Schuetz, B., M. Schicklberger, *et al.* (2009). Periplasmic electron transfer via the *c*-type cytochromes MtrA and FccA of *Shewanella oneidensis* MR-1. Appl Environ Microbiol **75**(24): 7789-7796.
Schwanhausser, B., D. Busse, *et al.* (2011). Global quantification of mammalian gene expression control. Nature **473**(7347): 337-342.
Schwarz, A. O., H. Urrutia, *et al.* (2012). Chlorate reduction capacity and characterisation of chlorate reducing bacterial communities in sediments of the rio Cruces wetland in southern Chile. Water Res **46**(10): 3283-3292.
Shapiro, J. A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc Nat Acad Sci USA **76**(4): 1933-1937.
Shingler, V. and T. Moore (1994). Sensing of aromatic compounds by the DmpR transcriptional activator of phenol-catabolizing *Pseudomonas sp*. strain CF600. J Bacteriol **176**(6): 1555-1560.
Siegel, J. M. (1957). The dark anaerobic metabolism of acetone and acetate by the photosynthetic bacterium *Rhodopseudomonas gelatinosa*. J Biol Chem **228**(1): 41-47.
Simon, J., M. Sänger, *et al.* (2004). Electron transport to periplasmic nitrate reductase (NapA) of *Wollinella succinogenes* is independent of a NapC protein. Mol Microbiol **49**: 69-79.
Singh, H. B., M. Kanakidou, *et al.* (1995). High concentrations and photochemical fate of oxygenated hydrocarbons in the global troposphere. Nature **378**(6552): 50-54.
Skerman, V. B. D. and I. C. MacRae (1957). The influence of oxygen availability on the degree of nitrate reduction by *Pseudomonas denitrificans*. Can J Microbiol **3**(3): 505-530.
Sluis, M. K., R. A. Larsen, *et al.* (2002). Biochemical, molecular, and genetic analyses of the acetone carboxylases from *Xanthobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10. J Bacteriol **184**(11): 2969-2977.
Smaczniak, C., N. Li, *et al.* (2012). Proteomics-based identification of low-abundant signalling and regulatory protein complexes in native plant tissues. Nat Protoc **7**(12): 2144-2158.
So, C. M., C. D. Phelps, *et al.* (2003). Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. Appl Environ Microbiol **69**(7): 3892-3900.

Soldin, O. P., L. E. Braverman*, et al.* (2001). Perchlorate clinical pharmacology and human health: a review. Ther Drug Monit **23**(4): 316-331.
Solomonson, L. P. and B. Vennesland (1972). Nitrate reductase and chlorate toxicity in *Chlorella vulgaris* Beijerinck. Plant Physiol **50**(4): 421-424.
Spormann, A. M. and F. Widdel (2000). Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation **11**(2): 85-105.
Staats, M. , M. Braster*, et al.* (2011). Molecular diversity and distribution of aromatic hydrocarbon-degrading anaerobes across a landfill leachate plume. Environ Microbiol **13**(5): 1216-1227.
Steinberg, L. M., J. J. Trimble*, et al.* (2005). Enzymes responsible for chlorate reduction by *Pseudomonas* sp. are different from those used for perchlorate reduction by *Azospira* sp. FEMS Microbiol Lett **247**(2): 152-159.
Stenklo, K., H. Danielsson Thorell*, et al.* (2001). Chlorite dismutase from *Ideonella dechloratans*. J Biol Inorg Chem **6**(5): 601-607.
Stepanyuk, V. V., G. F. Smirnova*, et al.* (1992). *Acinetobacter thermotoleranticus* sp. nov., a new species of *Acinetobacter* genus. Mikrobiologiya **61**: 490-500.
Stetter, K. O., G. Lauerer*, et al.* (1987). Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of archaebacteria. Science **236**(4803): 822-824.
Stolz, J. F. and P. Basu (2002). Evolution of nitrate reductase: molecular and structural variations on a common function. Chembiochem **3**(2-3): 198-206.
Streit, B. R. and J. L. DuBois (2008). Chemical and steady-state kinetic analyses of a heterologously expressed heme dependent chlorite dismutase. Biochemistry **47**(19): 5271-5280.
Suenaga, H., Y. Koyama*, et al.* (2009). Novel organization of aromatic degradation pathway genes in a microbial community as revealed by metagenomic analysis. ISME J **3**(12): 1335-1348.
Sun, W., X. Sun*, et al.* (2013). Presence, diversity and enumeration of functional genes (bssA and bamA) relating to toluene degradation across a range of redox conditions and inoculum sources. Biodegradation: DOI:10.1007/s10532-10013-19651-10534.
Suzuki, M. T., O. Béjà*, et al.* (2001). Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environ Microbiol **3**(5): 323-331.
Suzuki, M. T. and S. J. Giovannoni (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol **62**(2): 625-630.
Tago, K., S. Ishii*, et al.* (2011). Phylogenetic and functional diversity of denitrifying bacteria isolated from various rice paddy and rice-soybean rotation fields. Microb Environ **26**(1): 30-35.
Tarlera, S. and E. B. M. Denner (2003). *Sterolibacterium denitrificans* gen. nov., sp. nov., a novel cholesterol-oxidizing, denitrifying member of the β-Proteobacteria. Int J Syst Evol Microbiol **53**(4): 1085-1091.
Taylor, D. G., P. W. Trudgill*, et al.* (1980). The microbial metabolism of acetone. J Gen Microbiol **118**(1): 159-170.
Taylor, M. C., J. F. Whitte*, et al.* (1940). Sodium chlorite properties and reactions. Ind Eng Chem **32**(7): 899-903.
Terpolilli, J. J., G. A. Hood*, et al.* (2012). Chapter 5 - What determines the efficiency of N_2-fixing *Rhizobium*-legume symbioses? Advances in Microbial Physiology. K. P. Robert, Academic Press. **60:** 325-389.
Thauer, R. K., K. Jungermann*, et al.* (1977). Energy conservation in anaerobic chemotrophic bacteria. Bacteriol Rev **41**(1): 100-180.
Thompson, J. D., T. J. Gibson*, et al.* (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res **25**(24): 4876-4882.
Thony-Meyer, L. (1997). Biogenesis of respiratory cytochromes in bacteria. Microbiol Mol Biol Rev **61**(3): 337-376.
Thorell, H. D., J. Karlsson*, et al.* (2002). Cloning, characterisation, and expression of a novel gene encoding chlorite dismutase from *Ideonella dechloratans*. Biochim Biophys Acta **1577**(3): 445-451.

Thorell, H. D., K. Stenklo*, et al.* (2003). A gene cluster for chlorate metabolism in *Ideonella dechloratans*. Appl Environ Microbiol **69**(9): 5585-5592.
Trudgill, P. W. (1984). Microbial degradation of the acyclic ring. Microbial degradation of organic compounds. D. T. Gibson. New York, NY, USA, Marcel Dekker.
Urbansky, E. T. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremed J **2**(2): 81-95.
Vaillancourt, F. H., J. T. Bolin*, et al.* (2006). The ins and outs of ring-cleaving dioxygenases. Crit Rev Biochem Mol Biol **41**(4): 241-267.
Vizcaíno, J. A., R. G. Côté*, et al.* (2013). The Proteomics Identifications (PRIDE) database and associated tools: status in 2013. Nucl Acids Res **41**(D1): D1063-D1069.
Vogt, C., S. Kleinsteuber*, et al.* (2011). Anaerobic benzene degradation by bacteria. Microb Biotechnol **4**(6): 710-724.
Wallace, W., T. Ward*, et al.* (1996). Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*. J Indust Microbiol Biotechnol **16**(1): 68-72.
Weelink, S., M. van Eekert*, et al.* (2010). Degradation of BTEX by anaerobic bacteria: physiology and application. Rev Environ Sci Biotechnol **9**(4): 359-385.
Weelink, S. A. B., N. C. G. Tan*, et al.* (2008). Isolation and characterization of *Alicycliphilus denitrificans* strain BC that grows on benzene with chlorate as electron acceptor. Appl Environ Microbiol **72**(21): 6672-6681.
Weelink, S. A. B., N. C. G. Tan*, et al.* (2007). Physiological and phylogenetic characterization of a stable benzene-degrading, chlorate-reducing microbial community. FEMS Microbiol Ecol **60**(2): 312-321.
Weelink, S. A. B., W. Van Doesburg*, et al.* (2009). A strictly anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. FEMS Microbiol Ecol **70**(3): 575-585.
Weingarten, R. A., M. E. Taveirne*, et al.* (2009). The dual-functioning fumarate reductase is the sole succinate:quinone reductase in *Campylobacter jejuni* and is required for full host colonization. J Bacteriol **191**(16): 5293-5300.
Wentzel, A., T. Ellingsen*, et al.* (2007). Bacterial metabolism of long-chain *n*-alkanes. Appl Microbiol Biotechnol **76**(6): 1209-1221.
Westra, E. R., D. C. Swarts*, et al.* (2012). The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. Annu Rev Genet **46**: 311-339.
Wicker, T., F. Sabot*, et al.* (2007). A unified classification system for eukaryotic transposable elements. Nat Rev Genet **8**(12): 973-982.
Widdel, F. and R. Rabus (2001). Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol **12**(3): 259-276.
Wijk, D. J. v. and T. H. Hutchinson (1995). The ecotoxicity of chlorate to aquatic organisms: a critical review. Ecotoxicol Environ Saf **32**(3): 244-253.
Wöhlbrand, L., J. H. Jacob*, et al.* (2012). Complete genome, catabolic sub-proteomes and key-metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. Environ Microbiol **15**(5): 1334-1355.
Wolff, J. (1998). Perchlorate and the thyroid gland. Pharmacol Rev **50**(1): 89-106.
Wolterink, A., S. Kim*, et al.* (2005). *Dechloromonas hortensis* sp. nov. and strain ASK-1, two novel (per)chlorate-reducing bacteria, and taxonomic description of strain GR-1. Int J Syst Evol Microbiol **55**(5): 2063-2068.
Wolterink, A. F. W. M., A. B. Jonker*, et al.* (2002). *Pseudomonas chloritidismutans* sp. nov., a non-denitrifying, chlorate-reducing bacterium. Int J Syst Evol Microbiol **52**(6): 2183-2190.
Wolterink, A. F. W. M., E. Schiltz*, et al.* (2003). Characterization of the chlorate reductase from *Pseudomonas chloritidismutans*. J Bacteriol **185**(10): 3210-3213.

Worm, P., A. J. M. Stams*, et al.* (2011). Growth- and substrate-dependent transcription of formate dehydrogenase and hydrogenase coding genes in *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. Microbiology **157**(1): 280-289.
Wu, M. L., K. F. Ettwig*, et al.* (2011). A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium *Candidatus 'Methylomirabilis oxyfera'*. Biochem Soc Trans **39**: 243-248.
Xu, J., Y. Song*, et al.* (2003). Microbial degradation of perchlorate: principles and applications. Environ Eng Sci **20**(5): 405-422.
Xu, J., J. J. Trimble*, et al.* (2004). Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA. Water Res **38**(3): 673-680.
Yan, Y., J. Yang*, et al.* (2008). Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. Proc Nat Acad Sci **105**(21): 7564-7569.
Yu, H., M. Yuan*, et al.* (2011). Complete genome sequence of the nitrogen-fixing and rhizosphere-associated bacterium *Pseudomonas stutzeri* strain DSM4166. J Bacteriol **193**(13): 3422-3423.
Zaan, B. M. v. d., F. Talarico Saia*, et al.* (2012). Anaerobic benzene degradation under denitrifying conditions: *Peptococcaceae* as dominant benzene degraders and evidence for a syntrophic process. Environ Microbiol **14**(5): 1171-1181.
Zaar, A., W. Eisenreich*, et al.* (2001). A novel pathway of aerobic benzoate catabolism in the bacteria *Azoarcus evansii* and *Bacillus stearothermophilus*. J Biol Chem **276**(27): 24997-25004.
Zedelius, J., R. Rabus*, et al.* (2011). Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. Env Microbiol Rep **3**: 125-135.
Zerbino, D. R. and E. Birney (2008). Velvet: algorithms for *de novo* short read assembly using de Bruijn graphs. Genome Res **18**(5): 821-829.
Zhao, B. and C. L. Poh (2008). Insights into environmental bioremediation by microorganisms through functional genomics and proteomics. Proteomics **8**(4): 874-881.
Zoetendal, E. G., C. C. G. M. Booijink*, et al.* (2006). Isolation of RNA from bacterial samples of the human gastrointestinal tract. Nat Protocols **1**(2): 954-959.
Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev **61**(4): 533-616.

Publications of the author

Oosterkamp, M. J., S. Boeren, C.M. Plugge, P.J.Schaap and A.J. Stams (2013). Metabolic response of *Alicycliphilus denitrificans* strain BC toward electron acceptor variation. Proteomics 13(18-19): 2886-2894.

Oosterkamp, M. J., T. Veuskens, F. Talarico Saia, S.A. Weelink, L.A. Goodwin, H.E. Daligault, D.C. Bruce, J.C. Detter, R. Tapia, C.S. Han, M.L. Land, L.J. Hauser, A.A. Langenhoff, J. Gerritse, W.J. van Berkel, D.H. Pieper, H. Junca, H. Smidt, G. Schraa, M. Davids, P.J. Schaap, C.M. Plugge and A.J. Stams (2013). Genome analysis and physiological comparison of *Alicycliphilus denitrificans* strains BC and K601^{T}. PLoS One 8(6): e66971.

Oosterkamp, M. J., T. Veuskens, C.M. Plugge, A.A. Langenhoff, J. Gerritse, W.J. van Berkel, D.H. Pieper, H. Junca, L.A. Goodwin, H.E. Daligault, D.C. Bruce, J.C. Detter, R. Tapia, C.S. Han, M.L. Land, L.J. Hauser, H. Smidt and A.J. Stams (2011). Genome sequences of *Alicycliphilus denitrificans* strains BC and K601^{T}. J Bacteriol 193(18): 5028-5029.

Oosterkamp, M. J., F. Mehboob, G. Schraa, C.M. Plugge and A.J. Stams (2011). Nitrate and (per)chlorate reduction pathways in (per)chlorate-reducing bacteria. Biochem Soc Trans 39(1): 230-235.

Oudejans, C.B., M. van Dijk, **M. Oosterkamp**, A. Lachmeijer and M.A. Blankenstein (2007). Genetics of preeclampsia: paradigm shifts. Hum Genet 120(5): 607-612.

About the author

Margreet Jeannet (Marjet) Oosterkamp was born in Emmeloord, Noordoostpolder, The Netherlands, on September 12 in 1980. She graduated from the high school (VWO) Emelwerda College (Emmeloord, The Netherlands) in 1999. Marjet studied Biology at the the Rijksuniversiteit Groningen (The Netherlands). She did her internal study in the Microbial Physiology group with Dr. Geertje van Keulen and Prof. Lubbert Dijkhuizen in 2003-2004. This research focused on the anaerobic metabolism of the filamentous bacterium *Streptomyces coelicolor*. Her external study was performed at the University College Dublin (Ireland) in the Molecular Microbiology group with Dr. Ruth Fahey and Prof. Wim Meijer in 2004-2005. She focused on the oxidative stress response of the equine pathogen *Rhodococcus equi*. Marjet obtained her drs (or MSc) degree in 2005. That same year she started to work at the VUmc (Free University medical center) in Amsterdam (The Netherlands). She joined the Molecular Biology Laboratory of the department of Clinical Chemistry and worked with Dr. Marie van Dijk and Prof. Cees Oudejans. The research aimed to reveal the genetic cause of the HELLP-syndrome, a pregnancy-related disease. Marjet joined an international conference (in Kingston, Canada) and several national conferences. She decided to change from this human genetic to microbiological research and obtained a position as a doctoral student at Wageningen University (The Netherlands) in the Microbial Physiology group in 2008. She was supervised by Prof. Fons Stams, Dr. Caroline Plugge and Dr. Peter Schaap. Marjet went to several European and national conferences and joined a PhD trip to universities and institutes at the East coast of the USA. The research project 'Application of chlorate reduction as a novel technique for bioremediation of anaerobic soils' that Marjet took part in and that is summarized in this thesis, was funded by the technology foundation STW and ended in 2012. In 2013, Marjet became postdoctoral researcher at the Department of Animal Sciences and Institute of Genomic Biology of the University of Illinois at Urbana-Champaign (USA), where she is currently working. Her project is funded by the Energy Biosciences Institute and focuses on methane production from wastes of industrial ethanol distillation.

Acknowledgements

It was a pleasure for me to work on the project that is summarized in this thesis and in the Laboratory of Microbiology. I met many challenges, but had a great time during my PhD period. The project amazed me, made me run around, often tired me out, shrunk hours, made me enthusiast, always wanted me to do more, always wanted me to dive into the literature just a little bit longer. It was great fun. The labtrips, BBQs, the Veluweloop, the bowling-and-wokken trips and 'Christmas'dinners with micfys, Wednesday-evening film nights, kestrels on the screen in the canteen and other work-related parties and dinners and activities that I have forgotten to include, helped to make working a much greater pleasure than I could have imagined when I started the project. In this part of the thesis I will take the opportunity to thank people who helped me during this project and the ones who contributed to the project otherwise in the past time.

First of all, **Fons**, my supervisor and promotor. It has been a pleasure for me to work with you. Thank you for giving me the opportunity to join your excellent group in the beginning of 2008. Thank you so much for your support, listening ear, supervision and advice along the road of this doctoral project. Unforgettable for me is the moment that you were working in the lab and I asked you 'Do you have enough space Fons?', and you replied: 'Yes, I am a micro<u>biologist</u>! I do not need a lot of space.' Thank you for always being open and within reach, *in vivo* or *in silico* if you were elsewhere. Lots of success and good luck. **Caroline**, my co-supervisor and co-promotor, thank you for the gezelligheid, the discussions, help, advice and for sharing your stories, about so many things like sailing and Amstelveen. I appreciated your direct approach, it allowed me to still change direction or perspective before it was too late. Thank you for giving me the opportunity to be a supervisor in the MIB30303-course, I enjoyed that and it gave me the opportunity to get more experience in supervising students in a great way. Good luck for the future. **Peter**, thank you for your contribution and advice and thank you for becoming my co-promotor. Maybe the computer is indeed 'a stupid machine'. Still, I find it remarkable what you can do with it and I appreciate it very much that you were willing to help me out during my project. I hope that the amazing computer of yours will give you lots of fun and success. Good luck! Thanks to **STW** and the companies and foundations involved that took care of the financial aspects of this doctoral project. Thanks to the **users committee** of STW-chlorate for their contributions at the half-yearly meetings and for the many good discussions (that usually created anaerobic conditions in the meeting room). Thanks also for the help offered besides the meetings (especially **Hauke, Jan, Alette** and **Bart**). **Teun**, also doctoral student in this STW-project, good luck with everything!

Ton and **Aura**, thanks for your help, for answering my many questions. I am really happy that you accepted my invitation to become my paranimphs. Thanks for all help related to that and for the gezelligheid, for all stories, for all tips and advice. Without Ton I never would have known how useful an elastic band can be! Thanks for helping me with the GCs and HPLCs and with other things. Thanks for your elaborate explanation about the do's and don'ts of the micfys lab, it has been a perfect start for the work in the lab that saved me lots of searching and questioning. Aura it was a pleasure that you joined me in the HitChip lab. The racks for gloves were indeed very handy! I really enjoyed to discuss my issues with you. Thanks for your help with the qPCR and for all kinds of other advices that you gave me. Ton and Aura, lots of success. **Hans**, **Ineke**, **Wilma**, and **Philippe**, thanks for all help and for answering my questions, mainly about DGGE and the RNA/qRT-PCR work. Thanks for the gezelligheid in the HitChip lab. I liked to see how you were working, how you keep up-to-date with all kinds of handy tools and machines and how you keep a systematic and clear overview of the work for the chips, with all the schedules taped on the cupboards. Lots of success. **Sjon** thank you for helping me out when I had questions, with help for finding and ordering things related to molecular biology techniques, but also for your stories. I really liked to hear about the music you prefer to listen to and also the festivals and concerts that you visit. Once in a while I also like to hear that kind of music, although clearly not as much as you. Your knowledge about the music and the people involved is amazing! Thanks to **Ruud** for help with the experion and to motorbiker **Tom** (you see, I did not forget to put you in the acknowledgements) for answering my questions. **Anja,**

Nees and **Carolien**, thanks for your gezelligheid and help and special thanks to Anja for help with the reading version of the thesis. **Wim**, you are someone that I certainly should not forget. You helped me out so many times! Thanks for the gezellige conversations and for help with the computer, the network and all that has to do with that and also for all the rest of your help. For example, one day you helped me out of misery with a tool that could be used to remove the rotor that was stuck in the Sorvall centrifuge. Lots of success!

Rozelin, thank you so much for your friendship. I am very grateful for our conversations about friendship and all other important things in life. I'm storing them in an imaginary pink box. Thank you for your support and for allowing me to enjoy your excellent (Turkish) cooking skills every now and then. **Ana**, thank you for the many nice discussions. Thanks for your friendship, for the stories of fado and codfish. Thanks for bringing lovely Portugese cakes. Thanks for enjoying talking, for making difficult matters easier to bare. Thanks for your support and I hope that one day you will have a beautiful, big and fat cat. **Petra**, thanks for your friendship, for the gezelligheid, for your help and support. It was nice to organize the symposium with you. Finally, thanks for the guppies, I really enjoyed taking care of them. **Jeanine** and **Katrin**, thanks for the nice conversations and advice. Jeanine, thanks for your help, support and (hiking)stories. Katrin thanks for having me in Nottingham, I had a great time, thanks. Thanks for your happiness, for all the nice (I love apples) conversations and for your help. **Vicente**, it was nice to have you (and the plant) in the office. All the best for you and your PhD. I would also like to thank **Teun, Karen**, **Pierpaulo** and **Siavash** who I shared the office with in the basement for allowing me to be there during the last couple of months that I was working in Wageningen.

Thanks to the 'chlorate-team'. To **Flávia** and **Farrakh**, who I will never forget! You are two special people. I was very sad when you had to leave. You were always having fun. Farrakh with the many jokes, Flávia with her happy personality and goodmóóórnings. Thanks for all your help and support. Thanks also to **Martin**, **Teun**, **Bart**, **Gosse**, **Hauke**, **Caroline**, and **Fons** who were also part of the 'chlorate-team', for help and advice.

Martin, **Alona**, **Vicente** and **Samet**, it was nice to share supervision in the MIB30303 practical course with you. **Eve**, **Bas** and **Jueeli** thank you so much for helping me out in this project. I enjoyed teaching you. All the best of luck for developing your talents. Jueeli, it was nice to work with you, good luck for your PhD project at the Laboratory of Microbiology!

Wetsus bedankt voor de gastvrijheid, thanks to **Urania**, **Elsemiek** and **Astrid** for help with the EM pictures of strains BC and K601^{T}. These were initially for DOE JGI and are now also decorating the cover of this thesis. Urania, thank you for allowing me to crash at your place in Leeuwarden. Dat was heel gezellig! Thanks to Biqualis, and more specifically to **Sjef**, for the proteomics work.

I only mentioned now the people who were very close and/or important to me during my project, but there have been so much more other great people. It is difficult to fit all of your names and a couple of lines of writing in this acknowledgement. I hope you can understand that it is more convenient for me to thank you in person than here, in the thesis. People of micfys, people of moleco and bacgen, people of SSB, the many students that have been doing a project in microbiology, people of the OR AFSG, other staff and people associated to the Laboratory of Microbiology:

Thank you all so much!

There are also people from my private life who I will thank for their help and support during my PhD project.

Mijn vriendinnen **Mirjan** uit Haren en **Jeannet** uit Emmeloord, bedankt dat jullie me altijd zijn blijven steunen. Ik vind het geweldig dat alles goed is gebleven, ondanks dat onze levens in de loop van de tijd zo veranderd zijn en ondanks dat we elkaar veel minder vaak zien.

Mijn neef **Jeroen** en ook **Roelof ten Brinke** van **drukkerij Ten Brink in Meppel** wil ik heel hartelijk danken voor alle hulp bij het drukken van mijn proefschrift.

Schoonouders (**Henk** en **Thea**) en zwager **Mathijs**, dank voor alle gezelligheid en voor jullie belangstelling voor dit project. Dank voor de lekkere broden van de bakker die maar zo opeens in de vriezer lagen als we thuiskwamen na het werk. Dank dat we regelmatig mochten komen genieten van die lekkere zelfgemaakte erwtensoep, boerenkool en alle andere met zoveel zorg gemaakte gerechten en het barbecueën op de plaat. Jullie zijn me erg dierbaar.

Mark en **Lara**, my brother and his girlfriend. You are the most perfect guides for Spain that I know. I wish you all the best and luck for life and work and hope that, next to all the many courses and things you do, you keep making music and performing. Thanks for your support during the past years and although it is not easy to meet you. Somehow it always feels like you are around. **Irene**, zus, jouw kracht zal mij altijd versteld doen blijven staan. Dank voor alle leuke vakanties samen, dank dat je ook volhield tijdens de Nijmeegse vierdaagse en we samen konden eindigen. Dank dat je me altijd zo hebt gesterkt om mijn eigen pad te volgen. Dank voor alles, dank voor je steun. Ik vind het geweldig om te zien wat je hebt bereikt en hoe jij je doelen behaalt. Heel veel liefs, geluk en succes! Lieve pap en mam (**Henk** en **Willy**), bedankt dat jullie me altijd hebben gesteund. Mam, bedankt voor de zorgvuldig verpakte en vaak zeer nuttige voedselvoorraadjes die je me vaak meegaf als ik na een bezoek in het weekend weer terug ging naar mijn 'prutjes', zoals je mijn cultures omschreef. Dank dat je er altijd voor me was als ik het moeilijk had en het even niet meer zag zitten. Pap, dank voor je interesse in mijn opleiding en werk, dank dat je me altijd zo gestimuleerd hebt om daarvoor te gaan, dat je me liet zien hoe belangrijk dat is. Ik heb bewondering voor je loopbaan. Je hebt er veel voor gegeven en misschien nog wel meer voor gelaten. Ik vind het mooi om te zien dat je wat dat laatste betreft nu met een inhaalslag bezig bent. Ik hoop dat je meer en meer (be)rust(ing) kunt vinden. Dank voor het ophalen en wegbrengen van de gedrukte exemplaren van mijn proefschrift. Lastig om volledig te zijn in een aantal zinnen als er zoveel is om dankbaar voor te zijn, pap en mam oneindig veel dank voor alles door de jaren heen, dank voor jullie liefde en aandacht. Dit werk draag ik op aan jullie, omdat jullie me alle jaren van mijn leven gestimuleerd hebben om me verder te ontwikkelen. Jullie gaven me moed door me op pad te sturen met de gedachte: 'Doe je niets, dan heb je niets. Je kun altijd proberen, lukt het niet, dan is het jammer, maar dan heb je het in ieder geval geprobeerd.' Jullie hebben het goed gedaan en ga nu nog maar weer eens wat leuks doen, want jullie hebben nog een hoop 'quality time' in te halen!

En tenslotte mijn partner **Frank**. Dank dat je altijd op me wachtte tot ik weer thuiskwam van het lab, zelfs als het al laat was. Dank dat je me ongestoord mijn gang liet gaan als ik thuis aan het werk ging. Jij bracht mij rust, in mijn hoofd en in mijn leven en hebt thuis weer gezellig gemaakt. Dank voor je hulp bij de lay-out van het boekje, zo is dat ook een beetje van jou. Dank voor al je steun, dank voor al je liefde, dank dat je met me mee bent gegaan, 'Wel ver hoor, helemaal naar Amerika', voor mijn volgende project. Ben vol vertrouwen dat we elkaar blijven meenemen, op pad om onze (grote) dromen te realiseren. Met jou durf ik de toekomst aan!

This PhD was hard, but above that a pleasure!

Thanks to you all!

Marjet Oosterkamp.

Certificate of educational PhD programme

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Margreet Jeannet Oosterkamp

born on 12 September 1980 in Emmeloord, The Netherlands

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 18 December 2013

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Rik Leemans	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Ms. Margreet Jeannet Oosterkamp** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 54 ECTS, including the following activities:

SENSE PhD Courses

- Environmental Research in Context
- Research Context Activity: Organization of SENSE symposium '*Innovative techniques for a sustainable environment*', Wageningen, 19-20 February 2009
- Biological processes in environmental technology

Other PhD Courses

- Writing English for publication
- Guide to scientific artwork
- Teaching and supervising thesis students
- Project and time management
- Phylogeny using the softwarepackage ARB of SILVA

Management and Didactic Skills Training

- Co-supervising practical BSc course *Microbial physiology*
- Supervising practical MSc course *Microbiology*
- Supervising MSc course *Research methods in microbiology*
- Thesis supervision of two MSc students and a BSc student
- Participation in co-management employees' council Wageningen University

Oral Presentations

- *Chlorate reduction as a novel concept for bioremediation of heavily polluted anaerobic soils.* The University of Massachusetts PhD study trip, 16-27 April 2009, Amherst, USA
- *The anaerobic nitrate and chlorate reduction pathways of Alicycliphilus denitrificans strain BC: friends or foes.* Conference 'Enzymology and ecology of the nitrogen cycle', 15-17 September 2010, Birmingham, UK
- *Nitrate-enhanced bioremediation: towards a healthy environment.* SENSE symposium 'Microbes for sustainability', 4-5 April 2012, Wageningen, The Netherlands

SENSE PhD Education Coordinator

Dr. ing. Monique Gulickx

This research project was financially supported by the Technology Foundation, the Applied Science Division (STW) of the Netherlands Organization for Scientific Research (NWO), project number 08053.