Degradation of benzene and other aromatic hydrocarbons by anaerobic bacteria

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Voor Pa en Ma

Abstract

Accidental spills, industrial discharges and gasoline leakage from underground storage tanks have resulted in serious pollution of the environment with monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (so-called BTEX). High concentrations of BTEX have been detected in soils, sediments and groundwater. The mobility and toxicity of the BTEX compounds are of major concern. *In situ* bioremediation of BTEX by using naturally occurring microorganisms or introduced microorganisms is a very attractive option. BTEX compounds are known to be transformed (or degraded) by microorganisms under aerobic and anaerobic conditions. As BTEX compounds are often present in the anaerobic zones of the environment, anaerobic bioremediation is an attractive remediation technique. The bottleneck in the application of anaerobic techniques is the lack of knowledge about the anaerobic biodegradation of benzene. In particular, little is known about the bacteria involved in anaerobic benzene degradation and the anaerobic benzene degradation pathway has still not been elucidated. The aim of the research presented in this thesis was to gain more insight in the degradation of benzene and other aromatic hydrocarbons by anaerobic bacteria. In particular, the physiology and phylogeny of the bacteria responsible for the degradation were studied and the results have been presented in this thesis.

Anaerobic benzene and toluene degradation was studied with different electron acceptors in batch experiments inoculated with material from an aquifer polluted by BTEX-containing landfill leachate (Banisveld landfill near Boxtel, The Netherlands). Benzene was not degraded during one year of incubation. Toluene degradation, on the other hand, was observed with nitrate, MnO_2 and Fe(III)NTA as electron acceptors. After further enrichment and several isolation attempts, a novel betaproteobacterial bacterium, strain G5G6, was obtained in pure culture. Strain G5G6 is able to grow with toluene as the sole electron donor and carbon source, and amorphous and soluble Fe(III)-species, nitrate and MnO_2 as electron acceptors. Strain G5G6 has several other interesting physiological and phylogenetic characteristics, which will be subject of future research. Strain G5G6 represents a novel species in a novel genus for which we propose the name *Georgfuchsia toluolica*.

In general, aerobic degradation of BTEX is a faster process than anaerobic BTEX degradation. However, for a number of reasons application of oxygen-dependent processes in the subsurface are technically and financially often not appealing. Therefore, an alternative bioremediation strategy would be to introduce oxygen in an alternative way, e.g. by *in situ* production. Chlorate reduction is a way to produce molecular oxygen *in situ* under anaerobic conditions. The formation of oxygen during chlorate reduction may result in rapid oxidation of compounds which are slowly degraded under anaerobic conditions; an example of such a compound is benzene. Therefore, benzene degradation coupled to the reduction of chlorate (ClO_3^-) was studied in this thesis. With mixed material from a wastewater treatment plant and soil samples obtained from a location contaminated with benzene, a benzene-degrading chlorate-reducing stable enrichment culture was obtained. This stable enrichment consisted of about five different bacterial species. Cross feeding involving interspecies oxygen transfer is a likely mechanism in this enrichment. One of these species, strain BC, was obtained in pure culture. Phylogenetic analysis showed that strain BC is a *Alicycliphilus denitrificans* strain. Strain BC is able to degrade benzene in conjunction with chlorate reduction. Oxygenase genes putatively encoding the enzymes performing the initial steps in aerobic degradation of benzene, were detected in strain BC. This demonstrates the existence of aerobic benzene bacterial biodegradation pathways under essentially anaerobic conditions. Thus, aerobic pathways can be employed under conditions where no external oxygen is supplied.

The new insights into toluene degradation under anaerobic conditions and benzene degradation coupled to chlorate reduction, as described in this thesis, can be applied for the improvement or development of *in situ* bioremediation strategies for BTEX contamination.

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General introduction

Sander A.B. Weelink, Miriam H.A. van Eekert and Alfons J. M. Stams

1.1 Introduction

Aromatic compounds are the second most abundant family of organic constituents present in nature after carbohydrates. The most important natural sources of aromatic compounds are poorly biodegradable polymers such as lignin, condensed tannins and humus (Field *et al*., 1995). Since the start of the industrial revolution a wide variety of aromatic compounds has also been introduced into the environment through anthropogenic activity. Millions of tons of aromatic compounds are manufactured worldwide each year. With such huge quantities of these compounds being made, transported and used, it is inevitable that a substantial amount will be lost in the environment. Aromatic compounds are important constituents of crude oil (petroleum) and oil derivatives, such as gasoline. Gasoline is rich in monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (BTEX) (Fig. 1). Furthermore, these compounds are used as industrial solvents and they provide the starting materials for the production of pharmaceuticals, agrochemicals, polymers, explosives and many other everyday products (Smith, 1990). Environmental contamination with these aromatic compounds can be found at many places and are a major concern because of the mobility and toxicity of these compounds. Therefore, the cleanup of BTEX pollution in soil and groundwater has gained much attention the last decades. BTEX compounds are known to be transformed (or degraded) by microorganisms and this is an interesting and promising cleanup strategy. In this chapter, the existing knowledge on the microbial degradation of BTEX compounds will be summarized and discussed in more detail.

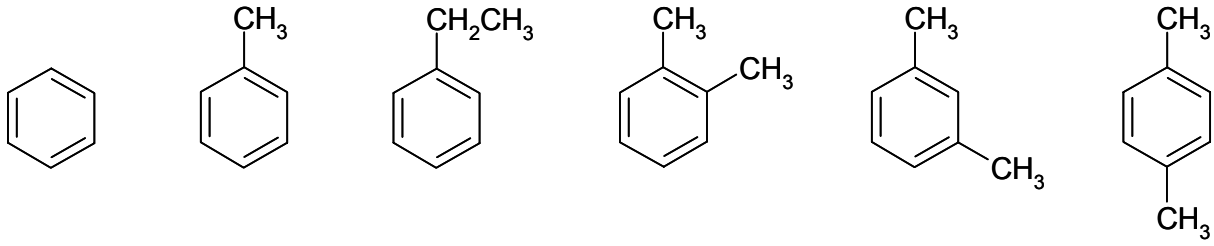

Figure 1. Chemical structures of benzene, toluene, ethylbenzene, *ortho*-xylene, *meta*-xylene and *para*-xylene.

1.2 BTEX contamination

It has been estimated that around 270,000 sites in the Netherlands are potentially heavily contaminated and that at around 11,000 sites urgently measurements have to be taken because of human or ecological risks or risks of spreading of the contaminants (RIVM, 2008). Contamination with BTEX compounds is often encountered at these sites. Accidental spills and industrial discharges have resulted in serious pollution of the environment with BTEX. Furthermore, gasoline leakage from underground storage tanks has been identified as an important source of groundwater contamination with BTEX. High concentrations of BTEX have been detected in soils, sediments and groundwater. The mobility and toxicity of the BTEX compounds are of major concern (several physical-chemical properties of BTEX compounds are summarized in Table 1). Compared with other oil hydrocarbons, BTEX are relatively water-soluble and therefore these compounds will rapidly produces a plume of contamination within the groundwater (Coates *et al*., 2002; Chakraborty and Coates, 2004). Benzene is the most hazardous of the BTEX compounds since it is a known human carcinogen (leukaemogenic potential) (Badham and Winn, 2007). Toluene and xylene are not carcinogenic, but toluene can enhance carcinogenesis by other compounds (Dean, 1978).

In pristine, aerobic aquifers contaminated with organic pollutants such as landfill leachate, generally a sequence of redox zones has developed as a result of organic contamination. Often, near the source of the organic pollutants methanogenic conditions are observed, whereas downstream of the

contaminant source zone in the plume sulfate-reducing and iron-reducing conditions may exist. Further downstream and at the fringes of the plume, nitrate and manganese(IV)-reducing conditions prevail (Christensen *et al*., 2001). Therefore, BTEX contamination is often present in the anaerobic zones of the environment (Lovley, 1997). In general, the degradation of BTEX compounds under anaerobic conditions is more difficult than under aerobic conditions. Therefore the use of (per)chlorate-reducing bacteria, which produce oxygen during the reduction of (per)chlorate, for the aerobic microbial degradation of compounds that are not or slowly degraded under anaerobic conditions, such as BTEX (in particular benzene), is an interesting remediation strategy. (Per)chlorate metabolism is also summarized and discussed in more detail in this chapter.

Table 1. Properties of BTEX compounds, according to Van Agteren *et al*. (1998).

Name	Molecular formula	Molecular weight (g/mol)	Density (kg/l)	T_m (°C)	T_b (°C)	Vapour pressure (kPa)	Aqueous solubility (mg/l)	Henry's Law constant (Pa m^3/mol)	Log K_{ow} (-)
Benzene	C_6H_6	78.1	0.878	5.5	80.1	10.13	1780	547	2.13
Toluene	C_7H_8	92.1	0.867	-95	110.8	2.93	515	669	2.65
Ethylbenzene	C_8H_{10}	106.2	0.867	-95	136.2	0.93	152	588	3.20
ortho-Xylene	C_8H_{10}	106.2	0.880	-25	144.4	0.67	175	496	2.95
meta-Xylene	C_8H_{10}	106.2	0.864	-48	139.0	0.80	200	699	3.20
para-Xylene	C_8H_{10}	106.2	0.860	13	138.4	0.87	198	709	3.18

Density, vapour pressure, aqueous solubility are at 20 °C. T_m: melting point, T_b: boiling point.

1.3 BTEX biodegradation

Much research on BTEX degradation under aerobic and anaerobic conditions has been initiated to develop biological remediation techniques for BTEX-contaminated sites. All aromatic compounds possess a relative resistance to degradation due to the large (negative) resonance energy. This large resonance energy is caused by the stability of the π-electron cloud (Aihara, 1992). Before the 1980s, microbial BTEX degradation studies were all performed under aerobic conditions. The last two decades many studies have been performed to investigate the ability of microorganisms to biodegrade BTEX components in the absence of oxygen. Aerobic bacteria use mono- and dioxygenases to incorporate oxygen atoms into the ring to destabilize the resonance structure, making ring cleavage possible. Under anaerobic conditions, oxygen is not available for the initial attack of the ring and therefore other pathways are involved in the BTEX degradation under anaerobic conditions. The degradation of BTEX, in particular benzene, under aerobic and anaerobic conditions will be discussed in more detail hereafter.

1.4 Aerobic BTEX degradation

Aerobic biodegradation of BTEX compounds has been studied since the sixties and has been reviewed several times (Gibson and Subramanian, 1984; Dagley, 1985, 1986; Smith, 1990). Many bacteria, especially *Pseudomonas* species, have been isolated that can use benzene as sole carbon and energy source. Under aerobic conditions, oxygen does not only serve as a terminal electron acceptor, but it is also used in the initial enzymatic activation of aromatic compounds. Oxygen is incorporated into the aromatic ring and these reactions are catalyzed by mono- or dioxygenases (Gibson and Subramanian, 1984). Hence, the biochemical strategy for aromatic hydrocarbon activation under oxic conditions is to introduce a hydroxyl group (monohydroxylation by monooxygenase) or hydroxyl groups (dihydroxylation by dioxygenases) into the aromatic ring. The aerobic degradation of toluene, ethylbenzene and xylene may involve mono- or dioxygenases, but other pathways have also been described (Van Agteren *et al*., 1998).

1.4.1 Dioxygenases

Dioxygenases catalyse the incorporation of both atoms of dioxygen into their substrates. These enzymes are widely distributed in nature and they are involved in both anabolic and catabolic processes (Eltis and Bolin, 1996). One important catabolic process is the aerobic degradation of aromatic compounds by bacteria wherein dioxygenases catalyse two critical reactions: ring dihydroxylation and ring cleavage. Dioxygenases may be considered to fall into two groups:
(1) dioxygenases involved in ring hydroxylation (ring-hydroxylating dioxygenases). These all require reduced cofactors, either NADH or NADPH, in addition to oxygen. They dihydroxylate aromatic substrates to produce cis-diols, e.g. the conversion of benzene to *cis*-benzenedihydrodiol (Fig. 2).
(2) Dioxygenases involved in ring fission (ring-cleavage dioxygenases). These have no reduced cofactor requirement and cleave the benzene ring of hydroxylated (di- or tri-) aromatic substrates, e.g. the conversion of catechol to 2-hydroxymuconic semialdehyde (by catechol 2,3-dioxygenase)

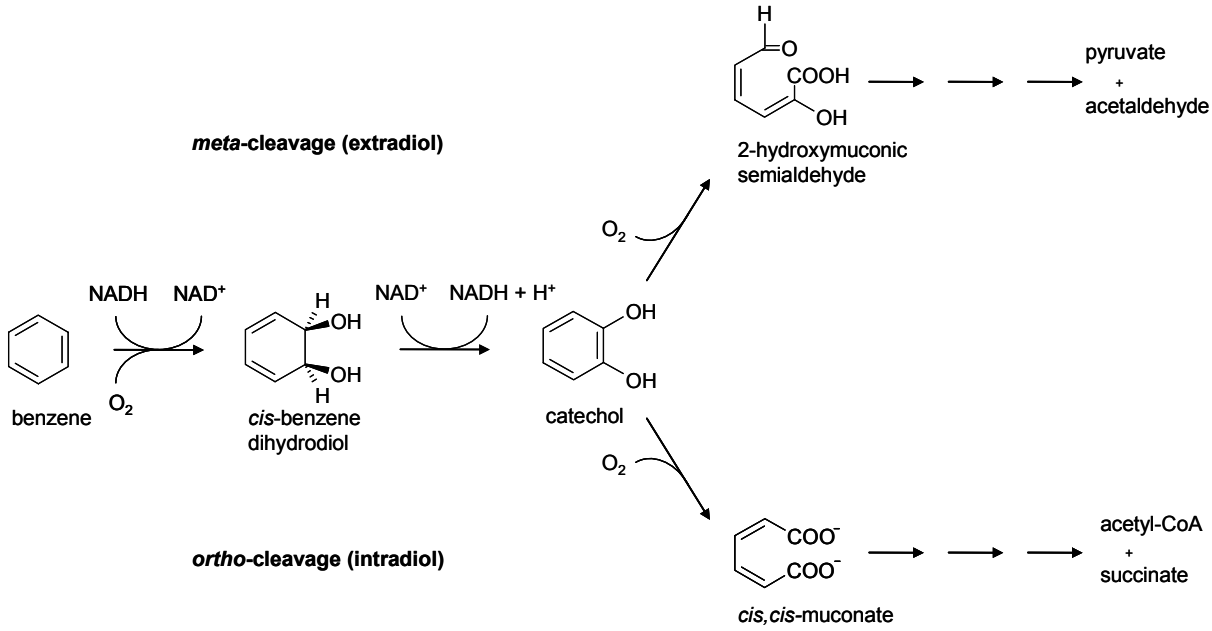

Figure 2. The aerobic biodegradation of benzene by dioxygenases.

Ring-hydroxylating dioxygenases

Ring-hydroxylating dioxygenases, also called Rieske nonheme iron oxygenases, are soluble, multi-component enzyme complexes composed of a terminal oxygenase component (iron-sulfur protein) and different electron transport proteins (a ferredoxin and a reductase or a combined ferredoxin-NADH-reductase) (Butler and Mason, 1997). The catalytic iron-sulfur proteins are heteromultimers, and comprise a large (α) and a small (β) subunit. The large subunit contains a Rieske-type [2Fe-2S] cluster, a mononuclear nonheme iron oxygen activation center, and a substrate-binding site, which also determines substrate specificity (Gibson and Parales, 2000). A comparison of the amino acid sequences of the terminal oxygenase α subunits (ISPα) revealed that they form a family of diverse but evolutionarily related sequences, and four distinct major lineages have been identified (Gibson and Parales, 2000). Although none of the enzymes is completely specific, a broad correlation exists between the grouping in toluene/biphenyl, naphthalene, benzoate, or phthalate subfamilies and the native substrates converted by the subfamily members. The toluene/biphenyl family includes enzymes for the degradation of toluene, benzene, isopropylbenzene, chlorobenzenes, and biphenyl from both Gram-negative and Gram-positive organisms. The naphthalene family consists of enzymes for the degradation of naphthalene and phenanthrene, but also enzymes for nitrobenzene and nitrotoluene

degradation belong to this family. All enzymes in the naphthalene family are from Gram-negative organisms. The benzoate family is a diverse group consisting of enzymes that oxidize aromatic acids (benzoate, toluate, anthranilate, 2-chlorobenzoate, trichlorophenoxyacetate, and isopropylbenzoate). The phthalate family is a large and diverse group of enzymes and this family contains enzymes that oxidize aromatic acids (vanillate, phthalate, 3-chlorobenzoate, phenoxybenzoate, and p-toluene sulfonate).The phthalate family comprises both dioxygenases and monooxygenases and represents the most diverse group in terms of amino acid sequence and primary substrate (Gibson and Parales, 2000).

Ring-cleavage dioxygenases

Aromatic-ring-cleavage dioxygenases perform ring fission of substrates (e.g. catechol) that carry two or more hydroxyl groups. Ring cleavage by ring-cleavage dioxygenases occurs either between the two hydroxyl groups (intradiol) or at a bond proximal to one of the two hydroxyl groups. Catechol can be degraded by either catechol 1,2-dioxygenase (intradiol or *ortho*-cleavage pathway) or catechol 2,3-dioxygenase (extradiol or *meta*-cleavage pathway). The *ortho*-cleavage pathway leads via *cis,cis*-muconate in the production of acetyl-CoA and succinate (Fig. 2) , which both enter the tricarboxylic acid cycle. The *meta*-cleavage pathway pathway leads via 2-hydroxymuconic semialdehyde to pyruvate and acetaldehyde (Fig. 2), which are also intermediates of the tricarboxylic acid cycle.

Catechol dioxygenases have been reviewed extensively (Eltis and Bolin, 1996; Broderick, 1999; Vaillancourt *et al*., 2006). In general, intradiol dioxygenases depend on nonheme Fe(III) as cofactor, whereas extradiol dioxygenases typically depend on nonheme Fe(II). Interestingly, a few Mn(II)-containing extradiol dioxygenases with strong sequence similarity to Fe(II)-containing ones have also been reported (Vaillancourt *et al*., 2006). Although the distinctions between intradiol and extradiol dioxygenases may appear to be minor, they are in fact a manifestation of enzymes that have completely different structures and employ different mechanisms (Harayama and Kok, 1992). Overall, extradiol dioxygenases appear to be more versatile than their intradiol counterparts. Thus, the former cleave a wider variety of substrates, have evolved on a larger number of structural scaffolds, and occur in a wider variety of pathways, including some biosynthetic pathways and pathways that degrade non-aromatic compounds. This increased versatility might ultimately reflect the apparent requirement of intradiol enzymes for substrates possessing vicinal hydroxyl groups (Vaillancourt *et al*., 2006).

Intradiol and extradiol enzymes share no significant sequence or structural similarities and thus belong to evolutionary distinct classes of proteins. Sequence and structural analyses further indicate that all intradiol dioxygenases characterized to date belong to a single evolutionary lineage. In contrast to the intradiol dioxygenases, extradiol and extradiol-type dioxygenases belong to at least three evolutionarily independent families and a phylogeny-based classification system for extradiol dioxygenases (type I, II and III) was proposed in 1996 (Eltis and Bolin, 1996). However, this system is currently obsolete due to the lack of upgrading and the obvious increase in the last 10 years of gene member representation in the databases. There are conformed new evolutionary branches in the whole family, and between these two subfamilies in particular (Howard Junca, personal communication).

1.4.2 Monooxygenases

One important group of bacterial oxygenases consists of the soluble di-iron monooxgenases, which can be divided into four groups based on the structural, biochemical, and genetic information that is available: (1) methane monooxygenases (sMMOs), (2) the Amo alkene monooxygenases, (3) the phenol hydroxylases, (4) the four component alkene/aromatic monooxygenases. All these enzymes are soluble, multicomponent enzymes which utilize dioxygen to catalyze the initial hydroxylation or

epoxidation step in pathways for the oxidation of their respective hydrocarbon substrates, and require NAD(P)H as an electron donor. With respect to aerobic benzene degradation, enzymes belonging to the phenol hydroxylases and the four component alkene/aromatic monooxygenases monooxygenases could play a role in the degradation pathway. Therefore, groups 1 and 2 will not be discussed further. The group of phenol hydroxylases consists of three-component oxygenases that hydroxylate phenolic substrates to the corresponding catechols. These enzymes catalyze the oxidation of phenol and certain methyl-substituted phenols. Some of these enzymes, such as the toluene *o*-monooxygenase (TOM) of *Burkholderia cepacia* G4, are also able to hydroxylate benzene, toluene, and other unactivated aromatic hydrocarbons, as well as a variety of other substrates, including diethyl ether, TCE, the three isomers of dichloroethylene, vinylchloride, and naphthalene (Leahy *et al*., 2003). The group of four-component alkene/aromatic monooxygenases contain a mixed assemblage of four-component alkene monooxygenases and aromatic ring monooxygenases that exhibit overlapping substrate specificities. The archetypal member of this group is the toluene 4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1, identified by Pikus *et al*. (1996) as the first aromatic oxygenase in this family of enzymes (Pikus *et al*., 1996). This enzyme has a fairly broad substrate specificity, oxidizing besides toluene, also acetanilide, chlorobenzene, ethylbenzene, TCE, 1,2-dichloroethane, chloroform, and C3-C8 alkenes, but not phenolic compounds (Leahy *et al*., 2003). Aromatic monooxygenases related to T4MO have since been identified in other *Pseudomonads*; these include the toluene 3-monooxygenase (T3MO) of *Ralstonia pickettii* PKO1 and the toluene/o-xylene monooxygenase (ToMo) of *Pseudomonas stutzeri* OX1. Based on sequence analyses, all were deduced to have the same four-component structure as T4MO, and all have attracted interest because of their ability to degrade TCE and other chlorinated aliphatic hydrocarbons (Leahy *et al*., 2003).

Monooxygenases involved in benzene degradation catalyze the oxygen dependent hydroxylation of the aromatic ring in a complex reaction scheme to form phenol in the overall reaction. The direct insertion of the activated oxygen into an C-H bond of the aromatic ring with subsequent rearrangement or the formation of an epoxide by oxygen addition to the aromatic ring has been proposed as initial reaction step (Hinson *et al*., 1985; de Visser and Shaik, 2003; Mitchell *et al*., 2003). Tao *et al*. (2004) studied the ability of aromatic monooxygenases to hydroxylate benzene. They found that the toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1, the toluene 3-monooxygenase (T3MO) of *Ralstonia pickettii* PKO1, and the toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4, convert benzene to phenol, as well as catechol and 1,2,3-trihydroxybenzene in successive hydroxylation reactions (Tao *et al*., 2004). Recently, a oligonucleotide microarray on the basis of benzene monooxygenase gene diversity was developed, as a new technology for simultaneous detection of the functional gene diversity in environmental samples. Phylogenetic analysis of the sequences obtained suggested the large diversity of the benzene monooxygenase genes. (Iwai *et al*., 2007)

1.5 Anaerobic BTEX degradation

As BTEX compounds are often present in the anaerobic zones of the environment, anaerobic bioremediation is an attractive remediation technique. Anaerobic degradation of aromatic hydrocarbons was first described by Kuhn and colleagues (1985) (Kuhn *et al*., 1985). Since then, the degradation pathways of toluene and ethylbenzene by denitrifying and sulfate-reducing microorganisms in particular have been characterized. Anaerobic degradation of BTEX has been reviewed in the past as well (Schink *et al*., 1992; Heider *et al*., 1999; Spormann and Widdel, 2000; Phelps and Young, 2001; Widdel and Rabus, 2001; Chakraborty and Coates, 2004; Heider, 2007; Fuchs, 2008)]. Aromatic compounds, such as benzene and toluene, are thermodynamically favorable electron donors for growth, because of the large Gibbs free energy released during the oxidation of

these compounds and the reduction of the different electron acceptors (Table 2 and 3). The differences in Gibbs free energy of the transformation of benzene and toluene do not reflect the differences in biodegradability of the two compounds. All studies regarding anaerobic BTEX degradation have indicated that anaerobic benzene degradation is most difficult and that toluene is one of the aromatic compounds, which is relatively easy to degrade anaerobically.

1.6 Anaerobic toluene biodegradation

Among the BTEX components, the anaerobic biodegradation of toluene is probably most extensively studied. Toluene can be biodegraded with nitrate, Mn(IV), Fe(III), sulfate or CO_2 as terminal electron acceptors (Chakraborty and Coates, 2004). More recently, it has been demonstrated that anaerobic toluene degradation can also be coupled to the reduction of humic substances (Cervantes *et al*., 2001), chlorine oxyanions, such as perchlorate or chlorate (Coates *et al*., 2001b), or arsenate (Liu *et al*., 2004). Moreover, toluene can also be assimilated as a carbon source by anoxygenic phototrophs (Zengler *et al*., 1999). Anaerobic toluene degradation has been found in field studies, column studies, enrichment cultures and microcosms and in pure cultures (Phelps and Young, 2001). Here, mainly pure cultures studies will be discussed in more detail.

1.6.1 Anaerobic toluene-degrading isolates

Several bacteria have been isolated, which couple the degradation of toluene to the reduction of nitrate (Table 4). Most of these bacteria belong either to the *Azoarcus* or *Thauera* genus, e.g. *Thauera aromatica* T1 (Evans *et al*., 1991), *Thauera aromatica* K172 (Anders *et al*., 1995), *Azoarcus tolulyticus* Tol4 (Fries *et al*., 1994) and *Azoarcus* sp. EbN1 (Rabus and Widdel, 1995). Recently, four *Magnetospirillum* strains, which belong to the *Alphaproteobacteria,* were described that can degrade toluene with nitrate as the electron acceptor (Shinoda *et al*., 2005). The toluene-oxidizing nitrate-reducing *Thauera* and *Azoarcus* species are facultative anaerobes and are members of the *Betaproteobacteria*. Most of the these organisms were isolated from anaerobic sludge or (freshwater) sediments (Anders *et al*., 1995). Several of the *Azoarcus* and *Thauera* species were originally described as *Pseudomonas* species, but were subsequently reclassified into their current taxonomic positions. Probably, the *Azoarcus* and *Thauera* species that have these aromatics-degrading capacities will be reclassified soon and separated from the other *Azoarcus* and *Thauera* species, which do not have these capacities (Kühner *et al*., 2005). Recently, the genome of strain EbN1 has been sequenced and this bacterium was renamed as *Aromatoleum aromaticum* (Rabus *et al*., 2005; Wöhlbrand *et al*., 2007).

One of the first reports about anaerobic toluene degradation, dealt with the degradation of toluene coupled to the reduction of Fe(III) by *Geobacter metallireducens* GS-15 (Lovley and Lonergan, 1990; Lovley *et al*., 1993). This bacterium belongs to the *Deltaproteobacteria* and can completely oxidize toluene to CO_2 coupled to the reduction of Fe(III). More recently, *Geobacter grbiciae* and *Geobacter* sp. TMJ1 that can oxidize toluene with Fe(III) were described (Coates *et al*., 2001a; Winderl *et al*., 2007). *Geobacter metallireducens* can also use nitrate, Mn(IV) or humic substances as the electron acceptor (Lovley *et al*., 1993; Coates *et al*., 2001a). In another study, toluene oxidation coupled to Mn(IV) reduction was also demonstrated in an enrichment culture, but a pure culture was not obtained (Langenhoff, 1997). In general, *Geobacter* species are often found to be dominant in the Fe(III)-reducing zone of environments contaminated with hydrocarbons (Rooney-Varga *et al*., 1999; Botton *et al*., 2007). Several bacteria have been described capable of degrading toluene with sulfate (Table 4), such as *Desulfobacula toluolica* and *Desulfotignum toluenicum* (Rabus *et al*., 1993; Beller *et al*., 1996; Harms *et al*., 1999b; Meckenstock, 1999; Morasch *et al*., 2004; Ommedal and Torsvik, 2007). These sulfate-reducing bacteria all belong to the *Deltaproteobacteria*. Toluene degradation with other less common electron acceptors has also been reported. Toluene degradation can be coupled to

(per)chlorate respiration by *Dechloromonas aromatica* RCB (Coates *et al*., 2001b), to the reduction of arsenate by strain Y5 (Liu *et al*., 2004) and to the reduction humic substances by an enrichment culture (Cervantes *et al*., 2001). Finally, toluene can also be assimilated as a carbon source by the anoxygenic phototroph, *Blastochloris sulfoviridis* (Zengler *et al*., 1999).

1.6.2 Anaerobic toluene degradation pathway

The biochemical pathway of anaerobic toluene degradation has been intensively studied over the last decade (Heider *et al*., 1999; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Especially, *Azoarcus* strain T, *Thauera aromatica* K172 and *Thauera aromatica* T1 were studied (Biegert *et al*., 1996; Beller and Spormann, 1997b, 1998; Coschigano *et al*., 1998; Heider *et al*., 1998; Leuthner and Heider, 1998; Leuthner *et al*., 1998; Beller and Spormann, 1999; Krieger *et al*., 1999). These studies revealed that the first step in the catabolism of toluene is the addition of fumarate onto the toluene methyl group to form benzylsuccinate by benzylsuccinate synthase (BssABC) (Fig. 3) (Leuthner *et al*., 1998). Benzylsuccinate is then activated to CoA-thioester by a succinyl-CoA-dependent CoA-transferase (BbbsEF), and benzylsuccinyl-CoA is subsequently oxidized to benzoyl-succinyl-CoA and benzoyl-CoA via beta-oxidation. Benzoyl-CoA reductase (BcrCABD) initiates the degradation of benzoyl-CoA, which is thereafter further oxidized via reductive ring cleavage to carbon dioxide. Benzoyl-CoA has been recognized as central intermediate in the anaerobic degradation of many aromatic compounds (Harwood *et al*., 1999).

Although the Bss pathway was first identified in *Azoarcus* and *Thauera* species growing under nitrate-reducing conditions, it is now considered as the common mechanism for activation of toluene under various (anaerobic) redox conditions by phylogenetically diverse bacteria. The Bss pathway was also found in the toluene-oxidizing Fe(III)-reducing *Geobacter metallireducens* (Kane *et al*., 2002) and in sulfate-reducing bacteria, PRTOL1 (Beller and Spormann, 1997a) and *Desulfobacula toluolica* (Rabus and Heider, 1998). Furthermore, this pathway was demonstrated in the toluene-utilizing phototrophic *Blastochloris sulfoviridis* (Zengler *et al*., 1999). Recently, Bss genes were also identified in a methanogenic toluene-degrading culture (Washer and Edwards, 2007). It is possible that some bacteria use other pathways for the initial toluene conversion, such as a pathway involving direct methyl group hydroxylation to benzyl alcohol or hydroxylation to cresol (Frazer *et al*., 1995; Langenhoff *et al*., 1997a). These pathways have not been studied as thoroughly as the Bss pathway. Therefore, involvement of fumarate addition cannot be ruled out (Phelps and Young, 2001).

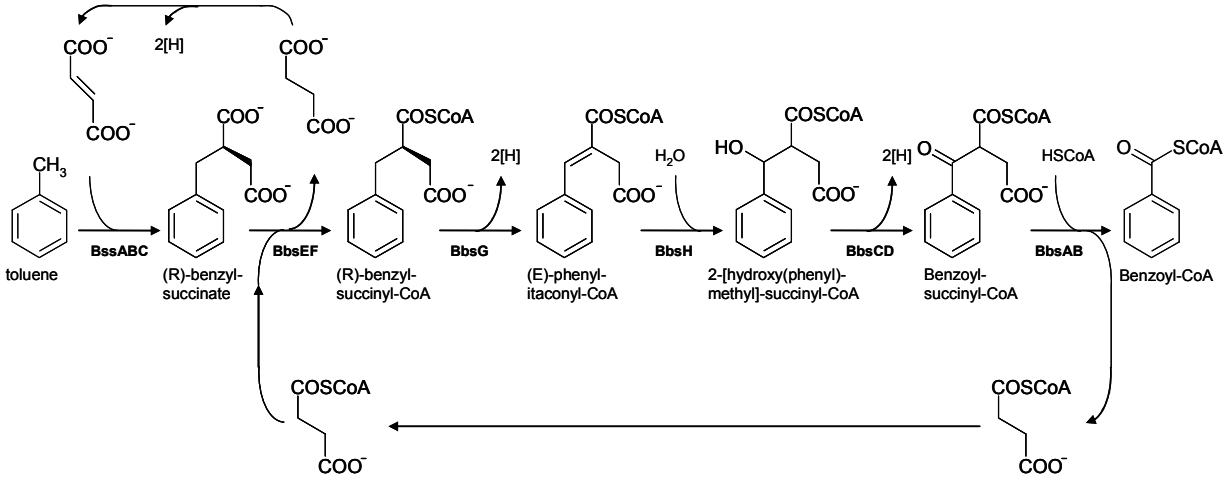

Figure 3. Anaerobic toluene degradation route, according to Kube *et al*. (2004). BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:(R)-benzylsuccinate CoA-transferase; BbsG, (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase.

Table 2. Stoichiometric equations and standard free energy changes ($\Delta G^{0'}$) for benzene (C_6H_6) and toluene (C_7H_8) oxidations with various electron acceptors.

Electron acceptor (ox/red)	Stoichiometric equation			$\Delta G^{0'}$ (kJ/mol) [a]
ClO_3^-/Cl^-	$C_6H_6 + 5\ ClO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 5\ Cl^- + 6\ H^+$	-3,813
	$C_7H_8 + 6\ ClO_3^- + 3\ H_2O$	→	$7\ HCO_3^- + 6\ Cl^- + 7\ H^+$	-4,557
O_2/H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O$	→	$6\ HCO_3^- + 6\ H^+$	-3,173
	$C_7H_8 + 9\ O_2 + 3\ H_2O$	→	$7\ HCO_3^- + 7\ H^+$	-3,789
NO_3^-/N_2	$C_6H_6 + 6\ NO_3^-$	→	$6\ HCO_3^- + 3\ N_2$	-2,978
	$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+$	→	$7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$	-3,555
NO_3^-/NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	-2,061
	$C_7H_8 + 18\ NO_3^- + 3\ H_2O$	→	$7\ HCO_3^- + 18\ NO_2^- + 7\ H^+$	-2,455
Fe^{3+}/Fe^{2+}	$C_6H_6 + 30\ Fe^{3+} + 18\ H_2O$	→	$6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	-3,040
	$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O$	→	$7\ HCO_3^- + 36\ Fe^{2+} + 43\ H^+$	-3,630
SO_4^{2-}/H_2S	$C_6H_6 + 3.75\ SO_4^{2-} + 3\ H_2O$	→	$6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 0.375\ H^+$	-186
	$C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O$	→	$7\ HCO_3^- + 2.25\ H_2S + 2.25\ HS^- + 0.25\ H^+$	-205
CO_2/CH_4	$C_6H_6 + 6.75\ H_2O$	→	$2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	-124
	$C_7H_8 + 7.5\ H_2O$	→	$2.5\ HCO_3^- + 4.5\ CH_4 + 2.5\ H^+$	-131

[a] The data for calculating standard free energy changes ($\Delta G^{0'}$) are from McCarty (1971), Thauer *et al*. (1977), and Stumm and Morgan (1981).

Table 3. Stoichiometric equations and standard free energy changes for benzene oxidations with various electron acceptors with and without biomass ($C_5H_7O_2N$) formation taken into account.

Electron acceptor (ox/red)	Stoichiometric equation with and without biomass production [a]			$\Delta G^{0'}$ (kJ/mol) [b]
ClO_3^-/Cl^-	$C_6H_6 + 5\ ClO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 5\ Cl^- + 6\ H^+$	- 3,813
	$C_6H_6 + 1.81\ ClO_3^- + 0.13\ H_2O + 0.96\ NH_4^+$	→	$1.21\ HCO_3^- + 1.81\ Cl^- + 0.96\ C_5H_7O_2N + 2.17\ H^+$	
O_2/H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O$	→	$6\ HCO_3^- + 6\ H^+$	- 3,173
	$C_6H_6 + 3.04\ O_2 + 0.32\ H_2O + 0.89\ NH_4^+$	→	$1.54\ HCO_3^- + 0.89\ C_5H_7O_2N + 2.43\ H^+$	
NO_3^-/N_2	$C_6H_6 + 6\ NO_3^-$	→	$6\ HCO_3^- + 3\ N_2$	- 2,978
	$C_6H_6 + 2.52\ NO_3^- + 0.87\ NH_4^+$	→	$1.65\ HCO_3^- + 1.26\ N_2 + 0.87\ C_5H_7O_2N + 0.87\ H_2O$	
NO_3^-/NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	- 2,061
	$C_6H_6 + 7.76\ NO_3^- + 0.83\ H_2O + 0.72\ NH_4^+$	→	$2.38\ HCO_3^- + 7.76\ NO_2^- + 0.72\ C_5H_7O_2N + 3.11\ H^+$	
Fe^{3+}/Fe^{2+}	$C_6H_6 + 30\ Fe^{3+} + 18\ H_2O$	→	$6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+$	-3,040
	$C_6H_6 + 12.41\ Fe^{3+} + 6.57\ H_2O + 0.72\ NH_4^+$	→	$1.60\ HCO_3^- + 12.41\ Fe^{2+} + 0.88\ C_5H_7O_2N + 14.90\ H^+$	
SO_4^{2-}/H_2S	$C_6H_6 + 3.75\ SO_4^{2-} + 3\ H_2O$	→	$6\ HCO_3^- + 1.875\ H_2S + 1.875\ HS^- + 0.375\ H^+$	-186
	$C_6H_6 + 3.44\ SO_4^{2-} + 2.63\ H_2O + 0.12\ NH_4^+$	→	$5.38\ HCO_3^- + 1.72\ H_2S + 1.72\ HS^- + 0.12\ C_5H_7O_2N + 0.34\ H^+$	
CO_2/CH_4	$C_6H_6 + 6.75\ H_2O$	→	$2.25\ HCO_3^- + 3.75\ CH_4 + 2.25\ H^+$	-124
	$C_6H_6 + 6.30\ H_2O + 0.08\ NH_4^+$	→	$2.04\ HCO_3^- + 3.54\ CH_4 + 0.08\ C_5H_7O_2N + 2.13\ H^+$	

[a] Stoichiometric coefficients, including cell yields, were calculated using method of McCarty (1971).

[b] The data for calculating standard free energy changes ($\Delta G^{0'}$) are from McCarty (1971), Thauer *et al*. (1977) and Stumm and Morgan (1981).

Table 4. Overview of all bacterial strains isolated with benzene, toluene, ethylbenzene and xylene under anaerobic conditions. The electron acceptor that the bacterium uses for growth is indicated in the table.

Strains	Full name or closest relative (with % similarity based on 16S rRNA)	Growth on: Benzene	Toluene	Ethylbenzene	Xylene	References
Strain T	*Azoarcus* sp. strain T		NO_3^-		NO_3^- (m) [a]	(Dolfing *et al*., 1990)
GS-15	*Geobacter metallireducens*		Fe^{3+} [c]			(Lovley and Lonergan, 1990)
K172	*Thauera aromatica*		NO_3^-			(Schocher *et al*., 1991)
T1	*Thauera aromatica* T1		NO_3^-			(Evans *et al*., 1991)
Tol-2	*Desulfobacula toluolica*		SO_4^{2-}			(Rabus *et al*., 1993)
8 strains	*Azoarcus tolulyticus* Tol4, *Azoarcus tolulyticus* Td15, *Azoarcus toluvorans* Td21		NO_3^-		NO_3^- (m)	(Fries *et al*., 1994)
4 strains	*Azoarcus* sp. EbN1, ToN1, PbN1, mXyN1		NO_3^-	NO_3^-		(Rabus and Widdel, 1995)
EB1	*Azoarcus* sp. strain EB1			NO_3^-		(Ball *et al*., 1996)
PRTOL1	*Desulforhabdus amnigenus* (96%)		SO_4^{2-}			(Beller *et al*., 1996)
63 strains	*Azoarcus toluclasticus*		NO_3^-			(Fries *et al*., 1997)
14 strains	*Azoarcus tolulyticus* (97-98%)		NO_3^-		NO_3^- (m)	(Hess *et al*., 1997)
TRM1			SO_4^{2-}			(Meckenstock, 1999)
oXyS1	*Desulfosarcina variabilis* (98.7%)		SO_4^{2-}		SO_4^{2-} (o)	(Harms *et al*., 1999b)
mXyS1	*Desulfococcus multivorans* (86.9%)		SO_4^{2-}		SO_4^{2-} (m)	
pCyN1	*Azoarcus* sp. EbN1 (100%)		NO_3^-			(Harms *et al*., 1999a)
TACP	*Geobacter grbiciae* TACP		Fe^{3+}			(Coates *et al*., 2001a)
RCB, JJ	*Dechloromonas aromatica* RCB	NO_3^- [b]	NO_3^-	NO_3^-	NO_3^- (m,o,p)	(Chakraborty *et al*., 2005)
	Dechloromonas sp. JJ	NO_3^-	NO_3^-			(Coates *et al*., 2001b)
S2	*Thauera aminoaromatica* S2		NO_3^-			(Mechichi *et al*., 2002)
EbS7	Strain mXyS1 (96%)			SO_4^{2-}		(Kniemeyer *et al*., 2003)
OX39	*Desulfotomaculum* strain R-acetonA170 (96%)		SO_4^{2-}		SO_4^{2-} (m,o)	(Morasch *et al*., 2004)
Y5	*Desulfosporosinus meridiei* (97%)		AsO_4^{3-}			(Liu *et al*., 2004)
DNT-1	*Thauera aminoaromatica* (99%)		NO_3^-			(Shinoda *et al*., 2004)
4 strains	*Magnetospirillum magneticum* AMB-1 (99-100%)		NO_3^-			(Shinoda *et al*., 2005)
DN11, AN9	*Azoarcus evansii* (99%), *Azoarcus* sp. ToN1 (99%)	NO_3^-				(Kasai *et al*., 2006)
H3	*Desulfotignum toluenicum*		SO_4^{2-}			(Ommedal and Torsvik, 2007)

[a] 'm' stands for growth with *m*-xylene, 'o' stands for *o*-xylene and 'p' stands for *p*-xylene.

[b] Besides nitrate (NO_3^-), *Dechloromonas aromatica* RCB is able to use perchlorate (ClO_4^-) and chlorate (ClO_3^-) as the electron acceptor.

[c] Nitrate and Mn(IV) can also serve as the electron acceptor for toluene oxidation (Lovley and Lonergan, 1990).

1.7 Anaerobic xylene degradation

The initial steps of anaerobic *meta*- and *ortho*-xylene degradation were elucidated in *Azoarcus* sp. strain T (Beller and Spormann, 1997b; Krieger *et al*., 1999). Research by Krieger *et al*. (1999) revealed that *meta*-xylene is converted to 3-methylbenzoate (or 3-methylbenzoyl-CoA) in a series of reactions that are analogous to those of anaerobic toluene conversion to benzoate (Krieger *et al*., 1999). The degradation of *para*-xylene is assumed to be proceed according to the same pathway (Widdel and Rabus, 2001).

1.8 Anaerobic ethylbenzene degradation

Anaerobic ethylbenzene degradation has been demonstrated in several nitrate-reducing bacteria and one sulfate-reducing bacterium (Table 4). Although ethylbenzene is chemically very similar to toluene, it is usually degraded via a completely different pathway. The denitrifying bacterial strains EB1, EbN1 and PbN1 degrade ethylbenzene to CO_2. These bacteria were used for to elucidate the anaerobic ethylbenzene degradation pathway (Ball *et al*., 1996; Rabus and Heider, 1998; Johnson *et al*., 2001; Rabus *et al*., 2002). These strains are closely related, and belong to the genus *Azoarcus* in the *Betaproteobacteria*. This metabolic pathway (Fig. 4) included an initial step catalysed by ethylbenzene dehydrogenase, a novel molybdenum/iron-sulfur/heme enzyme (Johnson *et al*., 2001; Rabus *et al*., 2002). It oxidizes the methyl group of ethylbenzene independently of oxygen, generating (S)-1-phenylethanol as first intermediate. Further metabolism of (S)-1-phenylethanol proceeds via oxidation to acetophenone, which is then carboxylated at the methyl group forming benzoylacetate. Benzoylacetate is converted via benzoylacetyl-CoA to benzoyl-CoA (Ball *et al*., 1996), the central intermediate in the anaerobic catabolism of aromatic compounds.

More recent studies on anaerobic ethylbenzene degradation under sulfate-reducing conditions resulted in the isolation of a novel organism, strain EbS7 (Kniemeyer *et al*., 2003). This strain is a member of the *Deltaproteobacteria*, most closely related to strain mXyS1, which can anaerobically oxidize toluene and m-xylene (Harms *et al*., 1999b). In contrast to the initial dehydrogenation reaction used by denitrifying ethylbenzene degraders, but similarly to toluene and xylene pathways, activation of ethylbenzene by strain EbS7 is achieved by a fumarate addition reaction at the secondary atom of the ethyl group to form 1-phenylethyl-succinate (Kniemeyer *et al*., 2003).

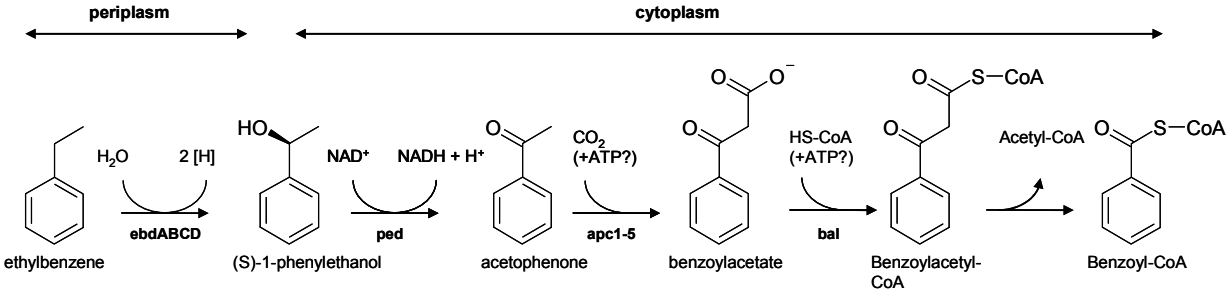

Figure 4. Anaerobic metabolism of ethylbenzene, according to Kühner *et al*. (2005). ebdABC, ethylbenzene dehydrogenase; ped: (S)-1-phenylethanol dehydrogenase; apc1-5, acetophenone carboxylase; bal, benzoylacetate CoA-ligase.

1.9 Anaerobic benzene degradation

For a long time, benzene was considered persistent under anaerobic conditions. Benzene is still considered as the most recalcitrant of all BTEX compounds under anoxic conditions. A summary of laboratory and field studies concerning anaerobic degradation of organic compounds in groundwater demonstrated that in many studies anaerobic benzene degradation did not occur (Aronson and

Howard, 1997). Anaerobic benzene degradation has been observed in some sediment, microcosm and column studies and microbial enrichments. Only recently, isolates capable of anaerobic benzene degradation have been described (Coates *et al*., 2001b; Kasai *et al*., 2006). Anaerobic benzene degradation has been extensively reviewed (Lovley, 2000; Phelps and Young, 2001; Coates *et al*., 2002; Chakraborty and Coates, 2004).

1.9.1 Anaerobic benzene degradation under different redox conditions

Anaerobic benzene degradation with nitrate has been demonstrated in microcosms (Major *et al*., 1988), in enrichment cultures (Burland and Edwards, 1999; Ulrich and Edwards, 2003) and in pure cultures (Coates *et al*., 2001b; Kasai *et al*., 2006). So far anaerobic benzene degradation with Fe(III) as the electron acceptor has not been observed in pure cultures, but only in microcosms (Lovley *et al*., 1994, 1996a; Anderson *et al*., 1998; Anderson and Lovley, 1999; Botton and Parsons, 2006) and in enrichment cultures (Rooney-Varga *et al*., 1999; Botton and Parsons, 2007; Kunapuli *et al*., 2007). Moreover, anaerobic benzene degradation with Fe(III) as the electron acceptor was stimulated by humic substances, which serve as electron shuttle compounds between Fe(III)-reducing bacteria and insoluble Fe(III) oxides (Lovley *et al*., 1996b). With sulfate as the electron acceptor, anaerobic benzene degradation has been found in a column study (Vogt *et al*., 2007), in microcosms (Edwards and Grbic-Galic, 1992; Lovley *et al*., 1995; Coates *et al*., 1996a; Coates *et al*., 1996b; Kazumi *et al*., 1997; Weiner *et al*., 1998; Anderson and Lovley, 2000), and in enrichments (Phelps *et al*., 1996; Phelps *et al*., 1998; Ulrich and Edwards, 2003; Musat and Widdel, 2007; Herrmann *et al*., 2008). Under methanogenic conditions, anaerobic benzene degradation has been demonstrated in microcosms (Kazumi *et al*., 1997; Weiner and Lovley, 1998) and in enrichments (Vogel and Grbic-Galic, 1986; Grbic-Galic and Vogel, 1987; Ulrich and Edwards, 2003; Chang *et al*., 2005). Furthermore, benzene was degraded with Mn(IV) as the electron acceptor in microcosms and sediment columns (Villatoro-Monzon *et al*., 2003; Villatoro-Monzon *et al*., 2008). Benzene degradation with (per)chlorate as the electron acceptor was shown in pure culture (Coates *et al*., 2001b) and in column studies (Tan *et al*., 2006). (Per)chlorate reduction will be discussed in more detail in paragraph 1.10.

1.9.2 Microbiology of anaerobic benzene degradation

Little is known about the microorganisms responsible for anaerobic benzene degradation. Most information on these microorganisms has been obtained by using molecular approaches. It was only recently that pure cultures of two *Dechloromonas* strains (RCB and JJ) and two *Azoarcus* strains (DN11 and AN9) were isolated, which are capable to degrade benzene anaerobically (Coates *et al*., 2001b; Kasai *et al*., 2006). *Dechloromonas* strain RCB (*Dechloromonas aromatica* RCB) and strain JJ are phylogenetically closely related (98.1% 16S rRNA sequence similarity) and both are members of the newly described *Dechloromonas* genus in the *Betaproteobacteria* (Achenbach *et al*., 2001; Coates *et al*., 2001b). Strain RCB was isolated from river sediment with 4-chlorobenzoate as the electron donor and chlorate as the electron acceptor. Strain JJ was isolated from lake sediment with anthrahydroquinone 2,6-disulphonate (AQDS, a humic substance analogue) as the electron donor and nitrate as the electron acceptor. Both strains are also able to oxidize benzene coupled to the reduction of nitrate. Benzene (160 µM) was completely degraded to CO_2 within 5 days (Coates *et al*., 2001b). So far, subsequent research was only carried out with strain RCB. In addition to benzene, this strain was able to use toluene, ethylbenzene and all three isomers of xylene as the electron donor with nitrate as the electron acceptor. Moreover, in addition to nitrate, strain RCB could degrade benzene with perchlorate, chlorate and oxygen (Chakraborty *et al*., 2005). The *Dechloromonas* species, together with the closely related *Dechlorosoma* species, are considered to represent the predominant (per)chlorate-reducing bacteria in the environment (Coates *et al*., 1999b) and they may be important in

the nitrate-dependent anaerobic degradation of benzene in the environment (Chakraborty and Coates, 2004). Interestingly, molecular analysis of a benzene-degrading nitrate-reducing enrichment culture indicated that the microbial population was dominated (70% of the cloned 16S rRNA gene sequences) by an organism 93% similar to *Dechloromonas* strain JJ. Furthermore, this organism was equally related (93%) to *Azoarcus* species, which are well known for their ability to anaerobically degrade toluene, ethylbenzene and xylene (Ulrich and Edwards, 2003).

Recently, two Azoarcus strains (DN11 and AN9) were isolated, which degrade benzene with nitrate as the electron acceptor (Kasai *et al*., 2006). Subsequently, the degradative capacities of strain DN11 and the potential for its application in bioaugmentation were investigated. Strain DN11 could grow on benzene, toluene, *m*-xylene and benzoate as the sole carbon and energy source under nitrate-reducing conditions, but *o*- and *p*-xylene were only cometabolically transformed in the presence of toluene. Phenol was not utilized under anaerobic conditions. Furthermore, strain DN11 could significantly enhance the benzene degradation after addition of the strain to laboratory batches containing benzene-contaminated groundwater (Kasai *et al*., 2007).

Under Fe(III)-reducing conditions, *Geobacter* species have often been associated with anaerobic benzene degradation. Benzene-degrading Fe(III)-reducing sediments and enrichments were enriched in organisms of the family *Geobacteraceae* (Anderson *et al*., 1998; Rooney-Varga *et al*., 1999; Botton *et al*., 2007). However, several species in the genus *Geobacter* have the ability to anaerobically degrade toluene and other aromatic compounds (Lovley *et al*., 2004), but none of the *Geobacter* species tested degrades benzene. Recently, a detailed functional and phylogenetic characterization of a benzene-degrading iron-reducing enrichment using stable isotope probing was presented. The authors obtained indications that benzene degradation in the enrichment involved an unusual syntrophy, in which members of the genus *Clostridium* primarily oxidize benzene and partially share electrons from benzene with members of the *Desulfobulbaceae* as syntrophic partners (Kunapuli *et al*., 2007). Syntrophic BTX degradation was also suggested to occur in iron-reducing enrichments (Botton *et al*., 2007).

Phelps *et al*. (1998) described the molecular characterization of a benzene-degrading sulfate-reducing enrichment. This enrichment received benzene as the only carbon and energy source for a period of 3 years, but repeated attempts to isolate the responsible bacteria failed. Molecular characterization revealed a diverse collection of phylotypes, i.e.16S rRNA genes belonging to *Proteobacteria*, Cytophageles and Gram-positive phyla as well as one deeply branching clone not closely related to any known bacterium were detected (Phelps *et al*., 1998). Four clones fell within the *Deltaproteobacteria,* in the family *Desulfobacteriaceae* and one of these clones was closely related to a known aromatic hydrocarbon degrader, *Desulfobacula toluolica*. The large diversity of organisms maintained over such a long period of time suggests that a consortium of organisms may be needed to degrade benzene anaerobically in this enrichment. Members of the family *Desulfobacteriaceae* have also been found in a methanogenic benzene-degrading enrichment culture. This enrichment was dominated (33% of the total population) by a clone belonging to the family *Desulfobacteriaceae* (Ulrich and Edwards, 2003). In another study, DGGE analysis demonstrated that a bacterium related to *Desulfobacterium* sp. clone OR-M2 dominated a methanogenic benzene-degrading enrichment (Da Silva and Alvarez, 2007). It was not proven that this *Desulfobacterium* was a benzene degrader, but it was suggested that it either initiates benzene degradation or is a critical (commensal) partner. Musat and Widdel (2007) demonstrated anaerobic benzene degradation by a marine sulfate-reducing enrichment culture. The dominant phylotype in this enrichment was closely related to a clade of *Deltaproteobacteria* that includes sulfate-reducing bacteria able to degrade naphthalene and other aromatic hydrocarbons. Cell hybridization with specifically designed 16S rRNA-targeted fluorescent oligonucleotide probes showed that the retrieved phylotype accounted for more than 85% of the cells detectable via DAPI staining in the enrichment culture (Musat and Widdel, 2007). Recently, a

benzene-degrading sulfate-reducing enrichment was obtained from *in situ* microcosms filled with solids (Herrmann *et al*., 2008). These microcosms had been initiated by exposing vials filled with different solids (sand, lava and Amberlite XAD-7) for 67 days in the sulfidic part of a groundwater monitoring well downstream of the source zone of a benzene-contaminated aquifer and were subsequently incubated in the laboratory. These vials had been placed in a perforated jar and had been opened before exposure in the monitoring well. In control microcosms without filling material, benzene was initially degraded, but the benzene-degrading capacity could not be sustained. The authors suggested that it could be favorable to use solids for the *in situ* enrichment of anaerobic benzene-degrading bacteria, a strategy that might be generally useful for the cultivation of bacteria that are considered to be hardly or not, cultivable (Herrmann *et al*., 2008). Liou *et al*. (2008) used laboratory incubations of coal-tar waste-contaminated sediment microbial communities under relatively controlled physiological conditions (anaerobic with or without sulfate or nitrate additions versus aerobic) to interpret results of a field-based stable isotope probing (SIP) assay. By using this SIP approach the authors were able to associate sets of active benzene-degrading taxa with consumption of particular electron acceptors (nitrate, sulfate or mixed aerobic/anaerobic metabolism) and then qualitatively compare these taxa with those retrieved from an uncontrolled *in situ* field experiment. ^{13}C-DNA clone libraries revealed a broad diversity of taxa involved in benzene metabolism and distinct libraries for each biodegradation treatment. Perhaps most importantly, in the field SIP experiment the clone libraries produced were dominated by *Pelomonas* (*Betaproteobacteria*) sequences similar to those found in the anaerobic benzene laboratory experiment (Liou *et al*., 2008).

1.9.3 Anaerobic benzene degradation pathway

Several mechanisms are known to cleave the aromatic ring anaerobically for aromatic compounds with functional groups such as carboxyl or hydroxyl groups. The anaerobic degradation of benzene, however, is more difficult due to the stability of benzene. The mechanisms of activation and further degradation of benzene are still unknown. The possible initial steps are hydroxylation, carboxylation and methylation, and subsequent transformation to the central aromatic intermediate benzoyl-CoA, which is further degraded to CO_2 (Fig. 5). Below, the different possible pathways will be discussed in more detail.

A. Benzene hydroxylation

In the late 1980s, the studies of Grbic-Galic and Vogel demonstrated that benzene could be degraded under methanogenic conditions (Grbic-Galic, 1986; Vogel and Grbic-Galic, 1986; Grbic-Galic and Vogel, 1987). Phenol, cyclohexanone, and propionate were detected as putative intermediates, suggesting initial hydroxylation of benzene to phenol and subsequent ring reduction of phenol to cyclohexanone. Experiments with ^{18}O-labeled water suggested that the oxygen incorporated into the aromatic ring was derived from water. However, the proportion of ^{18}O-labeled phenol that was produced was less than one third of the amount expected if all of the phenol had originated from anaerobic benzene oxidation with water supplying the oxygen (Vogel and Grbic-Galic, 1986).

An elegant way to investigate whether benzene is degraded via extracellular intermediates is isotope trapping. This approach involves the addition of potential intermediates to the sediments or enrichments to artificially increase the size of the pool of potential extracellular intermediates. If these added compounds are extracellular intermediates, then increasing their pool size will slow down the turnover rate of the intermediate pool. This means that any ^{14}C-labeled compound from ^{14}C-benzene metabolism that enters the intermediate pool, will be degraded more slowly, i.e. the production of ^{14}C-CO_2 is inhibited compared to the situation that the extracellular intermediate pool has not been increased (Lovley *et al*., 1995; Weiner and Lovley, 1998). Using this approach, Weiner and Lovley (1998) found phenol, propionate, and acetate as intermediates in a benzene-degrading methanogenic

enrichment. Furthermore, benzoate, phenol, *o*-hydroxybenzoate, and acetate were detected in a benzene-degrading enrichment derived from a mixed inoculum of cow dung and anaerobic digester sludge (Chaudhuri and Wiesmann, 1995). However, labeled substrate was not used. So these compounds could have been formed from other carbon sources. Another study used ^{13}C-labeled benzene and detection of intermediates with GC/MS (Caldwell and Suflita, 2000). Phenol was detected as intermediate in sulfate-reducing, Fe(III)-reducing and methanogenic enrichments, and benzoate was detected as intermediate in the sulfate-reducing enrichment. Mass spectral results indicated that the carboxyl group of the produced benzoate was ^{13}C-labeled during ^{13}C-benzene degradation. This suggested that another atom of ^{13}C was incorporated, presumably by carboxylation of the ring by a ^{13}C-labeled fragment liberated from the metabolism of the starting compound. Whether phenol is converted to benzoate in the sulfate-reducing enrichment is unknown, because 4-hydroxybenzoate was not found in the culture. Therefore, direct carboxylation of benzene to benzoate could not be ruled out. Under sulfate-reducing conditions, no extracellular intermediates were found in benzene-degrading sediments using isotope trapping experiments (Lovley *et al*., 1995). Phenol, benzoate, *p*-hydroxybenzoate, cyclohexane, catechol and acetate were tested as potential extracellular intermediates in this study. This could indicate that there was no extracellular production of any intermediates and that benzene was degraded to CO_2 within single cells of microorganisms.
It is possible, but difficult to hydrate molecules with double bonds that lack an adjacent carbon with a functional group, such as benzene (Coates *et al*., 2002). It is not known which enzymes are involved in this reaction, but a recently purified molybdenum-containing, iron-sulfur protein that adds a hydroxyl group to ethylbenzene may provide a model for hydroxylation of benzene (Johnson *et al*., 2001). In most prokaryotic systems studied, these oxomolybdenum enzymes catalyse the transfer of an oxygen atom from water to the substrate (Coates *et al*., 2002). Hydroxylation of the aromatic ring can also occur by a non-enzymatic mechanism involving highly reactive hydroxyl radicals (HO·), which can be formed by a Fenton-like reduction of hydrogen peroxide (H_2O_2) in the presence of ferrous iron complexes. These radicals can be incorporated into the aromatic ring. However, it is not apparent how hydrogen peroxide could be formed anaerobically though such a mechanism may be possible at interfaces between aerobic and anaerobic zones (Coates *et al*., 2002). The conversion of phenol to benzoate probably involves the carboxylation of phenol to form *p*-hydroxybenzoate (4-hydroxybenzoate). Several pure cultures degrade phenol anaerobically via initial carboxylation and this has been observed under nitrate-reducing, sulfate-reducing and methanogenic conditions (Heider and Fuchs, 1997). Anaerobic phenol degradation in *Thauera aromatica*, for instance, starts with the phosphorylation to form phenylphosphate, which is then carboxylated by phenylphosphate carboxylase, forming *p*-hydroxybenzoate (Schühle and Fuchs, 2004). The *p*-hydroxybenzoate is further activated by a specific CoA ligase and the hydroxyl group is reductively removed (Heider and Fuchs, 1997). However, in studies dealing with anaerobic benzene degradation, *p*-hydroxybenzoate has never been detected as intermediate. Evidence for benzene degradation via phenol formation and subsequent conversion to benzoate was found in a benzene-degrading methanogenic enrichment culture (Ulrich *et al*., 2005) and in a benzene-degrading iron-reducing enrichment (Botton and Parsons, 2007).

Recently, the anaerobic benzene degradation pathway was studied in *Dechloromonas aromatica* RCB using nitrate as terminal electron acceptor. Initial hydroxylation to phenol and subsequent carboxylation to *p*-hydroxybenzoate, and loss of the hydroxyl group to form benzoate (or the CoA derivative, benzoyl-CoA) was found in this organism (Chakraborty and Coates, 2005) (Fig. 5, pathway A). GC-MS analysis revealed the transient formation of phenol and benzoate in benzene-degrading cultures of strain RCB using nitrate as the electron acceptor. The concentrations of phenol and benzoate in filtered and unfiltered culture broths were identical, suggesting that both the hydroxylation

reaction and benzoate formation occurred at the outer membrane or in the periplasm of strain RCB. Probably, benzoate is transported into the cell for its subsequent conversion to benzoyl-CoA and further catabolism to CO_2 (Chakraborty and Coates, 2005). Hydroxylation of benzene to phenol can be performed by monooxygenase enzyme systems in the presence of oxygen (Gibson and Subramanian, 1984). To ensure that the benzene degradation with nitrate as the electron acceptor in strain RCB was not due to a similar mechanism involving molecular oxygen, chemical reductants (0.5 mM sodium ditionite or 0.1 mM sodium ascorbate) were added to active benzene-degrading cultures to remove any traces of oxygen. In the presence of the reductants, ^{14}C-benzene was still rapidly oxidized to $^{14}CO_2$. Formation of phenol was detected in cultures amended with sodium dithionite, however, no phenol was detected in benzene-degrading cultures of strain RCB with oxygen as the electron acceptor. Although the benzene metabolism was not inhibited, addition of dithionite (0.5 mM) retarded the benzene degradation rate and subsequent phenol formation. Higher concentrations of dithionite (>1 mM) completely inhibited benzene degradation. However, even at a dithionite concentration of 0.5 mM, the phenol degradation rates remained unaffected. It was suggested that dithionite inhibited the initial hydroxylation step of benzene degradation rather than the subsequent oxidation of phenol to CO_2 by strain RCB (Chakraborty and Coates, 2005).
To investigate the origin of the hydroxyl group of phenol, experiments with ^{18}O-labeled H_2O were performed. In a previous study, indications were obtained that the hydroxyl group of phenol originated from water (Vogel and Grbic-Galic, 1986). However, hardly any incorporation of ^{18}O label into the hydroxyl group of phenol was found in benzene-degrading culture of strain RCB and it was concluded that H_2O is not the source of the hydroxyl group. Unfortunately, it was not investigated whether the hydroxyl group of phenol could originate from nitrate, for instance using ^{18}O-labeled NO_3^-. Chakraborty *et al*. (2005), on the other hand, investigated whether highly reactive hydroxyl free radicals (HO·) were involved in the hydroxylation of benzene to phenol in strain RCB. They found that hydroxyl free radical scavengers, such as sodium iodide (0.5 mM), propyl iodide (0.5 mM), 5,5-dimethyl-1-pyrroline-*N*-oxide, or mannitol (10 mM), inhibited benzene degradation with nitrate as the electron acceptor. Phenol degradation and acetate oxidation under nitrate-reducing conditions remained unaffected in the presence of these scavengers, demonstrating that these compounds were not toxic to strain RCB. These results suggested that hydroxyl free radicals play an important role in the benzene ring hydroxylation. Since phenol and benzoate have been detected in several studies as intermediates of anaerobic benzene degradation under different redox conditions, Chakraborty *et al*. (2005) suggested that a single universal pathway may exist for anaerobic benzene degradation and that it is similar to the pathway in strain RCB (Fig. 5, pathway A). Recently, the genome of *Dechloromonas aromatica* strain RCB has been sequenced and is available (GenBank, http://www.ncbi.nlm.nih.gov/, accession no. CP000089). *Dechloromonas aromatica* has a single circular DNA chromosome with a length of 4,501,104 bps and 4,204 predicted protein coding genes. A function can be assigned to 69% of the protein coding genes. Several putative genes can be found in the genome sequence that could be involved in benzene metabolism. However, up to now no detailed analysis on these (putative) genes (or proteins) in strain RCB has been published.

Azoarcus strain DN11 could not grow on phenol, indicating that this strain uses a different anaerobic benzene degradative pathway from the pathway of *Dechloromonas* strain RCB, but it is not known what kind op pathway (Kasai *et al*., 2007). The benzene-degrading sulfate-reducing enrichment described by Musat and Widdel (2007) did not show metabolic activity towards phenol or toluene. Based on this observation the authors suggested that benzene degradation by the enrichment does not proceed via anaerobic hydroxylation to phenol or methylation to toluene (Musat and Widdel, 2007). Recently, it was demonstrated that caution should be exercised in interpreting hydroxylated benzene derivatives as metabolic intermediates of anaerobic benzene degradation (Kunapuli *et al*., 2008) in a

study with $^{13}C_6$-labelled benzene as the growth substrate for a benzene-degrading iron-reducing enrichment culture. Phenol was also identified as an intermediate at high concentration. However, it was clearly shown that phenol was formed abiotically by autoxidation of benzene during the sampling and analysis procedure as a result of exposure to air. This results in the production of hydroxyl radicals which readily react with aromatic compounds such as benzene producing phenol and hydroxylation products of phenol (Kunapuli *et al*., 2008):

$C_6H_6 + OH\cdot \rightarrow C_6H_5OH + H\cdot$ (1)

The authors suggest that autoxidation during sampling could also be a possible reason for non-detection of label in the hydroxyl group of phenol when the anaerobic benzene-degrading, denitrifying *Dechloromona*s strain RCB was incubated with labelled $H_2{}^{18}O$ (Chakraborty and Coates, 2005). Chakraborty and Coates (2005) ruled out water or air as possible sources of the hydroxyl group, as only 1% label incorporation was observed when the culture was incubated with $H_2{}^{18}O$ (25% and 50% labelled) and also phenol production occurred when dithionite was added to scavenge any oxygen present. As water is the only reasonable oxygen source for microorganisms in the absence of molecular oxygen, the hydroxyl oxygen had to derive from air, perhaps by autoxidation during sampling (Kunapuli *et al*., 2008).

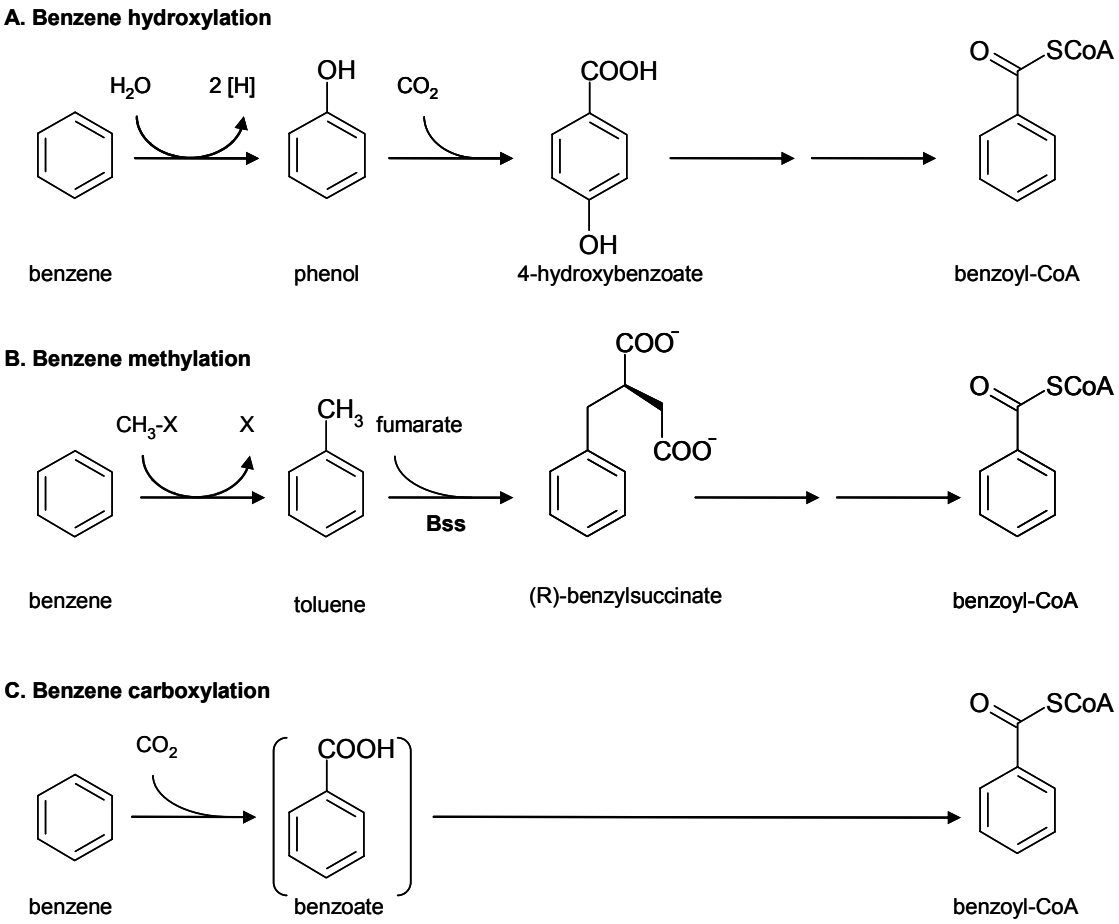

Figure 5. Possible mechanisms of the initial steps of benzene degradation under anaerobic conditions: A, benzene hydroxylation; B, benzene methylation; C, benzene carboxlation.

B. Benzene methylation

Methylation of benzene is an attractive option from a mechanistic point of view. Benzene is susceptible to substitution by strong electrophiles as in Friedel-Crafts alkylation. The electrophile could be the H_3C^+ unit derived from S-adenosylmethionine. Biological mediated methylation of benzene to toluene has previously been observed with human bone marrow incubated with *S*-adenosyl-methionine (Flesher and Myers, 1991). Methylation of benzene to toluene is energetically favorable using *S*-adenosyl-methionine as the methyl donor (Coates *et al*., 2002). Methylation has also been proposed for the anaerobic activation of another unsubstituted aromatic hydrocarbon, naphthalene. This reaction has been found in a sulfate-reducing enrichment culture and the methyl group might possibly be generated from bicarbonate via a reverse CO-dehydrogenase pathway (Safinowski and Meckenstock, 2005).

The first direct evidence for benzene methylation was recently found by Ulrich *et al*. (2005). In a nitrate-reducing and methanogenic enrichment culture [*ring*-^{13}C]-toluene was detected by GC/MS when ^{13}C-benzene was added to these cultures. The nature of the methyl group and the subsequent toluene degradation pathway are not known, but degradation via the benzylsuccinate synthase (Bss) pathway was hypothesized (Ulrich *et al*., 2005).

C. Benzene carboxylation

Carboxylation of benzene to form benzoate (or benzoyl-CoA) has been reported (Caldwell and Suflita, 2000; Phelps *et al*., 2001). In a sulfate-reducing enrichment, deuterated benzoate (D5) was detected when deuterated benzene (D6) was added to the enrichment, however, phenol was not detected (Phelps *et al*., 2001). The carboxyl group of benzoate was not labeled when ^{13}C-bicarbonate or ^{13}C-acetic acid were added to the enrichment. This is in contrast to the proposed pathway for anaerobic naphthalene and phenanthrene metabolism, where carboxylation of the aromatic ring by carbon dioxide (or bicarbonate) is the initial activation step (Zhang and Young, 1997; Meckenstock *et al*., 2000). The lack of incorporation of ^{13}C-labeled bicarbonate into the aromatic ring of benzene (Phelps *et al*., 2001) and the suggestion that the carboxyl group of benzoate may be derived from benzene itself (Caldwell and Suflita, 2000) could argue against a direct carboxylation mechanism. However, Kunapuli *et al*. (2008) demonstrated that in a benzene-degrading iron-reducing enrichment culture the carboxyl group of benzoate derived from the bicarbonate buffer, indicating a direct carboxylation of benzene. With $^{13}C_6$-labelled benzene as the growth substrate, C_7-labelled benzoate appeared, indicating that the carboxyl group of benzoate derived from CO_2 that was produced from mineralization of labelled benzene. This was confirmed by growing the culture in ^{13}C-bicarbonate-buffered medium with unlabelled benzene as the substrate, as the label appeared in the carboxyl group of benzoate produced. When using unlabelled buffer and high concentrations of ^{13}C-labelled benzene, a significant portion of the bicarbonate buffer became labelled by the release of $^{13}HCO_3$ from complete benzene oxidation. As the carboxyl group stems from the bicarbonate buffer, this eventually leads to the generation of $^{13}C_7$-benzoate. One could speculate that this finding might also explain why other authors could detect ^{13}C-carboxy-labelled benzoate when applying labelled benzene together with unlabelled bicarbonate buffer (Caldwell and Suflita, 2000).

1.9.4 Characterizing and assessing anaerobic benzene degradation: isotopic fractionation

In the previous paragraphs, it has already been shown that all BTEX compounds can be biodegraded under oxic and anoxic conditions. Biodegradation is the most significant process leading to a decrease in BTEX concentrations in soil and groundwater. Therefore, the evaluation of *in situ* BTEX biodegradation is essential for the implementation of groundwater management strategies such as natural attenuation (NA). Traditional methods used to confirm bioremediation in the field included monitoring decreases in contaminant concentrations and electron acceptors, and increases in

microbial (by)products (such as carbon dioxide). This is difficult for benzene because of e.g. high background HCO_3^- concentrations. However, the challenge is to prove biodegradation in the field, since other processes such as volatilization, dispersion, and sorption can cause loss of the contaminant, and accurate mass balances are difficult to obtain (Mancini *et al*., 2003). In recent years, stable isotope fractionation analysis has gained attention as a tool for characterizing and assessing in-situ biodegradation of organic pollutants in contaminated aquifers (Meckenstock *et al*., 2004; Fischer *et al*., 2007). This concept relies on the fractionation of stable isotopes during the microbial degradation, leading to an enrichment of heavier isotopes in the residual fraction of a pollutant. Isotopes of elements such as carbon (^{12}C and ^{13}C) and hydrogen (^{1}H and ^{2}H) react at slightly different rates during mass-differentiating reactions. During biodegradation, bonds containing the lighter isotopes are preferentially broken, causing the remaining contaminant to be enriched in the heavier isotopes compared to the original isotopic value. A large isotopic fractionation effect (primary isotope effect) can be observed if a bond (e.g. C-H bond) containing the element of interest is broken or formed in the rate-limiting step. Stable isotope fractionation has the ability to identify biodegradation of aromatic hydrocarbons in the field and to distinguish contaminant mass loss due to biodegradation versus that due to physical processes.

In studies concerning stable isotope fractionation of benzene, carbon (^{12}C and ^{13}C) and hydrogen (^{1}H and ^{2}H) isotope ratios are expressed in the delta notation ($\delta^{13}C$ and $\delta^{2}H$) in per mil (‰) units according to the following equation:

$$\delta^{13}C_{sample} \quad or \quad \delta^{2}H_{sample}\,[‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \cdot 1000 \qquad (1)$$

In this equation, R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ or $^{2}H/^{1}H$-ratio of the sample and an international standard, respectively. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope signature and Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios. For the description of isotope fractionation of biochemical reactions the Rayleigh equation can be used:

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad (2)$$

where R_t, C_t and R_0, C_0 are the stable isotope ratios and concentrations of a compound at a given point in time and at the beginning of a transformation reaction, respectively. The enrichment factor ε [‰] provides the link between the changes in stable isotope ratios (R_t/R_0) and the changes in the concentrations (C_t/C_0) (Fischer *et al*., 2007). In recently published laboratory experiments, carbon and hydrogen isotope discrimination were determined for aerobic and anaerobic benzene biodegradation (Hunkeler *et al*., 2001; Mancini *et al*., 2002; Mancini *et al*., 2003; Fischer *et al*., 2008) (see Table 5).

The biodegradation pathway of the aerobic cultures used in the study by Fischer *et al*. (2008), i.e. mono- or dihydroxylation, was known, and therefore a relation between enrichment factors and biodegradation pathway could be deduced. Their results indicate that carbon enrichments factors for dihydroxylation of benzene are significantly smaller compared to monohydroxylation and anaerobic degradation. No systematic differences are given for carbon enrichments factors of monohydroxylation and anaerobic degradation. The carbon enrichment factors for monooxygenase reaction are higher as benzene dihydroxylation, which can be expected when a cleavage of a C-H bond is involved to some extent as revealed in several studies (Hanzlik *et al*., 1984; Mitchell *et al*., 2003). Hydrogen enrichment factors for benzene monohydroxylation are higher compared to dihydroxylation. Hydrogen enrichment factors for benzene degradation under anaerobic (sulfate-reducing) conditions were significantly higher than for aerobic benzene degradation. The variability in the hydrogen isotope fractionation for the benzene biodegradation can be explained by different initial reaction mechanisms. Carboxylation,

methylation or hydroxylation of benzene might cause the cleavage of a C-H bond under anaerobic condition. In contrast, aerobic benzene biodegradation should only lead to secondary hydrogen isotope effects if a dihydroxylation reaction occurs, because no hydrogen bond is broken in the first irreversible reaction step of the transformation pathway (Hunkeler *et al*., 2001; Mancini *et al*., 2003) or a low isotope effect can be expected when a epoxylation is the initial reaction step (Mitchell *et al*., 2003). Similarly, the transformation of benzene by mammalian and methane monooxygenases exhibited a small or even negligible hydrogen isotope effect which was explained by the absence of C-H bond cleavage in the initial enzymatic degradation step (Hunkeler *et al*., 2001).

Table 5. Comparison of carbon and hydrogen enrichment factors (ε_C, ε_H) in aerobic and anaerobic benzene degradation.

Culture	Enzymatic pathway	ε_C [‰]	ε_H [‰]	Reference
Burkholderia sp. (aerobic)	unknown	-3.5 ± 0.3	-11 ± 2	(Hunkeler *et al*., 2001)
Acinetobacter sp. (aerobic)	unknown	-1.5 ± 0.1	-13 ± 1	(Hunkeler *et al*., 2001)
Rhodococcus opacus strain B-4	dihydroxylation	-1.3 ± 0.2	No enrichment, ±5 [(a)]	(Fischer *et al*., 2008)
Pseudomonas putida strain ML2	dihydroxylation	-0.7 ± 0.1	No enrichment, ±5 [(a)]	(Fischer *et al*., 2008)
Ralstonia picketii strain PKO1	monohydroxylation	-1.7 ± 0.2	-11 ± 4	(Fischer *et al*., 2008)
Cupriavidus necator ATCC 17697	monohydroxylation	-4.3 ± 0.4	-17 ± 11	(Fischer *et al*., 2008)
Nitrate-reducing enrichment	unknown, C-H bond cleavage expected	-2.2 ± 0.4 -2.4 ± 0.1	-29 ± 4 -35 ± 6	(Mancini *et al*., 2003)
Sulfate-reducing enrichment	unknown, C-H bond cleavage expected	-3.6 ± 0.3	-79 ± 4	(Mancini *et al*., 2003)
Methanogenic enrichment	unknown, C-H bond cleavage expected	-1.9 ± 0.1 -2.1 ± 0.1 -2.0 ± 0.1	-60 ± 3 -59 ± 4 -59 ± 3	(Mancini *et al*., 2003)
Sulfate-reducing mixed culture	unknown	-1.9 ± 0.3	-59 ± 10	(Fischer *et al*., 2008)

[a] Expected range for ε_H given by the uncertainty of hydrogen isotope analysis.

1.10 (Per)chlorate reduction

The last decade, the process of (per)chlorate reduction received a lot of attention, resulting in the isolation of various (per)chlorate-reducing bacteria and the characterization of the key enzymes. One of the most interesting aspects of (per)chlorate reduction is the fact that during (per)chlorate reduction molecular oxygen is produced. Oxygen formation during (per)chlorate reduction may be advantageous for the anaerobic biodegradation of persistent aromatic compounds, such as benzene. Therefore, below (per)chlorate reduction will be discussed in more detail.

1.10.1 Production, application and toxicity of (per)chlorate

The oxyanions perchlorate (ClO_4^-) and chlorate (ClO_3^-) mainly end up in the environment as a result of anthropogenic activities. So far, the only significant natural source of (per)chlorate is that associated with mineral deposits as found in Chile (Coates and Achenbach, 2004). However, there are indications for other natural sources, such as the formation of (per)chlorate in atmospheric processes (Dasgupta

et al., 2005). Recently, data gathered by NASA's Phoenix lander on Mars revealed that the red planet's soil could contain perchlorate. Approximately 90% of all perchlorate salts manufactured consist of ammonium perchlorate (NH_4ClO_4). It is predominantly used in the munitions industry as an energetics booster or oxidant in solid rocket fuel (Coates and Achenbach, 2004). Furthermore, perchlorate is also a component in fire-works. Chlorate is applied in agriculture as weed controller (herbicide) and as defoliant (Logan, 1998). Chlorate is also used for the production of chlorine dioxide (ClO_2), which is used as bleaching agent in the pulp and paper industries. Moreover, chlorate enters the environment due to the decomposition of hypochlorite (ClO^-), which is used as disinfectant.
Chemically, perchlorate is very stable in water, even in highly reducing environments. Chlorate is thermodynamically less stable than perchlorate. Nevertheless, aqueous chlorate concentrations were stable in various water systems, indicating that chlorate is chemically stable under most environmental conditions (Urbansky, 1998; Kang *et al*., 2006). Chlorite is very unstable, it can react with many reducing species and disproportionates under environmental conditions. Perchlorate contamination poses a significant health threat, because toxicological studies have demonstrated that it has a direct effect on iodine uptake by the thyroid gland. In addition, at higher concentrations (6 mg (kg body weight)$^{-1}$ d^{-1}) perchlorate can result in a lethal bone marrow disease. Both chlorate and chlorite cause hemolytic anemia in laboratory animals (Coates *et al*., 1999b). In 1998, perchlorate was added to the US Environmental Protection Agency (EPA) contaminant candidate list for drinking water supplies. In 1997, the EPA proposed a provisional recommended maximum concentration limit (MCL) of 18 µg/l for perchlorate in drinking water supplies. Currently, more toxicological and risk assessment studies are done to make the MCL more well founded. The World Health Organization (WHO) provided a (provisional) drinking water quality guideline for chlorate and chlorite of 0.7 mg/l; no values for perchlorate are given (World Health Organization (WHO), 2006). Perchlorate and chlorate have been detected as groundwater contaminants in the United States. Perchlorate contamination of ground water can be found especially in the southwestern states of California, Utah and Nevada. The highest perchlorate concentrations measured in the groundwater in the United States ranged from 630 mg/l to 3,700 mg/l. Surface water concentrations of chlorate in the Netherlands have been reported for the rivers IJssel, Meuse and Rhine, as 25 µg/l, 40 µg/l and 19 µg/l, respectively (Versteegh *et al*., 1993).
Removal of (per)chlorate from waste water or polluted sites can be achieved using different techniques. Typical water treatment technologies, such as ion exchange and carbon adsorption, so far have not been proven to be cost-efficient for the removal of perchlorate from drinking water (Logan, 1998; Urbansky, 1998). Biological treatment of (per)chlorate contaminated waste water or soil (bioremediation) seems more promising, because many microorganisms can use (per)chlorate as terminal electron acceptor for growth.

1.10.2. Microbial (per)chlorate reduction

It has been known for more than 50 years that microorganisms can reduce oxyanions of chlorine such as perchlorate and chlorate under anaerobic conditions. The high reduction potential of perchlorate (ClO_4^-/Cl^- E^0 = 1.287 V) and chlorate (ClO_3^-/Cl^- E^0 = 1.03 V) makes them ideal electron acceptors for microbial metabolism. When the reduction of (per)chlorate is coupled to electron-transport phosphorylation, microorganisms are able to grow by (per)chlorate reduction. Until 1996, little was known about the biochemical pathway of (per)chlorate reduction. Many denitrifying bacteria are able to reduce (per)chlorate, but in general, this reduction is not coupled to growth (Coates and Achenbach, 2004). Denitrifying bacteria likely cannot grow on chlorate because of the accumulation of toxic chlorite during (per)chlorate reduction (Fig. 6). Therefore, bacteria are able to grow by dissimilatory (per)chlorate reduction when: 1) these oxyanions can be used as electron acceptor and 2) the toxic intermediate chlorite is converted into chloride and oxygen.

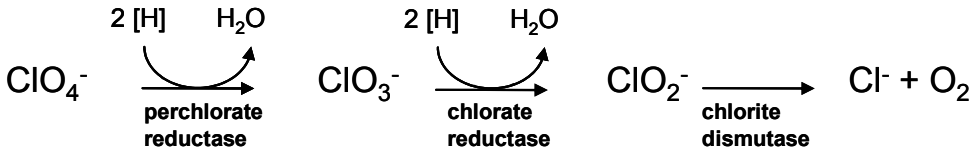

Figure 6. (Per)chlorate reduction scheme.

Many dissimilatory (per)chlorate reducing bacteria (DPRB) have been isolated and described. A comprehensive overview is given by Coates and Achenbach (2004) (Coates and Achenbach, 2004). DPRB are phylogenetically diverse and these bacteria can be found in the *Alpha-, Beta, Gamma* and *Epsilonproteobacteria*. The (per)chlorate-reducing species in the *Betaproteobacteria* represent two novel genera, i.e. the *Dechloromonas* and *Azospira* (formerly *Decholorosoma*). DPRB have been isolated from a broad diversity of environments, including both pristine and contaminated soils and sediments (Coates *et al*., 1999b). This was remarkable, because of the limited natural occurrence of (per)chlorate. However, these bacteria have diverse metabolic capabilities and this could account for their presence in environments in which (per)chlorate was not found. Phenotypic characterization revealed that the DPRB exhibited a broad range of metabolic capabilities, including the oxidation of hydrogen, simple organic acids and alcohols, aromatic hydrocarbons, hexoses, reduced humic substances, both soluble and insoluble ferrous iron, and hydrogen sulfide. All of the known DPRB are facultatively anaerobic or microaerophilic, which is understandable because oxygen is produced during (per)chlorate reduction. Some, but not all, DPRB can use nitrate as terminal electron acceptor for growth. So far, all microorganisms capable of perchlorate reduction can also use chlorate as the electron acceptor. However, microorganism capable of chlorate reduction are not always capable of using perchlorate as the electron acceptor (Coates and Achenbach, 2004).

1.10.3 Biochemistry and genetics of (per)chlorate reduction

Chlorite dismutation by DRPB is mediated by a chlorite dismutase. Van Ginkel *et al*. (1996) purified an enzyme from *Azospira oryzae* strain GR-1 which catalyzed the disproportionation of chlorite into chloride and oxygen(Van Ginkel *et al*., 1996). Since, some other chlorite dismutases have been isolated (Coates *et al*., 1999b; Stenklo *et al*., 2001; Bender *et al*., 2002). The obtained data in these studies suggest that the enzymes are homotetramers with a molecular mass ranging of approximately 120-140 kDa.

Initial studies suggested that chlorite dismutase is highly conserved among (per)chlorate-reducing bacteria. An immunoprobe with high affinity for chlorite dismutase was specific to (per)chlorate reducing bacteria; cross-reactivity with non-DRPB, which were phylogenetically related (16S rRNA similarity > 97%) was not observed (O'Connor and Coates, 2002). Recently, metabolic primers sets for *cld* genes were developed for the environmental detection of (per)chlorate-reducing bacteria and the first phylogenetic analysis of the *cld* genes was performed. Comparison of the *cld* and 16S ribosomal DNA (rDNA) gene trees indicated that the *cld* gene does not follow 16S rRNA phylogeny, indicating the possible role of horizontal transfer in the evolution of (per)chlorate respiration (Bender *et al*., 2004).

Kengen *et al*. (1999) purified and partially characterized a oxygen-sensitive (per)chlorate reductase enzyme from the (per)chlorate reducing bacterium *Azospira oryzae* strain GR-1. This enzyme, a heterodimer, is located in the periplasm of the bacterium. It has a total molecular mass of 420 kDa and contains iron, molybdenum and selenium (Kengen *et al*., 1999). In addition to perchlorate, this perchlorate reductase also catalyses the reduction of chlorate, nitrate, iodate and bromate. One enzyme is responsible for both reduction of perchlorate and chlorate (Kengen *et al*., 1999). Other

(per)chlorate reductases have been purified and characterized (Oltmann *et al*., 1976; Danielsson Thorell *et al*., 2003; Wolterink *et al*., 2003). The chlorate reductase of *Pseudomonas chloritidismutans* did not possess reductase activity with perchlorate (Wolterink *et al*., 2003). The effect of nitrate on (per)chlorate reduction by (per)chlorate-reducing bacteria is still not well understood, particularly with respect to the induction of pathways used to degrade these different chemicals. Perchlorate respiratory enzymes were inducible (by chlorate or perchlorate) and were different from those used for denitrification by perchlorate-reducing strain *Dechlorosoma* sp. KJ. Furthermore, they found that in *Pseudomonas* sp. PDA, a chlorate-reducing bacterium unable to grow with perchlorate or nitrate, chlorate reductase and chlorite dismutase were constitutively expressed under anaerobic and aerobic conditions independent of the presence of chlorate (Xu *et al*., 2004). Recently, the genes encoding perchlorate reductase (*pcrABCD*) in two *Dechloromonas* species were characterized. Expression analysis of the pcrA gene from *Dechloromonas agitata* indicated that transcription occurred only under anaerobic (per)chlorate-reducing conditions. The presence of oxygen completely inhibited pcrA expression regardless of the presence of perchlorate, chlorate, or nitrate. Deletion of the pcrA gene in *Dechloromonas aromatica* abolished growth with both perchlorate and chlorate but not growth with nitrate, indicating that the *pcrABCD* genes play a functional role in perchlorate reduction separate from nitrate reduction. Unlike perchlorate reductase, the chlorate reductase enzyme of *Ideonella dechloratans* and *Pseudomonas chloritidismutans* is a heterotrimer and was able to use chlorate but not perchlorate as substrate. The genes encoding this enzyme consist of a gene order of *clrABCD* (α-subunit, β-subunit, chaperone protein and γ-subunit) (Danielsson Thorell *et al*., 2003; Wolterink *et al*., 2003). This differs from the gene order for perchlorate reductase genes, which are organized *pcrABCD* (α-subunit, β-subunit, γ-subunit and chaperone protein) (Bender *et al*., 2005). The significance of the difference of the perchlorate reductase and chlorate reductase gene order is unknown (Coates and Achenbach, 2004).

1.10.4 Potential of (per)chlorate-reducing bacteria in bioremediation

(Per)chlorate reducing bacteria can be used for the treatment of perchlorate- and chlorate-containing waste streams and groundwater (Logan, 1998; Urbansky, 1998), e.g. the bioremediation of perchlorate-contaminated groundwater in a packed bed biological reactor (Losi *et al*., 2002) or the treatment of chlorate and perchlorate contaminated water using permeable barriers containing vegetable oil (Hunter, 2002). The ability of (per)chlorate-reducing bacteria to produce oxygen can also be applied in other bioremediation processes. The dismutation of chlorite by (per)chlorate-reducing bacteria in anaerobic environments can produce extracellular O_2. This O_2 can for instance be used by bacteria to degrade hydrocarbons. Especially, hydrocarbons that are slowly degraded or persistent under anaerobic conditions could be degraded by this mechanism (Coates *et al*., 1999a). An example of such a compound is benzene.

Molecular oxygen can be introduced into the anaerobic zones of a contaminated environment by injection of compressed air or O_2 below the water table (Coates *et al*., 1999a), but this is a costly and inefficient process due to the low solubility of oxygen. Hydrogen peroxide is often used as an additional soluble O_2 source, but this process has some disadvantages, such as toxicity of hydrogen peroxide to many bacteria. Also solid O_2-releasing compounds can be used, such as magnesium peroxide (MgO_2) or calcium peroxide (CaO_2). These compounds consist of powdery material and can also be used as injected slurries for *in situ* bioremediation. These O_2-releasing compounds have some advantages, but the high costs of the O_2-releasing compound and the difficulty to evenly distribute the compounds (i.e. the O_2) over a large area, for instance a contaminated aquifer, may be major disadvantages. Therefore, chlorite dismutation of by (per)chlorate-reducing bacteria offers a good alternative strategy to supply extracellular oxygen to the aerobic hydrocarbon-oxidizing population. Cell suspensions of (per)chlorate reducing cells of strain GR-1 showed formation of (extracellular)

oxygen upon the addition of chlorite (Rikken *et al*., 1996). The potential use of CRPB for bioremediation (of soils and sediments) has already been recognized in previous studies (Coates *et al*., 1999a; Logan and Wu, 2002). The amendment of (per)chlorate-reducing and chlorite to an anoxic soil led to complete degradation of ^{14}C-benzene to ^{14}C-carbon dioxide. Furthermore, the addition of chlorite in anoxic soils samples, inoculated with starved cells of the (per)chlorate reducer *Dechloromonas agitata* strain CKB, showed that ^{14}C-benzene was rapidly oxidized to ^{14}C-carbon dioxide. This observation further enhances the applicability of these kind or organisms to *in situ* bioremediation (Coates *et al*., 1999a). In another study, increased rates of toluene degradation were observed in sand columns inoculated with both (aerobic) toluene-degrading and chlorate-reducing enrichment cultures, which indicated a symbiotic relationship between the toluene-degrading bacteria and chlorate-reducing bacteria (Logan and Wu, 2002). Recently, addition of chlorate to a soil column polluted with benzene showed removal of benzene coupled with chlorate reduction (Tan *et al*., 2006).

1.11 Outline of the thesis

Several studies concerning anaerobic BTEX degradation have been performed and published (refer to the previous paragraphs in this chapter), but there are still gaps in the knowledge about anaerobic benzene degradation. In particular, the bacteria involved in anaerobic benzene degradation and the anaerobic benzene degradation pathway have still not been elucidated. The aim of the research presented in this thesis was to gain more insight in the degradation of benzene and other aromatic hydrocarbons by anaerobic bacteria. In particular, the physiology and phylogeny of the bacteria responsible for the degradation were studied.

Chapter 2 describes the anaerobic biodegradation of aromatic hydrocarbons in an aquifer polluted by BTEX-containing landfill leachate under different redox conditions. The degradation of benzene and toluene with different electron acceptors was studied in batch experiments with subsurface soil samples from the Banisveld landfill (Boxtel, The Netherlands) as inoculum. Toluene-degrading enrichments were established and molecular methods (PCR, DGGE, cloning and sequencing) were used to study the microbial composition of these enrichments. Subsequently, a novel toluene-degrading bacterium was isolated from these enrichments. The physiology and phylogeny of this bacterium was studied and the results are presented in chapter 3.

In chapter 4 the physiological and phylogenetic characterization of a benzene-degrading chlorate-reducing enrichment culture is presented. Molecular methods showed that this enrichment culture was stable and consisted of (at least) four different bacterial species. The species responsible for the benzene transformation was isolated and the physiological and phylogenetic characteristics of the bacterium were studied (chapter 5). In chapter 6 the physiological characteristics of this bacterium are compared with these of the closest related bacterium (*Alicycliphilus denitrificans* K601^{T}). Finally, in chapter 7 the main conclusions of this thesis are summarized and discussed in a broader context.

2

Anaerobic benzene and toluene biodegradation studies with different electron acceptors in contaminated aquifer microcosms

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To be submitted

Abstract

Benzene and toluene degradation was studied in microcosms under different electron accepting conditions. The microcosms were inoculated with soil samples from an aquifer, which was polluted by BTEX-containing landfill leachate (Banisveld landfill, The Netherlands). Benzene was not degraded during one year of incubation. Toluene degradation, on the other hand, was observed with nitrate, MnO_2 and Fe(III)NTA as electron acceptors. In transfers and a dilution series, toluene degradation could be sustained with MnO_2 and Fe(III)NTA. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments showed that in both toluene-degrading cultures the same bacterial phylotype became dominant. This organism is likely to be responsible for the toluene degradation. Cloning and sequencing of the 16S rRNA gene fragments revealed that this dominant phylotype is closest related to *Sterolibacterium denitrificans*, a cholesterol-oxidizing, denitrifying bacterium within the *Betaproteobacteria* (94.6% similarity based on 16S rRNA genes). This finding shows that besides *Geobacter*, *Thauera*, *Azoarcus* and *Magnetospirillum* other genera may be involved in anaerobic degradation of aromatic hydrocarbons under iron- and nitrate-reducing conditions.

Introduction

Aromatic hydrocarbons are widespread in nature and often cause severe pollution of soils, sediments and groundwater. Particularly, monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, xylene (BTEX) are often found in the environment. These contaminants are of environmental concern because they are relatively soluble, toxic, carcinogenic and can be very mobile in the environment (Coates *et al*., 2002; Badham and Winn, 2007). Contamination with BTEX is observed at many sites, especially those related with petrochemical activities like refinery and gasoline stations.

BTEX contamination is often present in the anaerobic zones of the environment (Lovley, 1997). Therefore, anaerobic bioremediation is an attractive remediation technique for these polluted environments. The bottleneck in the application of such approaches is the poor anaerobic biodegradability of benzene. However, evidence for anaerobic benzene degradation has recently started to accumulate, as anaerobic benzene degradation with different electron acceptors has been observed in some sediment, microcosm and column studies and microbial enrichments (Lovley, 2000; Phelps and Young, 2001; Coates *et al*., 2002; Chakraborty and Coates, 2004). Moreover, recently the first two anaerobic benzene-degrading *Dechloromonas* species and two *Azoarcus* species were described (Coates *et al*., 2001b; Kasai *et al*., 2006). However, the optimal physiological conditions for anaerobic benzene-degrading microorganisms and the corresponding biodegradation pathway(s) are still unknown.

Anaerobic toluene degradation, on the other hand, has been studied in considerable detail and reviewed (Frazer *et al*., 1995; Phelps and Young, 2001; Boll *et al*., 2002; Chakraborty and Coates, 2004). Toluene degradation has been observed with different electron acceptors and several anaerobic toluene-degrading bacteria have been isolated and studied. Biodegradation pathways, enzymes and genes involved in anaerobic toluene degradation have been described in the last years. These studies revealed that the first step in the catabolism of toluene is the addition of fumarate onto the toluene methyl group to form benzylsuccinate by benzylsuccinate synthase (Leuthner *et al*., 1998). In a series of enzymatic steps, benzylsuccinate is converted to benzoyl-CoA, which is then further oxidized after a reductive ring cleavage. Benzoyl-CoA has been recognized as a central intermediate in the anaerobic degradation of many aromatic compounds (Harwood *et al*., 1999).

In the Netherlands, BTEX compounds are also often encountered in landfills (Röling *et al*., 2001). In the past, landfilling was performed without the presence of appropriate liners to prevent percolation of leachate into underlying aquifers, and illegal dumping of petroleum waste often occurred. Remediation strategies have to be developed for these landfills, because BTEX compounds can cause a severe

health risk to humans and animals. For the successful application of remediation strategies, knowledge about the BTEX-degrading capacity of the bacteria in the landfill aquifer is required.
In this study, we have investigated benzene and toluene degradation in microcosms inoculated with soil samples from an aquifer polluted by BTEX-containing landfill leachate (Banisveld landfill near Boxtel, The Netherlands). Previous studies at this site showed that the BTEX concentration in the groundwater decreased in downstream direction. Microbiological degradation could be responsible for the decreases in organic contaminant concentrations. Initially this could not be ascertained because spatial and temporal heterogeneity could produce the concentration profiles as well (Röling *et al*., 2001). Recently, however, studies demonstrated BTX degradation under iron-reducing conditions in microcosms and enrichments incubated with soil and groundwater from the landfill leachate polluted aquifer (Botton and Parsons, 2006, 2007). BTX degradation under iron-reducing conditions was studied, since inside the leachate plume iron reduction is the dominant redox condition. Upstream of the landfill and above the plume, denitrification was shown to be the dominant redox process (Röling *et al*., 2001). In this study, we selected two of the BTEX compounds (i.e. benzene and toluene) to investigate the degradation of these compounds by microorganisms in the aquifer under different redox conditions in batch experiments. The following electron acceptors were tested: chlorate (ClO_3^-), nitrate (NO_3^-), manganese(IV)oxide (MnO_2), iron(III)nitrilotriacetate (Fe(III)NTA), amorphous iron(III) oxide (FeOOH), sulfate (SO_4^{2-}) and carbon dioxide/bicarbonate (CO_2/HCO_3^-), i.e. methanogenic conditions. When benzene or toluene degradation and reduction of the corresponding electron acceptor occurred in batch experiments, the culture was transferred to fresh medium and subsequently further enriched. Furthermore, the bacterial composition of the enrichments was studied by molecular methods, consisting of denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, cloning and sequencing.

Materials and Methods

Site description (general, biogeochemistry, microbial community)

A detailed description of the Banisveld landfill site has been presented by others (Röling *et al*., 2001; Van Breukelen *et al*., 2003). Briefly, Banisveld landfill site (6 ha), located southwest of Boxtel, The Netherlands, contains about 400,000 m^3 of primarily household refuse. Chemical waste may have been dumped illegally. Landfilling occurred between 1965 and 1977, and liners to prevent migration of landfill leachate were not installed. From the landfill body a leachate plume has developed in the direction of the groundwater flow. Groundwater flow (approximately 10 m/year) is directed towards a nature reserve with a unique oligotrophic ecosystem (Röling *et al*., 2001).
The biogeochemistry of the Banisveld landfill site has been studied in detail (Van Breukelen *et al*., 2003). The leachate plume contained high concentrations of dissolved organic carbon (DOC), methane, ammonium, iron, and other major ions (except sulfate and nitrate). DOC decreased from 9.2 to 5.4 mmol C/l in downstream direction, while no dilution took place as indicated by constant chloride (conservative tracer) concentrations along the flow path in the plume. Because the organic content of the soil was low (<0.1%), sorption could not explain the decreases. Therefore, microbial degradation must have lowered the DOC concentration downstream. Concentrations of aromatic compounds, such as benzene (maximum concentration 28 µg/l), ethylbenzene, xylene, and naphthalene also decreased in downstream direction. Concentrations of other organic contaminants (toluene and chlorinated aliphatics) were below 1 µg/l. BTEX compounds (< 221 µg/l) represent only 0,1 % of DOC in leachate from the landfill. In landfill sites the occurrence of redox zones is common (Christensen *et al*., 2001). At this site this phenomenon has not been observed. Denitrification was the dominant redox process upstream of the landfill and above the plume, whereas inside the plume iron reduction prevailed. Methane production and sulfate reduction did not occur inside the plume. Methane was present in the

leachate plume, but this was produced inside the landfill body and transported by groundwater flow (Van Breukelen *et al*., 2003).
Studies with groundwater samples showed a clear difference between the microbial community structures inside and outside the contaminant plume based on 16S rRNA gene-targeted analysis. Upstream of the landfill, members of the *Betaproteobacteria* dominated. Downstream of the landfill, the contribution of *Deltaproteobacteria* increased and other members of the *Betaproteobacteria* were present compared to upstream of the landfill. Most of the deltaproteobacterial sequences found were most closely related to those of iron-reducing members within the family *Geobacteraceae.* The dominance of members of the *Geobacteraceae* is in agreement with iron reduction as dominant redox process inside the plume (Röling *et al*., 2001; Lin *et al*., 2005). Recently, it was demonstrated that *Geobacteraceae* dominated in BTX-degrading iron-reducing enrichments inoculated with polluted soil or groundwater from the landfill leachate polluted aquifer, suggesting that these microorganisms play a key role in the natural attenuation of BTX *in situ* (Botton *et al*., 2007).

Inoculum
Samples from the aquifer downstream of the Banisveld landfill were kindly provided by Sabrina Botton and John Parsons (University of Amsterdam, Amsterdam, The Netherlands), and were collected in the framework of the Dutch TRIAS project “Resilience of the groundwater ecosystem in reaction to anthropogenic disturbances” (http://www.nwo.nl/trias/index.html/). In November 2002, soil cores were taken anaerobically with a core pushing device (Delft Geotechnics) at approximately 20 m downstream of the landfill body in the plume of the leachate. Cores were taken at three different depths: at the top of the plume (2.72-3.60 m, Poll-01), in the middle of the plume (3.90-4.90 m (Poll-02), and at the bottom of the plume (5.50-6.30, Poll-03); a schematic overview of aquifer and the cores is presented in the article of Botton and Parsons (2006). Core Poll-02 was used as inoculum in this study, as it was expected from biogeochemical data that this core would contain the most active BTEX-degrading microorganisms in comparison with the other two cores. After retrieval, the stainless steel cores were immediately capped, and the cores were stored in an anaerobic container flushed with N_2 gas. Soil cores were transferred to the laboratory and opened inside an anaerobic glove box. Soil was taken out of the cores and transferred to gas-tight glass flasks and stored at 4°C until used.

Chemicals
Amorphous iron(III) oxide (FeOOH) was prepared by neutralizing a 0.4 M $FeCl_3$ solution with NaOH until pH 7 (Lovley and Phillips, 1986a). Amorphous manganese(IV) oxide (MnO_2) was prepared by mixing equal amounts of 0.4 M $KMnO_4$ and 0.4 M $MnCl_2$ and adjusting the pH to 10 by adding NaOH (Burdige and Nealson, 1985). Both metal oxide suspensions were washed three times with demineralised water and were finally suspended in demineralised water. All other chemicals used were of the highest available purity and purchased from Sigma (Zwijndrecht, The Netherlands) and Merck (Amsterdam, The Netherlands). Gas mixtures (N_2/CO_2, H_2/CO_2) were obtained at Hoekloos (Schiedam, The Netherlands). DNA oligonucleotide primers used for PCR amplification were synthesized commercially by MWG Biotech AG (Ebersberg, Germany).

Medium and experimental setup
Benzene and toluene degradation in Banisveld landfill aquifer soil samples were studied under different redox conditions in batch experiments. Batch experiments were performed in 120 ml serum bottles filled with 40 ml sterile, anaerobic and phosphate-bicarbonate-buffered medium (pH 7.3) as described previously with the modification that cystein (1 mM) was used as reducing agent instead of sulfide (Holliger *et al*., 1993). About 5 g soil of core Poll-02 was added in an anaerobic glove box to these serum bottles. The bottles were closed with viton stoppers (Maag Technik, Dübendorf,

Switzerland) and aluminium crimp caps, and the headspace was replaced with N_2/CO_2 (80:20 (v/v); 170 kPa). Benzene (20 mM stock solution) or toluene (5 mM stock solution) was added from anaerobic sterile stock solutions resulting in final concentrations of 200 µM. Amorphous iron(III) oxide, FeOOH, (40 mM) and manganese(IV) oxide, MnO_2, (20 mM) were added from suspended solutions inside the anaerobic glove box before the bottles were closed. In batches with FeOOH the toluene concentration was 75 µM instead of 200 µM. Other electron acceptors, nitrate, sulfate, chlorate and Fe(III)NTA, were added from anaerobic sterile stock solutions resulting in final concentrations of 10 mM. All electron acceptors were tested in triplicate. Sterile controls were prepared by autoclaving soil containing batches twice for 20 min at 120°C. Abiotic controls were prepared by omitting soil from these batches. All serum bottles were statically incubated at 30°C in the dark. Concentrations of benzene and toluene were monitored over time. Reduction of the electron acceptors was also measured over time. Benzene and toluene were re-added when depleted. Whenever benzene or toluene degradation and reduction of the corresponding electron acceptor occurred, the culture was transferred (10% inoculum) to fresh medium and further enriched. Further enrichment of the cultures was achieved by preparing dilution series in liquid medium.

Analytical methods

Benzene and toluene were measured by headspace analysis using a gaschromatograph (Chrompack 436, Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector (FID) connected to a Sil 5CB capillary column (25 m x 0.32 mm, 1.2 µm film) and a split injection (ratio 1:50). The column, detector and injector temperatures were 50, 300 and 250ºC, respectively.

Nitrate (NO_3^-), nitrite (NO_2^-), chlorate (ClO_3^-), chloride (Cl^-), sulfate (SO_4^{2-}) were determined by suppressor mediated ion chromatography (Dionex, Breda, The Netherlands) and a conductivity detector. Eluent consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ was pumped through the system at a flow rate of 1 ml min^{-1}. The chromatograph was equipped with an IonPac® AS9-SC column (Dionex, Breda, The Netherlands). Mannitol (10 mM final concentration) was added to the samples for stabilization, and sodium fluoride (1 mM final concentration) was added as internal standard. Fe(II) production was determined by extracting Fe(II) in 0.5 N HCl. The extracted Fe(II) was measured by using the ferrozine technique and quantified spectrophotometrically at 562 nm (Lovley and Phillips, 1986a). Mn(II) production was determined by extracting Mn(II) in 0.5 N HCl as previously described (Lovley and Phillips, 1988). The extracted Mn(II) was measured with the colorimetric formaldoxime method and quantified spectrophotometrically at 450 nm (Armstrong *et al*., 1979).

DNA isolation and amplification of 16S rRNA genes

A bead-beat and phenol-chloroform based DNA extraction method was used to extract DNA from the enrichment cultures (Van Doesburg *et al*., 2005). The amplification of the total 16S rRNA genes was performed with the primers 7f and 1492r (Lane, 1991) using a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus, Norwalk, USA) as previously described (Van Doesburg *et al*., 2005). Size and yield of PCR products were estimated by 1.2% agarose gel (w/v) electrophoresis and ethidium bromide staining.

Cloning and sequencing

PCR products were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The purified 16S rRNA genes were cloned into *E. coli* JM109 (Invitrogen, Breda, The Netherlands) by using the pGEM-T Easy vector system (Promega, Madison, USA) with ampicillin selection and blue/white screening. Positive clones (white colonies) were picked with a sterile toothpick and transferred into 0.2 ml PCR tube containing 50 µl of Tris-EDTA buffer, which was heated for 15 min at 95°C to release the DNA. The inserts from recombinant clones were reamplified by PCR with the

vector specific primers T7 and Sp6 (Promega, Madison, USA). PCR products were screened by Restriction Fragment Length Polymorphism (RFLP), using the restriction endonucleases AluI, CfoI and, MspI (Promega, Madison, USA). Aliquots (5 µl) of crude reamplified rRNA PCR products were digested with 2.5 U each of the enzymes in 1x Buffer B (Promega, Madison, USA) in a final volume of 10 µl, for 1.5 h at 37°C. Digested products were separated by 4% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products with different RFLP patterns were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and used as a template in sequencing reactions. For sequence analysis the Sequenase sequencing kit (Amersham, Slough, UK) was used with IRD800 (MWG-Biotech, Ebersberg, Germany)-labeled sequencing primers Sp6, T7 (Promega, Madison, USA), 533f and 1100r (Lane, 1991). The sequences were automatically analysed on a LI-COR (Lincoln, USA) DNA sequencer 4000L and corrected manually. Sequences of 16S rRNA genes were compared to sequences deposited in publicly accessible databases using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al*., 1990; McGinnis and Madden, 2004).

DGGE analysis

Purified DNA from the enrichment cultures was used as PCR template. DGGE-suitable 16S rRNA gene fragments were generated by PCR with primers F-968-GC and R-1401 (Nubel *et al*., 1996), with 35 cycles of 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s. The PCR reaction mixtures were the same as for the full-length 16S rRNA gene PCR, except for the two primers. The PCR products were separated by DGGE according to the specifications described previously by using the Dcode system (Biorad Laboratories, Hercules, USA) (Muyzer *et al*., 1993). Linear gradients of 30-60% of denaturant (100% denaturant consisted of 7 M urea and 40% formamide) were used in 8% (v/v) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels according to the modifications described previously (Heilig *et al*., 2002). Electrophoresis was performed at a constant voltage of 85V and a temperature of 60°C for 16 h. After electrophoresis, the gels were stained with $AgNO_3$ (Sanguinetti *et al*., 1994). Besides cloning and sequencing, excision of DNA bands from the DGGE gel was applied to identify bacterial populations. Excised bands were incubated in 20 µl TE buffer at 37°C overnight for elution of the DNA from the gel. Subsequently, these samples were used as PCR template for re-amplification and subsequently subjected to DGGE analysis to check for purity prior to sequence analysis.

GenBank Accession Numbers

The 16S rRNA gene nucleotide sequence determined in this study has been deposited in the GenBank database under accession number EF219370.

Results and discussion

Benzene degradation with different electron acceptors

No anaerobic benzene degradation was observed after one year of incubation with the different terminal electrons that were tested (Table 1). In the abiotic and sterile controls, benzene and electron acceptor concentrations did not decrease (data not shown). It has been reported that rapid oxidation of benzene could be achieved by chelating iron(III) in soil with humic acids (Lovley *et al*., 1996a). In this study, the addition of either humic acids (1 g/l) or the humic acid analogue, anthraquinone-2,6-disulfonate (AQDS, 2.5 mM), to batches with Fe(III)NTA or FeOOH did not result in benzene degradation (data not shown).

In batches with nitrate, little nitrate reduction (decrease of 2.5 ± 0.2 mM) and nitrite production (1.1 ± 0.1 mM) were observed. Fe(II) production occurred in batches with Fe(III)NTA (6.9 ± 0.6 mM) and FeOOH (0.5 ± 0.1 mM). However, nitrate reduction and Fe(II) production were not coupled to the oxidation of benzene. Probably organic (or inorganic) compounds already present in the soil were used by the microorganisms as the electron donor for the reduction of nitrate and Fe(III). As we were

interested in anaerobic benzene degradation, in this study, control batches containing electron acceptors, but without the addition of benzene, were not included. However, it seems plausible to assume that in the absence of benzene a similar nitrate and Fe(III) reduction would have been obtained.

Benzene degradation coupled to FeOOH reduction has been previously demonstrated in microcosms with the same soil core Poll-02 as inoculum and in enrichments originating from these microcosms (Botton and Parsons, 2006, 2007). In that study, 20 µM of benzene was degraded in microcosms inoculated with polluted soil within approximately 230 days of incubation. Similar results were obtained in microcosms inoculated with polluted groundwater. When benzene was re-added, benzene was degraded again, and an increase in the degradation rate was observed in batches inoculated with groundwater, but no increase in the benzene biodegradation rate was found in batches inoculated with soil. The concentration of Fe(II) increased in all the soil microcosms by 0.9 to 3.4 mM. These values were higher than expected based on benzene degradation only, according to reaction 1:

$$C_6H_6 + 30\ Fe^{3+} + 18\ H_2O \rightarrow 6\ HCO_3^- + 30\ Fe^{2+} + 36\ H^+ \quad (1)$$

This indicates that the iron-reducing bacteria used other, endogenous carbon sources, similar to the results found in this study (Botton and Parsons, 2006).

The lack of benzene degradation in our study might be explained by the relatively high benzene concentrations applied. We used in our batch experiments 200 µM benzene, which is ten times higher than in previous batch experiments with the same soil (Botton and Parsons, 2006). The aim in our study was to selectively enrich benzene-degrading microorganisms by applying high benzene concentrations. It might be that the benzene concentrations were too high and therefore benzene degradation was inhibited due to toxicity. However, the benzene IC50 value (toxicant concentration that inhibited the species by 50%) for methanogens, which are often considered to be the most sensitive anaerobic microorganisms, was reported to be 6.7 mM (Blum and Speece, 1991). This value (6.7 mM) is much higher than benzene concentrations applied in this study (0.2 mM). In addition, iron-reduction did occur in our batches. Therefore, we consider toxicity effects of benzene on microorganisms rather unlikely. It might be that the 3-fold larger amount of inoculum (15 g soil) that was used by Botton and Parsons (2006) may have caused the observed differences.

Several other studies demonstrated that *Geobacteraceae* play a key role in the natural attenuation of BTX at the Banisveld landfill (Röling *et al*., 2001; Lin *et al*., 2005; Botton *et al*., 2007). However, there was no direct evidence to show that members of this family of organisms are capable of anaerobic benzene degradation. Recently, it was suggested that BTX could be synthrophically degraded by a bacterial consortium, originating from Banisveld landfill soil, in which *Geobacteraceae* utilized intermediate metabolites. This suggestion was based on the observation that *bssA* gene sequences in BTX degrading enrichments differed from those of *Geobacter* isolates, suggesting that the first steps of toluene degradation, but also benzene and xylene oxidation, are carried out by other members of the enrichments (Botton *et al*., 2007). Indications for syntrophic anaerobic benzene degradation were also found in a study, in which function and phylogeny of a benzene-degrading iron-reducing enrichment was investigated by using stable isotope probing. The authors hypothesized that *Clostridia* primarily oxidize benzene and partially share electrons derived from benzene oxidation with members of the *Desulfobulbaceae* as syntrophic partners (Kunapuli *et al*., 2007). If anaerobic benzene degradation in the Banisveld landfill soil indeed takes place by a syntrophic community, it is likely that the syntrophic relationship in our batches failed to develop, and that therefore no benzene degradation was observed.

Table 1. Benzene and toluene degradation in microcosms, inoculated with BTEX-polluted aquifer samples, under different terminal electron accepting conditions during one year incubation. +, benzene or toluene was degraded; -, no benzene or toluene degradation.

Electron acceptor	Benzene (200 µM)	Toluene (200 µM)
ClO_3^- (10 mM)	-	-
NO_3^- (10 mM)	-	+ [b]
MnO_2 (20 mM)	-	+
Fe(III)NTA (10 mM)	-	+
FeOOH (40 mM)	-	+ [c]
SO_4^{2-} (10 mM)	-	-
CO_2/HCO_3^- (50 mM) [a]	-	-

[a] The medium contained 50 mM HCO_3^- (44.4 mM $NaHCO_3$ and 5.6 mM NH_4HCO_3) and the headspace consisted of N_2/CO_2 (80/20%).
[b] Toluene degradation was observed in two of the triplicates.
[c] Toluene degradation was observed in one of the triplicates.

Toluene biodegradation with different electron acceptors

Toluene degradation was observed with nitrate, MnO_2, Fe(III)NTA and FeOOH as electron acceptors (Table 1). No decrease in toluene and electron acceptor concentrations in the abiotic and sterile controls was observed (data not shown). With nitrate and MnO_2, the initial amount of toluene (200 µM) was degraded within approximately 5 days and with Fe(III)NTA within approximately 12 days (Fig. 1). With FeOOH, however, toluene degradation was much slower and the initial amount of toluene (75 µM) was degraded within approximately 100 days (results not shown). Re-addition of toluene to batches with nitrate, MnO_2 and Fe(III)NTA resulted in an increased toluene degradation rate, but after approximately 15 days toluene degradation in the different batches ceased, irrespective of the electron acceptor present (Fig. 1). At that time, NO_3 was still present, but probably all Fe(III)NTA and MnO_2 were reduced. Unfortunately, Fe(II) and Mn(II) concentrations were not measured at that time (after 15 days). We only measured Fe(II) concentrations in the subsequent transfers. Our Mn(II) quantification method did not lead to reliable results, likely due to the formation of whitish precipitates in our media.

Toluene degradation was coupled to nitrate reduction (4.0 ± 0.3 mM) and the production of nitrite (1.5 ± 0.7 mM). The ratio of the amount of nitrate reduced to the amount of toluene degraded, corrected for electron acceptor reduction from endogenous electron donors, was 2.0 ± 0.3 (Table 2). Toluene degradation under Fe(III)-reducing conditions occurred in all three replicates with Fe(III)NTA and in only one of the triplicates with FeOOH as the electron acceptor (Table 1). During the reduction of Fe(III)NTA, the medium changed color from orange to colorless and sometimes black precipitates were observed. The formation of some black precipitates was also observed in batches with FeOOH. These precipitates are most likely composed of iron sulfide complexes, or insoluble Fe(II)–Fe(III) complexes as described previously (Botton and Parsons, 2006). In batches with FeOOH, the production of Fe(II) was measured. It should be noted that the above-mentioned Fe(II)-Fe(III) complexes are not measurable with the extraction method employed in this study (Lovley and Phillips, 1987). In the presence of such complexes, the final concentration of either form of iron (Fe(II) or Fe(III) would be underestimated. During Fe(III)NTA reduction, the Fe(II) concentrations were not measured, but it was assumed that all the Fe(III) (10 mM) was reduced to Fe(II) after approximately 15 days based on the color change and the observed cease in toluene degradation. Taking this into account, the corrected ratio of the amount of Fe(II) produced with the amount of toluene degraded was 11 ± 2.6 and 29.2, for Fe(III)NTA and FeOOH, respectively (Table 2). In batches with MnO_2, toluene degradation was accompanied by a color change of the precipitates from brown to white (MnO_2 has a brown color). The white precipitates were assumed to be rhodochrosite ($MnCO_3$). Rhodochrosite has

been observed in MnO_2 reducing cultures before (Lovley and Phillips, 1988). During MnO_2 reduction, the Mn(II) concentrations could not be measured, but it was assumed that all the Mn(IV) (20 mM) was reduced to Mn(II) after approximately 15 days based on the color change and the observed cease in toluene degradation. This assumption resulted in a Mn(II) to toluene ratio of 17.3 ± 1.4 (Table 2).
With nitrate and Fe(III)NTA as the electron acceptor, the observed corrected ratio of electron acceptor to electron donor is lower than the theoretical ratio. This could be explained by the formation of biomass, which would result in a lower electron acceptor to electron donor ratio. However, with MnO_2 and FeOOH, the observed and theoretical ratios are very similar. In previous studies using the same soil samples and FeOOH and toluene, similar results were obtained (Botton and Parsons, 2006, 2007). Another possibility could be that toluene was not completely mineralized resulting in lower electron acceptor to toluene ratios.

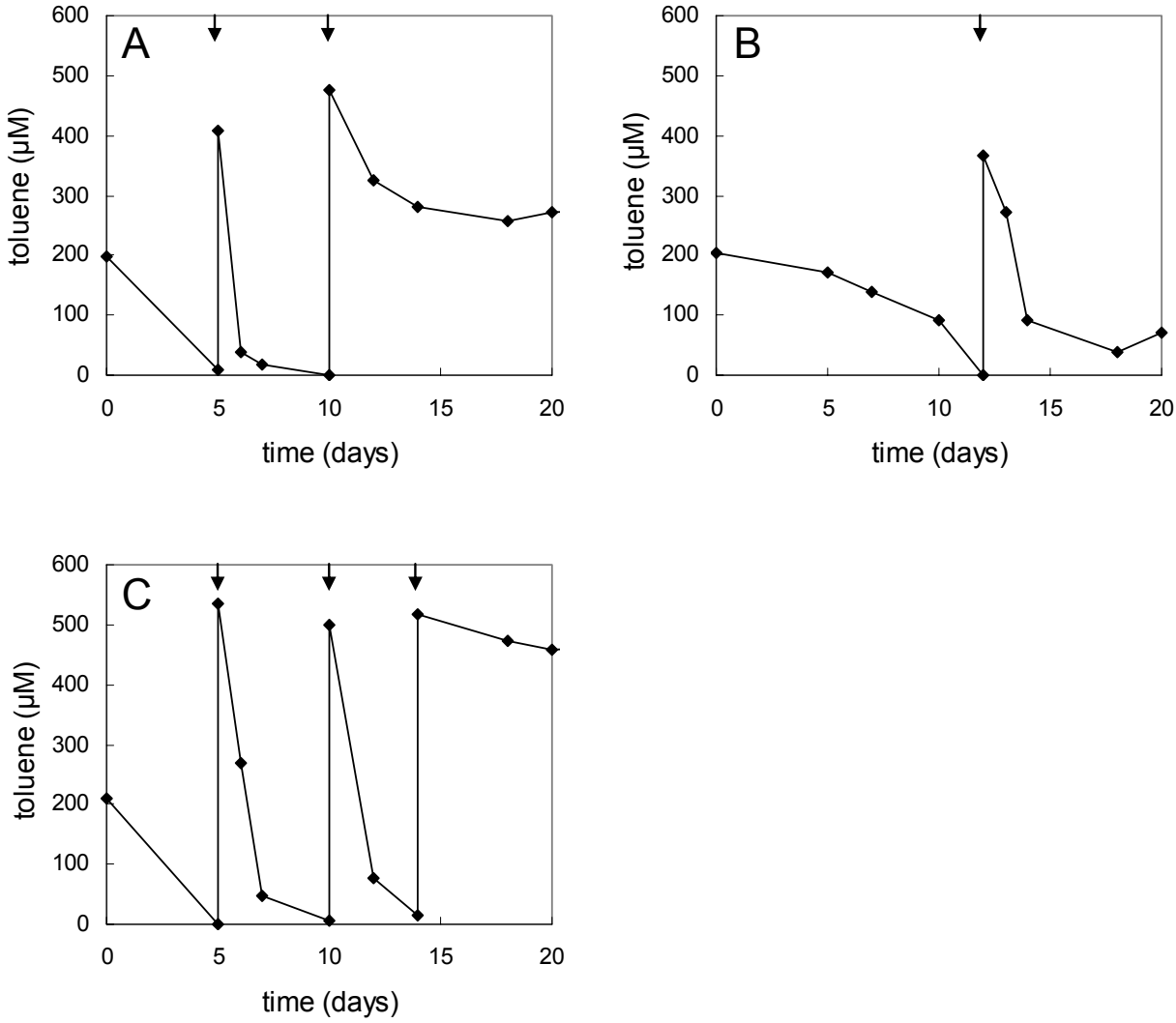

Figure 1. Toluene degradation in microcosms inoculated with Banisveld landfill aquifer soil samples with different electron acceptors: NO_3^- (A), Fe(III)NTA (B) and MnO_2 (C). Arrows indicate re-addition of toluene. Experiment was performed in triplicate batches and one of the (active) batches is shown; although in most cases similar results were obtained for the other (active) batches, averaging the data did not result in convenient figures due to small differences between the batches, e.g. the time for re-addition of toluene differed slightly.

Table 2. Ratio of the amount of electron acceptor reduced to the amount of toluene degraded in batch experiments with Banisveld landfill aquifer soil samples.

Electron acceptor	Toluene removed (mM)	Electron acceptor decrease (mM)	Nitrite (NO_2^-) produced (mM)	Ratio electron acceptor decrease/toluene removed		
				Uncorrected	Corrected [a]	Theoretical [b]
NO_3^-	0.7 ± 0.1	4.0 ± 0.3	1.5 ± 0.7	5.4 ± 0.3	2.0 ± 0.1	7.2
MnO_2	1.1 ± 0.1	20 [c]	-	17.3 ± 1.4	17.3 ± 1.4	18
Fe(III)NTA	0.5 ± 0.1	10 [d]	-	21.1 ± 5.0	11 ± 2.6	36
FeOOH	0.1	2.9	-	36.1	29.2	36

[a] The ratio was corrected for electron acceptor decrease not coupled to toluene degradation but coupled to other electron donors present in the soil. This was achieved by assuming the electron acceptor decrease observed in batches with benzene as the electron donor as endogenous electron acceptor decrease. Endogenous Mn(II) production could not be measured.

[b] Theoretical reactions (without taking into account the production of biomass from toluene):

$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$

$C_7H_8 + 18\ MnO_2 + 29\ H^+ \rightarrow 7\ HCO_3^- + 18\ Mn^{2+} + 15\ H_2O$

$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O \rightarrow 7\ HCO_3^- + 36\ Fe^{2+} + 43\ H^+$

[c] Assuming that all added MnO_2 was reduced to Mn(II), resulting in 20 mM) Mn(II).

[d] Assuming that all added Fe(III)NTA was reduced to Fe(II), resulting in 10 mM) Fe(II).

In similar batch experiments, toluene concentrations of 70-90 µM were degraded with FeOOH as the electron acceptor within 220-500 days with a lag-phase of 200-450 days (Botton and Parsons, 2006). In our study, toluene concentrations of 75 µM were degraded with FeOOH as the electron acceptor within 100 days with a lag-phase of 47-91 days. Toluene degradation with nitrate, MnO_2 and Fe(III)NTA occurred almost without a lag-phase and the initial concentration of toluene (200 µM) was completely degraded within 5-12 days. These results indicate that the adaptation time for the toluene-degrading bacteria is longer with FeOOH, compared to nitrate, MnO_2 and Fe(III)NTA. A possible reason for this could be that the bioavailability of nitrate, MnO_2 and Fe(III)NTA is higher than for FeOOH. Nitrate and Fe(III)NTA are soluble compounds and therefore highly bioavailable. Amorphous MnO_2 as prepared in this study, is bioavailable for microbial reduction with toluene as the electron donor (Langenhoff *et al*., 1997b). It seems that MnO_2 was more bioavailable than FeOOH in our batch experiments. On the other hand, amorphous FeOOH as prepared in this study, is considered to be a solid iron form with a rather high bioavailability for microbial reduction compared to other solid iron forms (Lovley and Phillips, 1986b).

Transfers of toluene degrading cultures

Toluene-degrading batches with nitrate, MnO_2 and Fe(III)NTA as the electron acceptor were transferred to fresh medium (10% inoculum). The toluene-degrading culture with FeOOH as the electron acceptor was not transferred to fresh medium, because toluene degradation was much slower than with the other electron acceptors. Transfers with nitrate as the electron acceptor did not degrade toluene within one month (data not shown). No further toluene concentration measurements were done after this period. Remarkably, toluene was degraded within 5 days in the initial nitrate batches with soil. An explanation for this difference is that formation of nitrite might have led to death of the toluene-degrading bacteria. Nitrite toxicity has often been reported for toluene-degrading denitrifying bacteria (Dolfing *et al*., 1990; Evans *et al*., 1991; Schocher *et al*., 1991).

Transfers with MnO_2 and Fe(III)NTA, on the other hand, degraded toluene within one week (Fig. 2). In transfers with Fe(III)NTA, toluene degradation was coupled to Fe(III) reduction as indicated by an increase in Fe(II) concentration. As observed before, during Fe(III)NTA reduction the medium changed color from orange to colorless and sometimes black precipitates were observed. In transfers with MnO_2, toluene degradation was accompanied by a color change of the precipitates from brown to white. An attempt was made to quantify Mn(IV) reduction by measuring the Mn(II) concentrations using the formaldoxime method as described previously (Armstrong *et al*., 1979). However, this method did not provide reliable results.

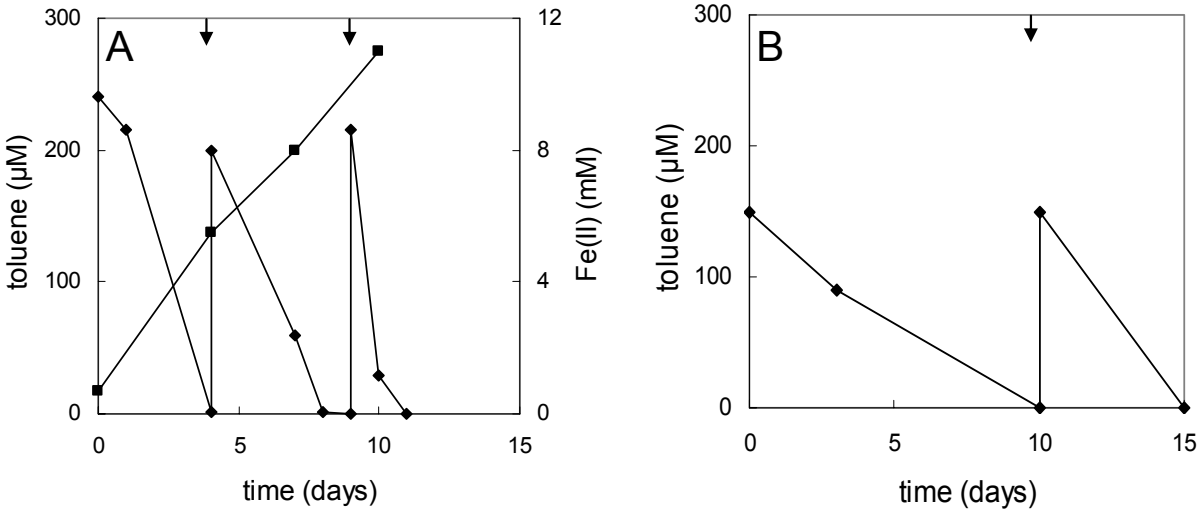

Figure 2. Toluene (♦) degradation coupled to the reduction of Fe(III)NTA (A; Fe(II) (■)) and $Mn(IV)O_2$ (B) by enrichments originated from Banisveld soil. Batch incubations performed in duplicate; both batches showed similar results, one batch is presented. Arrows indicate re-addition of toluene.

Biomolecular analyses of toluene-degrading enrichments

After four transfers, dilution series of both toluene-degrading cultures were prepared in liquid medium with their corresponding electron acceptor (i.e. Fe(III)NTA or MnO_2). Both cultures were diluted till 10^{-7}. Growth was observed in all dilutions. Denaturing gradient gel electrophoresis (DGGE) was used to determine the microbial diversity of the highest dilutions (Fig. 3). In the culture with Fe(III)NTA, three bands (A, B and D) were clearly present. Bands A and D were excised, used as PCR template for re-amplification and subsequently used in another DGGE. Re-amplification of band A failed, however, amplification of band D was successful. The excised band D showed an almost identical DGGE pattern (lane 3) as the toluene-Fe(III)NTA culture (lane 4). In the toluene-MnO_2 culture, three bands (B, C, E) were clearly present. Band C was successfully excised and re-amplified by PCR. Band C (lane 1) showed a DGGE pattern similar to that observed for the toluene-MnO_2 culture (lane 2). Amplification of band E failed; no PCR-product was observed after agarose gel electrophoresis. Probably, bands A, C, D and E are PCR-artifacts produced during the PCR amplification. Excision and re-amplification of bands C and D resulted in DGGE patterns similar to the multiple bands obtained with the original sample. This phenomenon has previously been observed by others (Speksnijder *et al*., 2001). These authors found that excision, re-amplification, and DGGE analysis of a single DGGE band from a multiple bands displaying sample, resulted in all the bands of the original sample. They demonstrated that this was caused by heteroduplex formation during PCR amplification of closely related DNA sequences.

Band B was present in both the toluene-Fe(III)NTA and the toluene-MnO_2 culture. Although DGGE analysis is not a quantitative method, the results suggested that both cultures are dominated by the

same species. To identify the dominant phylotype corresponding with band B, enrichment of the culture was continued by making liquid and agar dilution series. Since growth with Fe(III)NTA was faster than with MnO_2, dilution series were prepared with Fe(III)NTA only. After three consecutive dilution series, a highly enriched culture was obtained. Cloning and sequencing was applied to identify the bacteria present. After cloning and initial RFLP screening of 96 clones, yielding 2 discernible RFLP patterns, the DGGE pattern of 42 clones was checked. All clones showed one single band on DGGE, present at the same height as band B (lane 5), indicating that the difference in RFLP pattern was caused by opposite direction of the insert in the vector. Identical 16S rRNA gene sequences of 1496 bp were obtained from two randomly chosen clones. The 16S rRNA gene sequence was compared with sequences in the NCBI database. Closest relative was an uncultered clone belonging to the *Betaproteobacteria* (clone ctg_CGOF369 16S ribosomal RNA gene, GenBank accession nr. DQ395793), which showed 96% similarity. Closest cultured relative was *Sterolibacterium denitrificans* (AJ306683, 94.6 % similarity), which is a cholesterol-oxidizing, denitrifying bacterium (Tarlera and Denner, 2003). The species dominant in the two toluene-degrading cultures is probably responsible for toluene degradation coupled to the reduction of MnO_2 and Fe(III)NTA. Finally, this anaerobic toluene-degrading species was isolated in pure culture (Chapter 3, this thesis). The DGGE pattern of the pure culture indeed corresponded to band B (lane 6, Fig. 3). Interestingly, the microorganism represented by this 16S rRNA sequence was obviously not a member of the *Azoarcus* and *Thauera* genera within the *Betaproteobacteria*, which are known to contain toluene-oxidizing nitrate-reducing species. In addition to *Azoarcus* and *Thauera*, the third genus of denitrifying bacteria that can degrade toluene is *Magnetospirillum* in the *Alphaproteobacteria* (Shinoda *et al*., 2005). Under iron-reducing conditions, only members of the *Geobacter* genus have been reported to degrade toluene (Coates *et al*., 2001a).

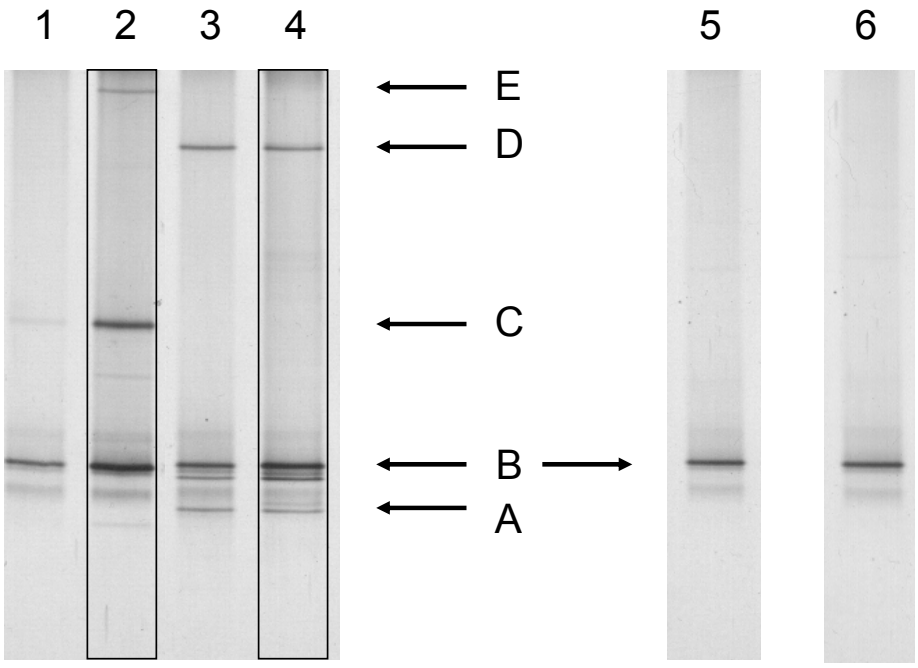

Figure 3. DGGE of PCR-amplified V6-V8 region of 16S rRNA gene fragments from the highest dilution (10^{-7}) of the toluene-degrading enrichment cultures. Lane 2: toluene-MnO_2 culture; lane 4: toluene-Fe(III)NTA culture; lane 1: DGGE of PCR-reamplified excised band C; lane 3: DGGE of PCR-reamplified excised band D; lane 5: DGGE of clone originated from the toluene-Fe(III)NTA culture (lane 4); lane 6: anaerobic toluene-degrading pure culture.

We have studied benzene and toluene degradation in Banisveld landfill aquifer under different electron accepting conditions in batch cultures. Benzene was not degraded during one year of incubation, while toluene was degraded with nitrate, MnO_2 and Fe(III) as the electron acceptor. In transfers and a dilution series, toluene degradation was sustained with MnO_2 and Fe(III)NTA. DGGE analysis showed that in the two toluene-degrading cultures the same phylotype is dominant. Cloning and sequencing of the 16S rRNA gene revealed that this dominant microorganism is closest related to *Sterolibacterium denitrificans*, a cholesterol-oxidizing, denitrifying bacterium of the *beta-Proteobacterium* (94.6% similarity). This anaerobic toluene-degrading species was isolated in pure culture and further characterized (Chapter 3, this thesis). This finding shows that besides *Geobacter*, *Thauera*, *Azoarcus* and *Magnetospirillum* other genera may be involved in anaerobic degradation of aromatic hydrocarbons under iron- and nitrate-reducing conditions.

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3

Anaerobic degradation of aromatic hydrocarbons with Fe(III), Mn(IV) or nitrate by the *Betaproteobacterium*, *Georgfuchsia toluolica*, gen. nov., sp. nov.

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Submitted

Abstract

A bacterium (strain G5G6) that grows anaerobically with toluene was isolated from an aquifer contaminated by BTEX-containing landfill leachate (Banisveld landfill, The Netherlands). The bacterium is able to use Fe(III), Mn(IV) and nitrate as terminal electron acceptor for growth on aromatic compounds. The bacterium is not able to grow on common substrates like sugars, lactate or acetate. Phylogenetic analysis of the 16S rRNA sequence indicated that strain G5G6 belonged to the *Betaproteobacteria*. Its closest cultured relative is *Sterolibacterium denitrificans* Chol-1S^T (94.6% similarity based on 16S rRNA genes), a cholesterol-oxidizing, denitrifying bacterium. Strain G5G6 possesses the *bssA* gene encoding the alpha subunit of benzylsuccinate synthase, which catalyses the first step in anaerobic toluene degradation. The *bssA* gene sequence is closest related to that of other *Betaproteobacteria* (72-81% similarity). Strain G5G6 is the first toluene-degrading, iron-reducing bacterium that does not belong to the *Geobacteraceae*, within the *Deltaproteobacteria*. Based on a thorough phylogenetic and physiological analysis, we consider the novel toluene-degrading bacterium strain G5G6 (DSMZ 19032^T = JCM 14632^T) as a hitherto unknown taxon of the *Betaproteobacteria*. We propose the name *Georgfuchsia toluolica* gen. nov. , spec. nov.

Introduction

Aromatic hydrocarbons are widespread in nature and often cause severe pollution of soils, sediments and groundwater. Particularly, monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, xylene (BTEX) are of environmental concern. They are relatively soluble, toxic, carcinogenic and very mobile in the environment (Coates *et al*., 2002; Badham and Winn, 2007). Contamination with BTEX is observed at sites with petrochemical activities like refinery and gasoline stations. BTEX contamination is often present in anaerobic settings (Lovley, 1997). Anaerobic bioremediation is an attractive remediation technique for such polluted environments. The occurrence of specific phylogenetic groups of bacteria and their specific catabolic genes are indicators for the biodegradation potential of a soil.

Anaerobic alkylbenzene degradation, in particular anaerobic toluene degradation, has been studied under different redox conditions. So far only members of the family *Geobacteraceae* have been reported to be capable of toluene degradation with Fe(III) as the the electron acceptor. *Geobacter metallireducens* GS-15 can completely oxidize toluene to CO_2 (Lovley and Lonergan, 1990; Lovley *et al*., 1993), while growth on toluene with Fe(III) was also demonstrated for *Geobacter grbiciae* and *Geobacter* sp. TMJ1 (Coates *et al*., 2001a; Winderl *et al*., 2007). *G. metallireducens* can also use Mn(IV), humic substances and nitrate as the electron acceptor (Coates *et al*., 2001a). However, most toluene-oxidizing, nitrate-reducing bacteria belong either to the *Azoarcus* or the *Thauera* genus, e.g. *Thauera aromatica* T1 (Evans *et al*., 1991), *T. aromatica* K172 (Anders *et al*., 1995), *Azoarcus tolulyticus* Tol4 (Fries *et al*., 1994) and *Azoarcus* sp. EbN1, which has recently been classified as *Aromatoleum aromaticum* (Rabus and Widdel, 1995; Wöhlbrand *et al*., 2007). Recently, four *Magnetospirillum* strains, which belong to the *Alphaproteobacteria*, were described that can degrade toluene with nitrate as the electron acceptor (Shinoda *et al*., 2005). Several bacteria have been reported to degrade toluene with sulfate, such as *Desulfobacula toluolica* and *Desulfotignum toluenicum* (Rabus *et al*., 1993; Beller *et al*., 1996; Harms *et al*., 1999b; Meckenstock, 1999; Morasch *et al*., 2004; Ommedal and Torsvik, 2007). These sulfate-reducing bacteria all belong to the *Deltaproteobacteria*. Toluene degradation with other less common electron acceptors also has been reported. Toluene degradation can be coupled to (per)chlorate respiration by *Dechloromonas aromatica* RCB (Coates *et al*., 2001b) and to arsenate reduction by strain Y5 (Liu *et al*., 2004). In addition, toluene can also be assimilated as a carbon source by the anoxygenic phototroph, *Blastochloris sulfoviridis* (Zengler *et al*., 1999).

In anaerobic toluene-degrading bacteria a key catabolic gene was identified, the *bssA* gene, which encodes for the alpha-subunit of benzylsuccinate synthase that catalyzes the first step of the anaerobic degradation of toluene and xylenes (Biegert *et al*., 1996; Beller and Spormann, 1999; Achong *et al*., 2001; Kane *et al*., 2002). Benzylsuccinate, formed by addition of fumarate to toluene, undergoes further transformation to benzoyl-CoA, which is then converted to CO_2 as described for many species (Biegert *et al*., 1996; Beller and Spormann, 1999; Beller and Edwards, 2000; Kane *et al*., 2002; Botton and Parsons, 2007).

Recent research on the detection and quantification of *bssA* genes has suggested the existence of a still unknown group of bacteria involved in the degradation of toluene in a BTEX-contaminated iron-reducing aquifer (Botton *et al*. 2007). Therefore, we started to enrich for toluene-degrading, iron-reducing bacteria using material from that aquifer. Here, we describe the isolation and characterization of strain G5G6 from this enrichment. This bacterium grows anaerobically on toluene with amorphous and soluble iron(III)-species, nitrate and MnO_2 as the electron acceptor. It can not degrade toluene or other organic compounds aerobically. We demonstrate that this bacterium cannot be assigned to any of the known genera with members that degrade aromatic hydrocarbons with iron or nitrate as the electron acceptor. We propose that strain G5G6 represents a member within a new genus within the *Betaproteobacteria*.

Materials and Methods

Inoculum

Strain G5G6 was isolated from a highly-enriched toluene-degrading iron-reducing enrichment culture. This enrichment culture was obtained from microcosms inoculated with soil samples from an iron-reducing aquifer polluted by BTEX-containing Banisveld landfill leachate (near Boxtel, The Netherlands). An extensive site description of the Banisveld landfill has been presented elsewhere (Van Breukelen *et al*., 2003). Soil cores were taken anaerobically with a core pushing device (Delft Geotechnics, Delft, The Netherlands) at approximately 20 m downstream of the landfill body in the plume of the leachate in November 2002 (Botton and Parsons, 2006). Microcosms were obtained by inoculating about 5 g soil of core Poll-02 in 120-ml serum bottles filled with 40 ml sterile, anaerobic and phosphate-bicarbonate-buffered medium (pH 7.3) as described below. Toluene (200 µM) and different electron acceptors were added to the microcosms. The following electron acceptors were tested: chlorate (ClO_3^-), nitrate (NO_3^-), manganese(IV)oxide (MnO_2), iron(III)nitrilotriacete (Fe(III)NTA), amorphous iron(III) oxide (FeOOH), sulfate (SO_4^{2-}) and carbon dioxide/bicarbonate (CO_2/HCO_3^-), i.e. methanogenic conditions. Concentrations of toluene were monitored over time. Reduction of the electron acceptors was also measured over time. Toluene was re-added when depleted. Whenever toluene degradation and reduction of the corresponding electron acceptor occurred, the culture was transferred (10% inoculum) to fresh medium and further enriched.

Enrichment and isolation procedures

Enrichment and isolation of strain G5G6 was performed in sterile, anaerobic phosphate-bicarbonate-buffered medium (pH 7.3) as described previously with the modification that cystein (1 mM) was used as reducing agent instead of 1 mM Na_2S (Holliger *et al*., 1993). Cultivation was done at 30°C in 120-ml serum bottles containing 40 ml medium. The bottles were closed with viton or butyl rubber stoppers, depending on the substrate used, and sealed with aluminum crimp caps. The gas phase of the bottles consisted of 20% CO_2 and 80% N_2 (170 kPa) to give a pH of 7.3. The medium was sterilized by autoclaving at 121°C for 30 min. The vitamin solution was filter-sterilized and added after heat sterilization. The cultures were incubated in the dark in an orbital shaker (50 rpm). Toluene (5 mM stock solution) was added from anaerobic sterile stock solution resulting in final concentrations of 200 µM. Fe(III)NTA solution was prepared by boiling and cooling distilled water under a stream of 100%

N_2, then adding sodium bicarbonate ($NaHCO_3$, 200 mM), nitrilotriacetic acid, trisodium salt (NTA, $C_6H_6NNa_3O_6 \cdot H_2O$, 100 mM) and ferric chloride ($FeCl_3 \cdot 6H_2O$, 100 mM) as described previously (Caldwell *et al*., 1999). The Fe(III)NTA solution was filter-sterilized and aliquots from this solution were added to batches resulting in final concentrations of 5 or 10 mM.
For strain isolation, dilution series of the highly-enriched culture were made with toluene (200µM) and either Fe(III)NTA (10 mM) or nitrate (10 mM) in 1.2% agar (Agar Noble, Difco, Becton Dickinson Microbiology Systems, Sparks, USA) to solidify the medium. Colonies of the highest dilutions were picked and transferred to new agar dilution series. This procedure was repeated several times. Purity of the culture was checked by growth on rich media (Wilkins-Chalgren anaerobe broth, Oxoid), microscopy and denaturing gradient gel electrophoresis (DGGE). Strain G5G6 was routinely grown with toluene (0.25 or 0.5 mM) and nitrate (10 mM).
The Gram type was determined using Gram-staining and electron microscopy as previously described (Plugge *et al*., 2000). Phase contrast micrographs were made with a Leica (Wetzlar, Germany) DMR HC microscope equipped with a Leica DC 250 digital camera. The Leica QWin computer programme was used to make digital micrographs.

Physiological studies
All growth parameters of strain G5G6 were determined in duplicate or triplicate incubations in phosphate-bicarbonate-buffered medium prepared as described above. The properties of strain G5G6 were compared to those of *Sterolibacterium denitrificans* strain Chol-1S^T (DSM 13999^T), which was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Sterolibacterium denitrificans* was cultivated in the phosphate-bicarbonate-buffered medium described above with the modification that sulfide (0.5 mM) was used instead of cysteine. This organism was routinely cultivated on cholesterol (2 mM), which was added to the medium prior to autoclaving, and nitrate (10 mM).
The growth rate of strain G5G6 was determined by measuring the increase of OD_{600} and cell numbers in time of triplicate batch cultures. Cell numbers were enumerated by phase-contrast microscopy using a Bürker-Türk counting chamber at 1000× magnification. Cell yields were determined by measuring the dry-weight of the biomass obtained from 400 mL cultures. This was done gravimetrically after the cell pellet was dried at 105°C over-night.
Late log-phase cells of strain G5G6 grown on toluene (250 or 500 µM) and nitrate (10 mM) were used as inoculum (5%) in experiments to determine the use of electron donors and acceptors. These experiments were performed in duplicate. To determine the substrate spectrum of strain G5G6 the following electron donors were tested with nitrate (10 mM) as the electron acceptor: benzene (25 and 50 µM), *o*-xylene, *m*-xylene, *p*-xylene (all 50 µM), ethylbenzene (50 µM), monochlorobenzene (50 µM), benzoate (500 µM), phenol (500 µM), catechol (500 µM), naphthalene (25 µM), *o*-cresol (500 and 1000 µM), *m*-cresol (500 and 1000 µM), *p*-cresol (500 and 1000 µM), benzaldehyde (500 and 1000 µM), *p*-hydroxybenzoate (500 and 1000 µM), glucose, fructose, xylose, acetate, pyruvate, propionate, butyrate, lactate, fumarate, succinate, malate, crotonate, citrate, pivilate, cysteine, glutamate, alanine, aspartate, ethanol, methanol, glycerol, mannitol (all 10 mM), H_2 (170 kPa, with the addition of 1 mM acetate) and yeast extract (1 g/l). Fermentative growth was tested with pyruvate, fumarate, lactate (all 10 mM) and yeast extract (1 g/l). Growth was monitored by visual observation of the turbidity of the culture and the decrease in nitrate concentration (initially 10 mM). All electron donors were tested in duplicate.The following electron acceptors were tested with toluene (250 µM) as the electron donor: oxygen (5 or 10% in headspace), perchlorate (5 and 10 mM), chlorate (5 and 10 mM), nitrate (10 mM), nitrite (2.5 and 5 mM), sulfate (5 and 10 mM), sulfite (5 and 10 mM), thiosulfate (5 and 10 mM), fumarate (5 and 10 mM), manganese(IV)oxide (MnO_2, 25 mMl), iron(III)pyrophosphate (10 and 20 mM), iron(III)NTA (5 and 10 mM), amorphous iron(III) oxide (FeOOH, 40 mM), iron(III)citrate (30 mM),

AQDS (anthraquinone-2,6-disulfonate, 4 mM), selenate (5 and 10 mM), arsenate (5 and 10 mM) and CO_2/HCO_3^- (methanogenic conditions, 50 mM). Growth was determined by visual observation and by measuring toluene concentration. When applicable, all electron donors and electron acceptors were added as sodium salts.
The pH optimum was determined with toluene (200 µM) and nitrate (10 mM) at 30°C using a pH range of 6.6-9.0. Different pH values of the medium were obtained by changing the percentage of CO_2 in the headspace, while the bicarbonate concentration of the medium was kept constant. The pH values were calculated using the Henderson-Hasselbach equation (Breznak and Costilow, 1994). In this way pH values of 9 (0% CO_2 v/v), 8.5 (1.2% CO_2 v/v), 8 (3.9% CO_2 v/v), 7.3 (20% CO_2 v/v), and 6.6 (100% CO_2 v/v) were obtained. The temperature optimum was determined with toluene (200 µM) and nitrate (10 mM) in the range of 21-45°C. Growth of the strain was followed by measuring the toluene concentration.

Analytical methods
Aromatic hydrocarbons were measured by headspace analysis using a gas chromatograph as described previously (Weelink *et al*., 2007). Nitrate (NO_3^-), nitrite (NO_2^-), perchlorate (ClO_4^-), chlorate (ClO_3^-) and chloride (Cl^-) were determined by high-pressure liquid chromatography (HPLC) as described before (Scholten and Stams, 1995). Fe(II) production was determined by extracting Fe(II) in 0.5 N HCl. The extracted Fe(II) was measured by using the ferrozine technique and quantified spectrophotometrically at 562 nm (Lovley and Phillips, 1986a). Ammonium was analyzed by the indophenol blue method as described previously (Hanson and Philips, 1981).
For lipid analyses, the freeze-dried cell material was saponified with 1 M KOH in 96% methanol by refluxing for 1 hr and subsequently neutralized and extracted with dichloromethane (DCM). After methylation and silylation, the fatty acids and hydroxy fatty acids were analyzed by GC and by gas chromatography-mass spectrometry (GC-MS). The double bond position of the monounsaturated fatty acids was determined on basis of the mass spectra of their dimethyldisulfide (DMDS) derivatives as described by (Nichols *et al*., 1986). To demonstrate that the C_{17} fatty acid methylester with molecular weight 282 is not monounsaturated, but contains a cyclopropyl ring, the fatty acids were hydrogenated with hydrogen using PtO_2 as a catalyst.

Molecular Biology techniques
DNA isolation and amplification of 16S rRNA genes
A bead-beat and phenol-chloroform based DNA extraction method was used to extract DNA from the enrichment cultures (Van Doesburg *et al*., 2005). The amplification of the total 16S rRNA genes was performed with the primers 7f and 1492r (Lane, 1991) using a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus, Norwalk, USA) as previously described (Van Doesburg *et al*., 2005). Size and yield of PCR products were estimated by 1.2% agarose gel (w/v) electrophoresis and ethidium bromide staining.

Cloning and sequencing
PCR products were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The purified amplicons were cloned into *E. coli* JM109 (Invitrogen, Breda, The Netherlands) by using the pGEM-T Easy vector system (Promega, Madison, USA) with ampicillin selection and blue/white screening. Positive clones (white colonies) were picked with a sterile toothpick and transferred into 0.2 ml PCR tubes containing 50 µl of Tris-EDTA buffer, which was heated for 15 min at 95°C to release the DNA. The rRNA gene inserts from recombinant clones were re-amplified by PCR with the vector specific primers T7 and Sp6 (Promega, Madison, USA). PCR products were screened by Restriction Fragment Length Polymorphism (RFLP), using the restriction endonucleases AluI, CfoI and, MspI

(Promega, Madison, USA). Aliquots (5 µl) of crude re-amplified rRNA genes were digested with 2.5 U of each of the enzymes in 1x Buffer B (Promega, Madison, USA) in a final volume of 10 µl, for 1.5 h at 37°C. Digested products were separated by 4% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products with different RFLP patterns were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and used as a template in sequencing reactions. For sequence analysis the Sequenase sequencing kit (Amersham, Slough, UK) was used with IRD800 (MWG-Biotech, Ebersberg, Germany)-labelled sequencing primers Sp6, T7 (Promega, Madison, USA), 533f and 1100r (Lane, 1991). The sequences were automatically analysed on a LI-COR (Lincoln, USA) DNA sequencer 4000L and corrected manually. Sequences of 16S rRNA genes were initially compared to sequences deposited in publicly accessible databases using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al*., 1990; McGinnis and Madden, 2004). Phylogenetic analysis was performed using different methods as implemented in the ARB software package, using the most recent version of the ARB-SILVA database (release 94, March 2008) (Ludwig *et al*., 2004; Pruesse *et al*., 2007).

Denaturing gradient gel electrophoresis (DGGE) analysis

Purified DNA from the enrichment cultures and the pure culture were used as template. A DGGE-suitable 16S rRNA amplicon was generated with 35 cycles of 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s. The PCR reaction mixtures were the same as for the total 16S rRNA PCR, except for the two primers; F-968-GC and R-1401 (Nubel *et al*., 1996). The PCR products were separated by DGGE according to the specifications described previously by using the Dcode system (Biorad Laboratories, Hercules, USA) (Muyzer *et al*., 1993). Linear gradients of 30-60% of denaturant (100% denaturant consisted of 7 M urea and 40% formamide) were used in 8% (v/v) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels according to the modifications described previously (Heilig *et al*., 2002). Electrophoresis was performed at a constant voltage of 85 V and a temperature of 60°C for 16 h. After electrophoresis, the gels were stained with $AgNO_3$ according to the method described previously (Sanguinetti *et al*., 1994).

Sequence analysis of the gene encoding the alpha subunit of benzylsuccinate synthase

Amplification of a 0.57 kb fragment of the *bssA* gene was performed with primers bssA_f (TCGA(C/T)GA(C/T)GGCTGCATGGA) and bssA_r (TTCTGGTT(T/C)TTCTGCAC), (Botton *et al*., 2007), which were added (0.4 µM each) to a 25 µl reaction vial along with 0.4 mM dNTPs, 10 µg BSA (Biolabs, UK) and 2.5U Taq polymerase (MRC Holland, Netherlands). Amplification was performed according to the following programme: 94°C for 4 min, followed by 35 cycles of 94°C for 0.5 min, 44°C for 1 min and 72°C for 1 min, with a final elongation phase at 72 °C for 5 min. Phylogenetic analysis of the obtained sequences was carried out by comparison with sequences deposited in the GenBank database and using the BLAST algorithm to attain the most closely related sequences (Altschul *et al*., 1990). The *bssA* gene sequences were aligned using CLUSTALW, distance analysis with the Jukes-Cantor correction, and bootstrap re-samplings (100 times) were carried out with the TREECON package (Van de Peer and De Wachter, 1994). The distance matrix was used to construct a tree by neighbour joining (Saitou and Nei, 1987).

GenBank accession numbers and culture collections

The 16S rRNA gene nucleotide sequence determined in this study has been deposited under GenBank accession number EF219370. Strain G5G6 has been deposited in two different collections of microorganisms, the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany), strain number DSM 19032 and Japanese Collection of Microorganisms (JCM, Riken BioResource Center, Japan), strain number JCM 14632.

Results

Isolation of the strain

Microcosms were inoculated with soil samples from the iron-reducing aquifer contaminated by BTEX-containing leachate of the Banisveld landfill (near Boxtel, The Netherlands). Toluene degradation was observed with nitrate, MnO_2 and Fe(III)NTA as electron acceptors (results not shown). In subsequent transfers and dilution series, toluene degradation could be sustained. After several serial dilution series, a highly enriched toluene-degrading culture Fe(III)-reducing culture was obtained. Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments showed that the same bacteria were enriched when Fe(III) was replaced by nitrate. From this enrichment, a pure culture was obtained by serial dilution series in anaerobic media with toluene and nitrate and supplemented with agar (1.2 %). Initially, dilution series were prepared with Fe(III)NTA as the electron acceptor, but colonies in these batches were smaller than in batches with nitrate and therefore more difficult to pick up.

Colonies in agar batches incubated with toluene and nitrate were round, lens-shaped, 0.5-1.0 mm in diameter and brown-reddish. Colonies were picked from the highest dilution with a sterile needle and directly transferred to new agar (and liquid) dilution series. This procedure was repeated until a pure culture was obtained, which was designated strain G5G6. Purity of the culture was shown in growth tests in rich medium (with Wilkins-Chalgren anaerobe broth no growth was observed) and microscopic observations of the toluene-grown culture. In addition, DGGE analysis of 16S rRNA gene fragments amplified with general bacterial primers (V6-V8 region) from DNA isolated of cultures of strain G5G6 showed one single band. The position of this band corresponded with the dominant band in the enrichment cultures (results not shown). Cells of strain G5G6 were straight to slightly curved small rods, 0.5-0.6 µm wide and 0.8-1.2 µm long, motile and stained Gram-negative. Cells of the isolate also had the tendency to form chains (Fig. 1).

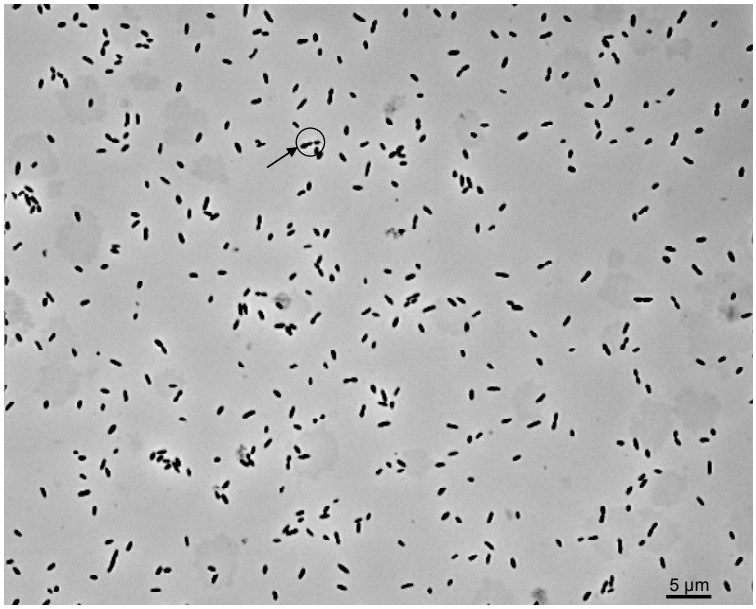

Figure 1. Phase-contrast micrograph of cells of strain G5G6 grown on toluene (500 µM) and nitrate (10 mM). 1,000× magnification. Arrow with circle indicates a chain of three cells of strain G5G6.

Toluene degradation with nitrate and Fe(III)NTA

Strain G5G6 degraded toluene with Fe(III)NTA and nitrate as electron acceptors and coupled this to growth as indicated by an increase in cell numbers and OD_{600} (Fig. 2A and 2B). With nitrate, toluene

(330 µM) was degraded in 6 days with a (maximum) toluene degradation rate of about 70 µM/day (2.9 µM/h). With Fe(III)NTA, toluene (215 µM) was degraded in 7 days with a (maximum) toluene degradation rate of about 50 µM/day (2.1 µM/h). In batches with Fe(III)NTA, growth was monitored by enumerating cell numbers, because OD_{600} measurements did not provide reliable results due to the presence of Fe(III)NTA. Cell number and OD_{600} increase coincided with toluene degradation. In batches with Fe(III)NTA, toluene degradation was coupled to Fe(II) production (Fig. 2A). During the reduction of Fe(III)NTA, the medium changed color from orange to colorless and sometimes grey-black precipitates were observed. During nitrate reduction, about 10 % of the nitrate reduced was converted to nitrite (Fig. 2B). No substantial toluene degradation was observed in controls without inoculum or without electron acceptor. No detectable nitrate or Fe(III)NTA reduction was observed in controls without inoculum or without toluene. No growth was observed in controls without toluene or electron acceptor. During toluene degradation culture samples were analyzed by HPLC for the presence of extracellular intermediates, but neither aromatic compounds nor organic acids were detected, suggesting that toluene is completely degraded to CO_2. The specific growth rate (µ) of strain G5G6 on toluene with Fe(III)NTA and nitrate was about 0.5 day^{-1} (doubling time 1.4 days) and about 0.2 day^{-1} (doubling time 3.5 days), respectively. The biomass yield of strain G5G6 determined in a 400 ml culture grown on toluene and nitrate was 30 g biomass per mol of toluene degraded. Growth of strain G5G6 occurred in a pH range of 6.6 till 9.0, with an optimum at pH 7.3. The optimal temperature for growth was 25-30°C with lower and upper limits of about 20 and 37°C.

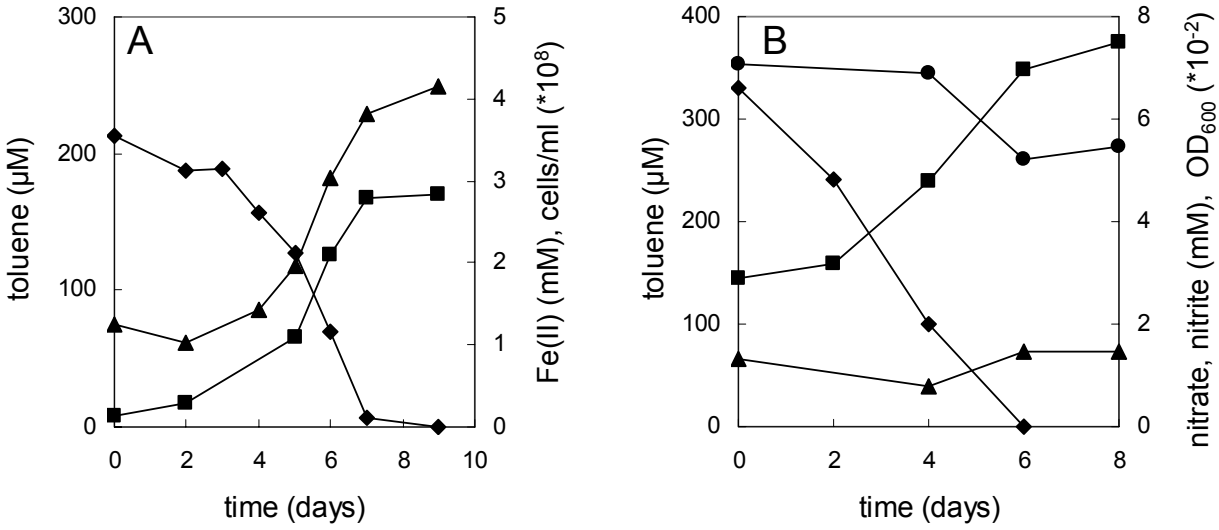

Figure 2. Toluene degradation by strain G5G6 coupled to Fe(III)NTA reduction (A) and to nitrate reduction (B). A. Toluene (♦), Fe(II) (▲) and cells/ml (■); B. Toluene (♦), nitrate (●), nitrite (▲) and cells/ml (■)

The observed ratio of electron acceptor reduced and toluene degraded was compared with the expected stoichiometry of complete oxidation of toluene coupled to nitrate and Fe(III)NTA reduction (Table 1 and 2). The observed ratio of nitrate reduced to toluene degraded (5.5) is somewhat different from the predicted ratio for toluene oxidation coupled to nitrate to dinitrogen gas (7.2) or ammonium (4.5). In separate experiments in which a total amount of about 0.9 mM toluene was degraded no measurable increase in the ammonium concentration was observed. This suggests that strain G5G6 is a denitrifying bacterium. The ratio of Fe(II) produced to toluene degraded in the enrichment culture and strain G5G6 isolated from it were almost equal, 15.7 and 13.6, respectively.

Table 1. Overview of toluene, electron acceptor and products produced during growth of strain G5G6 on toluene.

Electron acceptor	Toluene degraded (mM)	Electron acceptor decrease (mM)	Fe(II) produced (mM)	Nitrite produced (mM)	Ratio electron acceptor decrease/toluene removed
NO_3^-	0.33	1.87	-	0.14	5.5
Fe(III)NTA	0.21	N.A. [(a)]	2.89	-	13.6 [(a)]

[a] With Fe(III)NTA the amount of electron acceptor decrease is assumed to be equal to the Fe(II) produced.

Table 2. Stoichiometric equations and standard free energy changes ($\Delta G^{0'}$) for benzene (C_6H_6) and toluene (C_7H_8) oxidations with various electron acceptors.

Electron acceptor (ox/red)	E_0' (mV)	Stoichiometric equation			$\Delta G^{0'}$ (kJ/mol) [(a)]
NO_3^-/NH_4^+	-	$C_7H_8 + 4.5\ NO_3^- + 2\ H^+ + 7.5\ H_2O$	→	$7\ HCO_3^- + 4.5\ NH_4^+$	-2,220
NO_3^-/N_2	-	$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+$	→	$7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$	-3,555
NO_3^-/NO_2^-	+433	$C_7H_8 + 18\ NO_3^- + 3\ H_2O$	→	$7\ HCO_3^- + 18\ NO_2^- + 7\ H^+$	-2,455
Fe^{3+}/Fe^{2+}	+772 [(b)]	$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O$	→	$7\ HCO_3^- + 36\ Fe^{2+} + 43\ H^+$	-3,630

[a] The data for calculating standard free energy changes ($\Delta G^{0'}$) are from McCarty (1971), Thauer *et al*. (1977) and Stumm and Morgan (1981).

[b] In fact, the $\Delta G^{0'}$ for toluene oxidation coupled to Fe(III)NTA reduction is lower than reported in Table 2, because the redox potential of the redox pair Fe(III)/Fe(II) is lower when the iron is chelated or complexed. The redox potential (at pH 7.0 and 25°C) of redox couple Fe(III)NTA/Fe(II)NTA is +385 mV (Straub *et al*., 2001).

Benzylsuccinate synthase gene analysis

Benzylsuccinate synthase (Bss) is the key enzyme of anaerobic toluene degradation and has so far been found in all anaerobic toluene-degrading bacteria tested (Biegert *et al*., 1996; Winderl *et al*., 2007). Bss catalyzes the addition of fumarate to toluene. The gene encoding for the alpha-subunit of Bss is well-conserved and has been successfully demonstrated in mixed communities (Botton *et al*., 2007; Winderl *et al*., 2007). Amplification with previously designed *bssA*-specific primers revealed a 0.57 kb long fragment for strain G5G6. The sequence of this fragment was closest related to *bssA* genes from denitrifying *Betaproteobacteria* (*Thauera* and *Azoarcus* species; Fig. 3). However, the sequence clustered distinctly from the *bssA* genes of these *Betaproteobacteria* (72-81% similarity). The *bssA* sequence of strain G5G6 was closest associated (91-93% similarity) with sequences observed in toluene-oxidizing, iron-reducing enrichments obtained from the same aquifer from which isolate G5G6 was obtained (Botton *et al*., 2007). In addition, amplification with primers specific for the genes encoding benzoyl reductase (*bcrA*) and 6-ketocyclohex-1-ene 1-carbonyl-CoA hydrolase (*oah*), enzymes involved in anaerobic benzoyl-CoA degradation, revealed the presence of fragments of the expected length (data not shown).

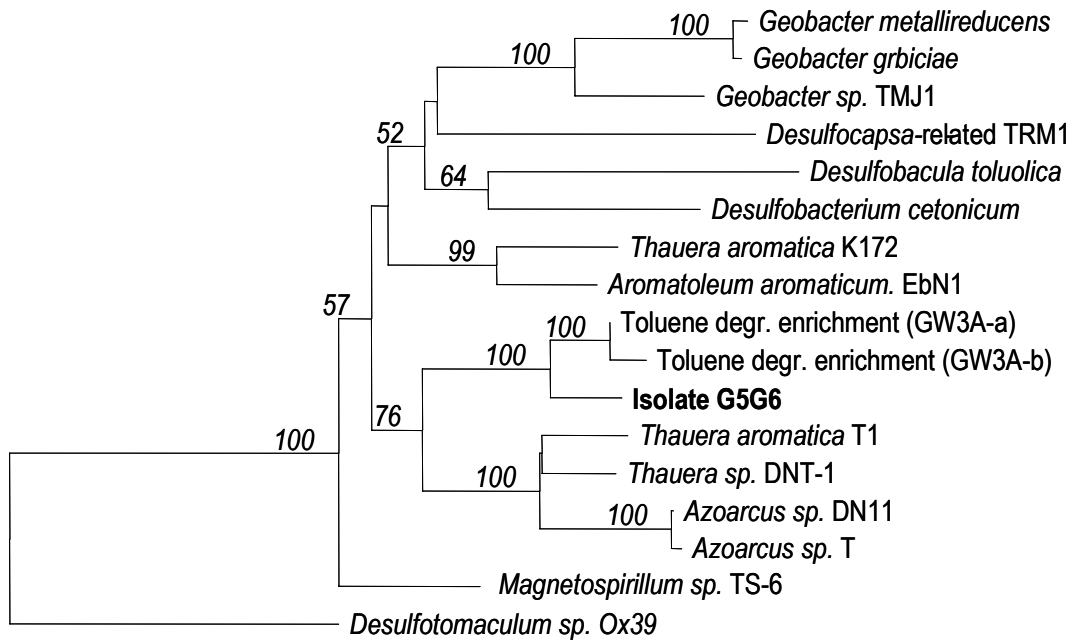

Figure 3. Neighbor joining tree of partial *bssA* gene sequences retrieved from strain G5G6, from anaerobic toluene degrading enrichments derived from the aquifer polluted by the Banisveld landfill, and from pure cultures of anaerobic, toluene-degrading bacteria. Only bootstrap values above 50% are shown.

Growth on other electron donors and electron acceptors

The ability of strain G5G6 to grow on other substrates was tested (Table 3). Strain G5G6 was not able to grow on carboxylic acids (e.g. pyruvate, acetate), amino acids (cysteine, glutamate, aspartate and alanine), sugar compounds (glucose, fructose, xylose and mannitol), alcohols (ethanol and methanol), cholesterol, hydrogen or yeast extract. Fermentative growth by strain G5G6 was not observed. Besides on toluene, strain G5G6 grew with ethylbenzene (Fig. 4), phenol, *p*-cresol, *m*-cresol, benzaldehyde and *p*-hydroxybenzoate.

Table 3. Overview of the electron donor use by strain G5G6 and *Sterolibacterium denitrificans* Chol-1S^T (DSM 13999^T). Data from strain G5G6 are from this study, whereas data for *Sterolibacterium denitrificans* are from Tarlera and Denner (2003) or this study. + = growth; - = no growth; n.d. = not determined.

	Strain G5G6	*Sterolibacterium denitrificans*
Carboxylic acids [(a)]	-	-
Glucose, fructose, xylose	-	-
Pivilate	-	n.d.
Cysteine	-	-
Glutamate	-	-
Alanine	-	n.d.
Aspartate	-	n.d.
Ethanol	-	-
Methanol	-	n.d.
Glycerol	-	n.d.
Mannitol	-	- [(b)]
H_2 (+acetate, 1 mM)	-	n.d.
Yeast extract	-	n.d.
Cholesterol	-	+
Pyruvate (fermentative)	-	- [(c)]
Fumarate (fermentative)	-	- [(c)]
Lactate (fermentative)	-	- [(c)]
Yeast extract (fermentative)	-	- [(c)]
Benzene	-	- [(b)]
Toluene	+	- [(b)]
o-Xylene, *m*-xylene, *p*-xylene	-	n.d.
Ethylbenzene	+	- [(b)]
Monochlorobenzene	-	n.d.
Benzoate	-	-
Phenol	+	-
Catechol	-	n.d.
Naphthalene	-	n.d.
o-Cresol	-	n.d.
p-Cresol, *m*-cresol	+	n.d.
Benzaldehyde	+	n.d.
p-Hydroxybenzoate	+	n.d.

[a] Carboxylic acids were: acetate, pyruvate, lactate, succinate, propionate, butyrate, malate, citrate, crotonate and fumarate.

[b] Data from this study, all other data with respect to *Sterolibacterium denitrificans* are from Tarlera and Denner (2003).

[c] Tarlera and Denner (2003) did not explicitly mention that these fermentative substrates were tested and not used, but they mentioned that the strain has a strictly respiratory metabolism.

Ethylbenzene (0.52 mM) degradation by strain G5G6 led to the reduction of nitrate (3.9 mM) and the production of nitrite (2.2 mM) (Fig. 4). The ratio of the amount of nitrate reduced to ethylbenzene degraded was 7.4. Part of the nitrate was recovered as nitrite (58%). The ratio of 7.4 is close to 8.4, which is based on the stoichiometric reaction of the degradation of ethylbenzene coupled to nitrate reduction to dinitrogen.

Strain G5G6 was tested for growth with several electron acceptors with toluene (250 µM) as the electron donor. In addition to nitrate and Fe(III)NTA, strain G5G6 used manganese(IV)oxide (MnO_2) and other iron(III) species, such as amorphous iron(III) oxide (FeOOH), Fe(III)citrate and

Fe(III)pyrophosphate. Oxygen (5, 10 and 20% in the headspace tested) and nitrite (2.5 and 5 mM tested) were not used as the electron acceptor for growth. Furthermore, perchlorate, chlorate, sulfate, sulfite, thiosulfate, fumarate, AQDS (anthraquinone-2,6-disulfonate), carbondioxide, selenate and arsenate were not used as the electron acceptor.

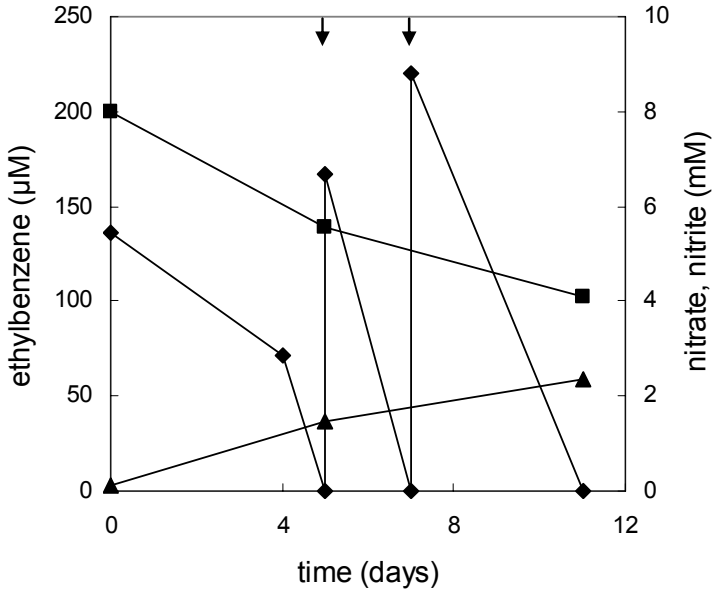

Figure 4. Ethylbenzene degradation by strain G5G6 coupled to nitrate reduction.
Ethylbenzene (♦), nitrate (■) and nitrite (▲). Arrows indicates re-addition of ethylbenzene.

Phylogenetic characterization

Cloning and sequencing of 16S rRNA gene fragments (1496 bp) of strain G5G6 showed that the sequence is most similar to those of species of the *Betaproteobacteria* (Fig. 5). Strain G5G6 is most closely related to an uncultured organism clone ctg_CGOF369 from an environmental sample (1534 bp, 96% similarity). The most closely related validly described cultured bacterium in the NCBI GenBank DNA database is *Sterolibacterium denitrificans* strain Chol-1S^T with a similarity of only 94.6%. This bacterium is a cholesterol-oxidizing, denitrifying member of the *Betaproteobacteria*. Strain G5G6 was also closely related (94.2%) to *Denitratisoma oestradiolicum* strain AcBE2-1^T, isolated on the natural steroid hormone 17 beta-oestradiol (E2) (Fahrbach *et al*., 2006). Interestingly, clone Cart-N1, which was obtained from a highly enriched benzene-degrading nitrate-reducing mixed culture, was also related to strain G5G6 with a similarity of 93.5% (Ulrich and Edwards, 2003). 16S rRNA sequence similarities of strain G5G6 with the nearest, but relatively distant, betaproteobacterial genera ranged from 90.1% (*Thauera terpenica,* DSM 12139^T) to 92.5% (*Zoogloea resiniphila*, ATCC 700687^T). Furthermore, 16S rRNA sequence similarities with the chlorate-reducing bacterium *Dechlorosoma suillum* (recently renamed as *Azospira oryzae*, DSM 13638) and the benzene-degrading bacterium *Dechloromonas aromatica* RCB were 92.2% and 90.5%, respectively. A comparison of the physiological properties of strain G5G6 and Chol-1-S^T is presented in Table 3.

The most abundant cellular fatty acids of strain G5G6, grown on toluene and nitrate, were $C_{16:0}$, $C_{16:1}$ ω7, $C_{17:0}$ cyclopropyl, $C_{18:0}$ and $C_{18:1}$ ω7 (Table 4). Similar fatty acid profiles have been described for *Sterolibacterium denitrificans* strain Chol-1S^T (Tarlera and Denner, 2003) and other betaproteobacterial genera *Thauera* and *Azoarcus* (Song *et al*., 2001) and *Zoogloea ramigera* (Hiraishi *et al*., 1992), although a direct comparison of fatty acid profiles may be difficult because of the different cultivation conditions (Table 4).

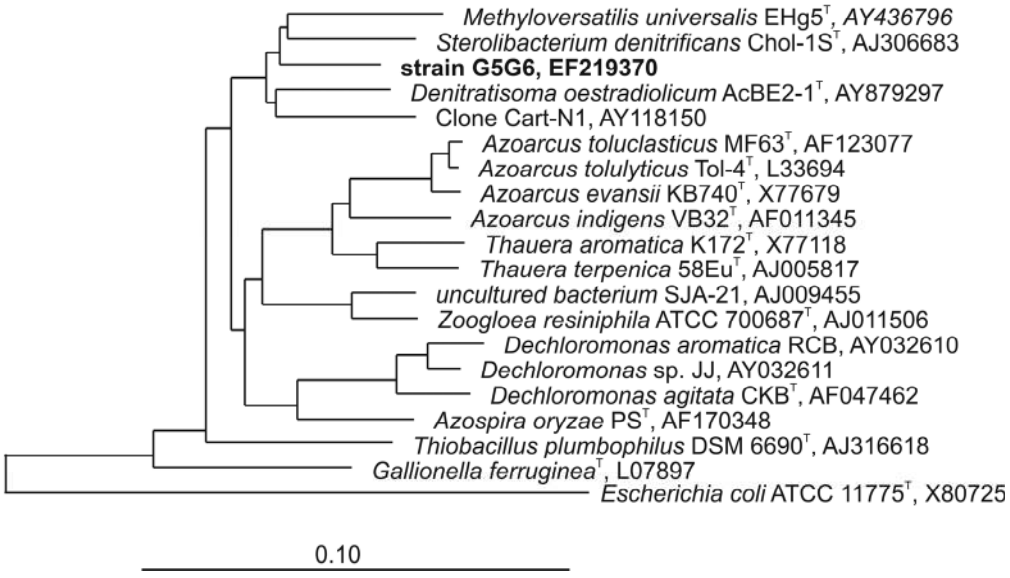

Figure 5. Neighbor joining tree of partial 16S rRNA sequences retrieved from strain G5G6 and other related *Betaproteobacteria*. Alignment and phylogenetic analysis were performed with the ARB software (Ludwig *et al*., 2004). The tree was constructed using the neighbor joining method based on *E. coli* positions 32-1480, using a *Bacteria* filter, as implemented in ARB. The reference bar indicates 10 % diversity. GenBank accession numbers of reference sequences are given.

Table 4. Overview of key characteristics of strain G5G6 and related genera (related means based on 16S rRNA genes). Data for the genera *Sterolibacterium, Thauera*, *Azoarcus* and *Zoogloea* were compiled from other studies (Hiraishi *et al*., 1997; Reinhold-Hurek and Hurek, 2000; Song *et al*., 2001; Tarlera and Denner, 2003). n.d. = not determined.

Characteristic	Strain G5G6	*Sterolibacterium*	*Zoogloea*	*Thauera*	*Azoarcus*
16S rRNA genes sequence similarity with strain G5G6	100%	94.6%	93% [(a)]	91% [(a)]	91% [(a)]
Utilization of toluene as carbon source	+	-	-	+/- [(b)]	+/- [(b)]
Major cellular fatty acids	$C_{16:0}$ $C_{16:1}$ ω7 $C_{17:0}$ cyclopropyl $C_{18:0}$ $C_{18:1}$ ω7	$C_{10:0}$ 3-OH $C_{16:1}$ 7ωc/iso $C_{15:0}$ 2-OH $C_{16:0}$ $C_{18:1}$ ω7c	$C_{10:0}$ 3-OH $C_{16:0}$ $C_{16:1}$	$C_{10:0}$ 3-OH $C_{16:0}$ $C_{16:1}$ $C_{12:0}$	$C_{10:0}$ 3-OH $C_{16:0}$ $C_{16:1}$ $C_{12:0}$

[a] Percentage is indication for the whole genus, exact similarity depends on strain.

[b] Some *Azoarcus* and *Thauera* species can use toluene as carbon source, but not all strains of these genera.

Discussion

A novel bacterium, strain G5G6, was isolated that grows anaerobically with toluene as the sole electron donor and carbon source and with nitrate or ferric iron as the electron acceptor. Strain G5G6 was isolated from an iron-reducing aquifer polluted by BTEX-containing leachate from a landfill (Banisveld, The Netherlands). Members of the iron-reducing *Geobacteraceae* were previously observed to dominate the plume (Röling *et al*., 2001; Lin *et al*., 2005). Recently, it was demonstrated that *Geobacteraceae* also dominated in BTX-degrading iron-reducing enrichments inoculated with polluted soil or groundwater from the landfill, suggesting that *Geobacteraceae* play a key role in the natural attenuation of BTX *in situ* (Botton *et al*., 2007). However, the sequence of the detected *bssA* in microcosms did not correspond to the described *bssA* genes of known members of the *Geobacteraceae*, suggesting that a still unknown group of bacteria is involved in BTX degradation. The toluene-degrading iron-reducing strain G5G6 isolated from this aquifer is the first representative of a novel group of aromatic-hydrocarbon degrading, iron-reducing bacteria that does not belong to the *Geobacteraceae*.

Toluene-oxidizing nitrate-reducing bacteria described thus far belong either to the *Azoarcus* and *Thauera* genus in the *Betaproteobacteria* or to the *Magnetospirillum* genus in the *Alphaproteobacteria*. Currently described bacteria able to degrade toluene coupled to Fe(III) reduction all belong to the *Geobacter* genus in the *Deltaproteobacteria*, which includes among others the well studied *Geobacter metallireducens* (Lovley and Lonergan, 1990; Lovley *et al*., 1993). The phylogenetic characterization showed that strain G5G6 belonged to the *Betaproteobacteria,* but it is not a member of the *Azoarcus* and *Thauera* genera. The closest related cultured bacterium is *Sterolibacterium denitrificans* strain Chol-1S^T, a cholesterol-oxidizing, denitrifying bacterium, with a 16S rRNA gene similarity of only 94.6%. Physiological properties of strain G5G6 and *Sterolibacterium denitrificans* differ considerably as indicated in Table 3. Both strains are able to denitrify using a restricted range of substrates. Substrates that are commonly used by denitrifying bacteria such as acetate or other volatile fatty acids, pyruvate, glucose and hydrogen are not consumed by these two strains. However, the substrate spectrum of the two strains is different. Strain G5G6 only grows with aromatic compounds, such as toluene, ethylbenzene and some other aromatic compounds. *Sterolibacterium denitrificans* only grows with cholesterol, cholesterol derivates and some long-chain fatty acids (palmitate and stearate) (Tarlera and Denner, 2003). *Sterolibacterium denitrificans* uses oxygen or nitrate as the electron acceptor. Strain G5G6 is not able to grow with oxygen, but is able to grow with nitrate, MnO_2 and Fe(III)-species as the electron acceptor. Comparative evolutionary distance analysis indicated that strain G5G6 represents a separate lineage of descent within the *Betaproteobacteria*. The nearest, but relatively distant, phylogenetic relatives were species of the genera *Sterolibacterium, Denitratisoma*, *Azoarcus*, *Thauera* and *Zoogloea* (16S rRNA gene sequence similarities ranging from 90-95%) (Table 4).

The ratio of Fe(II) produced to toluene degraded in the enrichment culture and strain G5G6 isolated from it were 15.7 and 13.6, respectively. These ratios are lower than the theoretical ratio (i.e. 36), which could be explained by the production of biomass from toluene resulting in lower observed ratios. It could also be that toluene was not completely degraded to CO_2, although we did not detect organic compounds in the culture liquid. Furthermore, it could be that Fe(II) precipitates formed during Fe(II) were not measured with the extraction method employed in this study, as also observed in a study by Botton and Parsons (2007), resulting in an underestimation of the Fe(II) to toluene ratio. The ratio of Fe(II) produced to toluene degraded was close to 36 for the toluene-degrading-Fe(III)-reducing bacterium *Geobacter metallireducens* during toluene oxidation with amorphous Fe(III) oxide (Lovley and Lonergan, 1990). In toluene degradation experiments with nitrate as the electron acceptor, some nitrite production was observed (less than 10% of nitrate reduced). However, the amount of nitrite produced varied somewhat in the different experiments that were performed. For example, during

growth on ethylbenzene and nitrate (Fig. 4), nitrite production was more than 50% of nitrate reduced. The ratio nitrate reduced to toluene degraded (5.5) was different from theoretical ratio for nitrate reduction to N_2 (7.2). When biomass production is taken into account, the theoretical ratio of nitrate reduced to toluene degraded will be significantly lower. Ammonium was not formed as product of nitrate reduction. This indicates that strain G5G6 is a denitrifying bacterium, like its phylogenetically most closely related relatives. Strain G5G6 was not able to grow with nitrite as sole electron acceptor. Likely, the concentration of 2.5 mM nitrite used in this study was too high. Nitrite toxicity has often been reported for toluene-degrading denitrifying bacteria (Dolfing *et al*., 1990; Evans *et al*., 1991; Schocher *et al*., 1991).

The specific growth rate (μ) of strain G5G6 on toluene with Fe(III)NTA and nitrate was 0.5 day^{-1} (doubling time about 1.4 days) and 0.2 day^{-1} (doubling time about 3.5 days). Reported doubling times for other toluene-degrading nitrate-reducing strains in the *Betaproteobacteria* ranged from 4.3 hours (*Azoarcus tolulyticus* strain Tol-4) to 11 hours (*Aromatoleum aromaticum* strain EbN1) (Rabus and Widdel, 1995; Chee-Sanford *et al*., 1996). To compare the biomass yield of strain G5G6 (i.e. 30 g biomass per mol of toluene degraded) with the amount of toluene degraded and nitrate reduced, the f_e (fraction of electrons from toluene used for energy production) and f_s (fraction of electrons from toluene used for cell synthesis) approach was used (Rittmann and McCarty, 2001). The cumulative amount of toluene degraded (0.33 mM) and the amount of nitrate reduced (1.87 mM) (Table 1) were used to calculate the f_s. This calculation provided an f_s value of 0.21, leading to the following overall balanced equation:

$$C_7H_8 + 5.67\ NO_3^- + 0.55\ H_2O + 0.38\ NH_4^+ + 1.51\ CO_2 \rightarrow$$
$$0.38\ <C_5H_7O_2N> + 2.83\ N_2 + 6.62\ HCO_3^- + 1.33\ H^+ \quad (1)$$

According to this approach, the biomass ($C_5H_7O_2N$) yield should 43 g biomass per mol toluene, which is in fairly good agreement with the measured yield (30 g biomass per mol toluene). For *Azoarcus tolulyticus* strain Tol-4 a biomass yield of 49.6 g biomass per mol toluene has been reported (Chee-Sanford *et al*., 1996).

The *bssA* gene sequence of strain G5G6 was closest related to *bssA* genes of denitrifying *Betaproteobacteria* (*Thauera* spp., *Azoarcus* spp., Fig. 3). However, the sequence clustered distinctly among the *bssA* genes of these *Betaproteobacteria* (72-81% similarity), in line with the phylogenetic separation between strain G5G6 and toluene-oxidizing denitrifiers on basis of their 16S rRNA gene (Fig. 5). The *bssA* sequence of strain G5G6 was closest associated (91-93% similarity) with sequences observed in toluene-oxidizing, iron-reducing enrichments obtained from the same aquifer from which isolate G5G6 was obtained, the aquifer downstream from the Banisveld landfill. However, as in that study the community was investigated by using primers specific that target *Geobacter* 16S RNA genes, phylotypes corresponding to isolate G5G6 were not detected (Botton *et al*., 2007). Genes encoding benzoyl reductase (*bcrA*) and 6-ketocyclohex-1-ene 1-carbonyl-CoA hydrolase (*oah*) were found to be present in strain G5G6, suggesting that it degrades aromatic compounds via benzoyl-coA as intermediate in a similar manner as other facultative and strict anaerobic bacteria (Barragan *et al*., 2004; Shinoda *et al*., 2005; Wischgoll *et al*., 2005) and distinct from the anaerobic phototropic *Rhodopseudomonas pallustris* (Egland *et al*., 1997).

Strain G5G6 has a number of unique features that need to be considered in future studies on anaerobic degradation of aromatic hydrocarbons

(1) it is a toluene-oxidizing, iron-reducing bacterium not belonging to *Geobacteraceae.* Within the family *Geobacteraceae* in the *Deltaproteobacteria*, *G. metallireducens, G. grbiciae* and *Geobacter* sp. TMJ1 have been described to oxidize toluene (Lovley and Lonergan, 1990; Lovley *et al*., 1993; Coates

et al., 2001a; Winderl *et al*., 2007). Like strain G5G6, *G. metallireducens* is able to use both iron(III) and nitrate. *G. grbiciae* is not able to use nitrate (Coates *et al*., 2001a).
(2) it is a toluene-oxidizing nitrate-reducing bacterium not belonging to the *Azoarcus* and *Thauera* (*Betaproteobacteria)* or to the *Magnetospirillum* genus (*Alphaproteobacteria*). Strain G5G6 is not able to use oxygen as the electron acceptor. The toluene-degrading denitrifying *Thauera* and *Azoarcus* species in the *Betaproteobacteria* are facultative anaerobic bacteria.
(3) only two other bacteria described so far are able to grow by the degradation of both toluene and ethylbenzene with nitrate. i.e. *Azoarcus* sp. EbN1 (recently renamed as *Aromatoleum aromaticum* (Rabus and Widdel, 1995; Wöhlbrand *et al*., 2007)) and *Dechloromonas aromatica* RCB (Chakraborty *et al*., 2005).
(4) Strain G5G6 only grows with aromatic compounds, not with more common growth substrates like acetate, hydrogen, lactate, pyruvate, and glucose. As far as we know, other anaerobic toluene-degrading bacteria do not have this limited substrate use, with the exception of the sulfate-reducing strain OX39. Strain OX39 does not use acetate, lactate, ethanol, methanol, glucose or H_2, but is able to grow with toluene, *m*-xylene, *o*-xylene, benzoate, *m*-toluic acid and *o*-toluic acid (Morasch *et al*., 2004).
(5) Strain G5G6 does not use AQDS as the electron acceptor, while most other iron-reducers do. An exception is the iron-reducer *Rhodoferax ferrireducens* T118^T (Finneran *et al*., 2003). Even iron-reducing hyperthermophilic *Archaea* species can use AQDS as the electron acceptor (Lovley *et al*., 1996b; Lovley *et al*., 2000).

Phylogenetic analysis of the 16S rRNA sequence indicated that strain G5G6 belonged to the *Betaproteobacteria*, with *Sterolibacterium denitrificans* Chol-1S^T as closest but rather distinct cultured relative (94.6% similarity based on 16S rRNA genes). Key characteristics of strain G5G6 and related genera are listed in Table 4. Based on phylogenetic and physiological data presented in this study, we consider the novel toluene-degrading bacterium strain G5G6 to be a hitherto unknown taxon of the *Betaproteobacteria*, for which we propose the name *Georgfuchsia toluolica* gen. nov. , spec. nov.

Description of *Georgfuchsia gen. nov.*
Ge.org.fuch' sia. N.L fem .n. *Georgfuchsia* named after professor Georg Fuchs, for his scientific contribution to our present understanding of anaerobic microbial growth on aromatic hydrocarbons.
Straight or slightly curved, small, rod-shaped, Gram-negative cells. Mesophilic. Strictly respiratory type of metabolism. Chemo-organoheterotrophic. Capability of degrading aromatic compounds. The type species of the genus is *Georgfuchsia toluolica* strain G5G6.

Description of *Georgfuchsia toluolica* sp. nov.
Georgfuchsia toluolica (to.lu.ol' ica. N.L. fem. adj. toluolica pertaining to toluene). Cells are short rods, 0.5-0.6 µm wide and 0.8-1.2 µm long, motile and stained Gram-negative. Cells had the tendency to form chains. Colonies in agar batches were round, lens-shaped, 0.5-1.0 mm in diameter and brown-reddish. The optimum pH for growth was pH 7.3 and the optimum temperature was 25-30°C. Electron acceptors used are nitrate, MnO_2 and various Fe(III)-species (both amorphous as soluble Fe(III)-species). Oxygen was not used as the electron acceptor (tested with toluene as electron donors). Range of substrates used for growth was very limited. Strain G5G6 only grows with aromatic compounds, not with common compounds like acetate, hydrogen, lactate, pyruvate, and glucose. Electron donors used are toluene, ethylbenzene, phenol, *p*-cresol, *m*-cresol, benzaldehyde and p-hydroxybenzoate. The specific growth rate (µ) on toluene with Fe(III)NTA and nitrate was 0.47 day^{-1} (doubling time 1.5 days) and 0.16 day^{-1} (doubling time 4.3 days), respectively. Major fatty acids are $C_{16:0}$, $C_{16:1}$ ω7, $C_{17:0}$ cyclopropyl, $C_{18:0}$ and $C_{18:1}$ ω7.

The type strain is strain G5G6^T (DSMZ 19032^T = JCM 14632^T). Isolated from an iron-reducing aquifer contaminated by BTEX-containing landfill leachate (Banisveld landfill, near Boxtel, the Netherlands).

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4

Physiological and phylogenetic characterization of a stable benzene-degrading, chlorate-reducing microbial community

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Abstract

A stable anoxic enrichment culture was obtained that degraded benzene with chlorate as the electron acceptor. The benzene degradation rate was 1.65 mM benzene per day, which is similar to reported aerobic benzene degradation rates, but 20-1650 times higher than reported for anaerobic benzene degradation. Denaturing gradient gel electrophoresis of part of the 16S rRNA gene, cloning and sequencing showed that the culture had a stable composition after the seventh transfer. Five bacterial clones were further analyzed. Two clones corresponded to bacteria closely related to *Alicycliphilus denitrificans* K601. The three other clones corresponded to bacteria closely related to *Zoogloea resiniphila* PIV-3A2w, *Mesorhizobium* sp. WG and *Stenotrophomonas acidaminiphila*. DGGE analysis of cultures grown with different electron donors and acceptors indicated that the bacterium related to *Alicycliphilus denitrificans* K601 is able to degrade benzene coupled to chlorate reduction. The role of the other bacteria could not be conclusively determined. The bacterium related to *Mesorhizobium* sp. WG can be enriched with benzene and oxygen, but not with acetate and chlorate, while the bacterium related to *Stenotrophomonas acidaminophila* grows with acetate and chlorate, but not with benzene and oxygen. As during chlorate reduction oxygen is produced, an aerobic pathway of benzene degradation is most likely.

Introduction

Anoxic bioremediation is attractive when anaerobic conditions prevail at a polluted soil site. Due to the relatively high solubility of monoaromatic hydrocarbons, these compounds are readily transferred to the anoxic zones of the environment. Thus far, anaerobic bioremediation techniques for soils polluted with aromatic hydrocarbons are not often applied. The bottleneck in the application of anaerobic techniques is the supposed poor anaerobic biodegradability of benzene. Anaerobic biodegradation of benzene under various redox conditions has been described, but only in a few studies the microorganisms involved were identified (Phelps *et al*., 1998; Rooney-Varga *et al*., 1999; Ulrich and Edwards, 2003; Chang *et al*., 2005; Kasai *et al*., 2006). Up to now, four anaerobic benzene-degrading bacteria were described; two *Dechloromonas* strains (RCB and JJ) that degrade benzene coupled with chlorate, perchlorate, nitrate or oxygen reduction (Chakraborty *et al*., 2005) and two denitrifying *Azoarcus* strains (DN11 and AN9) (Kasai *et al*., 2006). The optimal physiological conditions for anaerobic benzene-degrading bacteria and the biodegradation pathways are still unclear. Benzene is chemically very stable (Aihara, 1992) and therefore its degradation requires an initial destabilisation. Aerobic bacteria convert benzene first to phenol or catechol by means of mono- or dioxygenases (Yerushalmi *et al*., 2001). Under anoxic conditions, initial attack in benzene biodegradation has been suggested to occur via hydroxylation, fumarate addition, carboxylation or methylation (Grbic-Galic, 1986; Coates *et al*., 2002; Chakraborty and Coates, 2005; Ulrich *et al*., 2005). (Per)chlorate reducing microorganisms (ClRM) reduce (per)chlorate to chloride. Typically, during (per)chlorate reduction the intermediate chlorite is dismutated to molecular oxygen and chloride (Rikken *et al*., 1996; Van Ginkel *et al*., 1996; Logan, 1998). Oxygen formation during (per)chlorate reduction may be advantageous for the anaerobic biodegradation of persistent aromatic compounds, such as benzene. A previous study showed that amendment of ClRM and chlorite to an anoxic soil led to complete degradation of ^{14}C-benzene to ^{14}C-carbon dioxide (Coates *et al*., 1999a). Addition of chlorate in a soil column polluted with benzene also showed removal of benzene coupled with chlorate reduction, but we were not able to obtain an enrichment culture in batch culture from material of this column (Tan *et al*., 2006). Therefore, we started new experiments to study the bacteria involved in anaerobic benzene degradation with chlorate. This paper describes the physiological and phylogenetic properties of the microorganisms in a highly active benzene-degrading chlorate-reducing batch enrichment culture.

Materials and Methods

Inoculum and medium composition

Enrichment of the culture was started with material from a batch culture incubated in GR-1 medium (Rikken *et al*., 1996) under a N_2 atmosphere in the dark at TNO Built Environment and Geosciences. This TNO-culture was originally inoculated with mixed material from a wastewater treatment plant and soil samples obtained from a location contaminated with benzene. Benzene was added from water-saturated anoxic stock solutions. The TNO-culture was exposed to four sequential additions of 11, 5, 15 and 20 µM of benzene, respectively. Benzene was removed completely, with a half-lifetime of 12 days. Further enrichment was performed via inoculation of 5 % (v/v) of the TNO-culture in three different chlorate containing media: GR-1 medium, AW-1 medium with sulfide (AW-1-sulfide, 2 mM sulfide) and AW-1 medium with sulfate as sulfur source (AW-1-sulfate, 1.4 mM sulfate). GR-1 medium contains 0.4 mM sulfate as sulfur source and is phosphate-buffered, whereas AW-1 medium is both phosphate and bicarbonate buffered (Rikken *et al*., 1996; Wolterink *et al*., 2002). After the initial inoculation in three different media, further batch incubations were performed in 120-mL bottles filled with 40 mL of chlorate-containing AW-1-sulfate medium (Wolterink *et al*., 2002). The bottles were closed with Viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminum crimp caps. The headspace of the batches consisted of 170 kPa of 20 % CO_2 and 80 % N_2 to give a pH of 7.3.

Cultivation and experimental setup

Batch cultures were incubated at 30ºC in an orbital shaker (50 rpm). When degradation occurred, the culture was transferred (1-5 % v/v) into fresh medium (AW-1-sulfate) in dilution series. Perchlorate, chlorate and nitrate (10-20 mM, final concentrations), and oxygen (5-10 % v/v of the headspace) were tested as electron acceptors. The possible intermediates of benzene degradation (toluene, catechol, phenol, benzoate) were added from anaerobic stock solutions. Final concentrations were 0.5 mM except for toluene (0.25 mM). The intermediates were added individually, or in combination with benzene. In later experiments, we tested different combinations of electron donors and electron acceptors: benzene and chlorate, benzene and oxygen, benzene and nitrate, phenol and chlorate, phenol and nitrate, acetate and chlorate. In these experiments, fermented yeast extract (FYE, 0.125 g/L) was added to batches as a nutrient supplement to stimulate growth as was done in previous studies (Holliger *et al*., 1993; Van Doesburg *et al*., 2005).

Analytical procedures

Benzene was measured by headspace analysis using a gas chromatograph (Chrompack 436, Chrompack, Middelburg, The Netherlands) with a capillary column (SIL 5CB, 25 m * 0.32 mm, DF 1.2, 100 kPa, split flow of 28 mL min^{-1}, Chrompack, Middelburg, The Netherlands) and a flame ionisation detector. The column, detector and injector temperatures were 50, 300 and 250 ºC, respectively. Chlorate, chloride and nitrate were determined by suppressor mediated ion chromatography (Dionex, Breda, The Netherlands) and a conductivity detector. Eluent consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ was pumped through the system at a flow rate of 1 mL min^{-1}. The chromatograph was equipped with an IonPac® AS9-SC column (Dionex, Breda, The Netherlands). Mannitol (10 mM final concentration) was added to the samples for stabilization, and sodium fluoride (1 mM final concentration) was added as internal standard. Oxygen production was measured with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio, USA). Proteins for yield determination were determined according with a BCA protein assay kit (Pierce Biotechnology, Rockford, USA).

DNA isolation and 16S rRNA gene amplification

A bead-beat and phenol-chloroform based DNA extraction method was used to extract DNA from the enrichment cultures (Van Doesburg *et al*., 2005). The amplification with the primers 7f and 1492r and purification of the 16S rRNA genes was previously described (Van Doesburg *et al*., 2005).

Cloning and sequencing

The purified 16S rRNA genes were cloned into *E. coli* JM109 (Invitrogen, Breda, The Netherlands). After blue/white screening, positive colonies were picked with a sterile toothpick and transferred into 0.2 mL PCR tube containing 50 µL of Tris-EDTA buffer, which was heated for 15 min at 95°C to release the DNA. The 16S rRNA gene inserts from recombinant clones were reamplified by PCR with the vector specific primers T7 and Sp6 (Promega, Madison, WI). PCR products were screened by Restriction Fragment Length Polymorphism (RFLP), using the restriction endonucleases *AluI*, *CfoI* and, *MspI* (Promega, Madison, WI). Aliquots (5 µL) of crude reamplified 16S rRNA gene PCR products were digested with 2.5 U each of the enzymes in 1x Buffer B (Promega, Madison, WI) in a final volume of 10 µL, for 1.5 h at 37 °C. Digested products were separated by 4% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products with different RFLP patterns were purified by the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and used as a template in sequencing reactions.

For sequence analysis the Sequenase kit (Amersham, Slough, United Kingdom) was used according to the manufacturer's instructions with IRD800 (MWG-Biotech, Ebersberg, Germany)-labeled sequencing primers Sp6, T7 (Promega, Madison, WI), 533f and 1100r (Lane, 1991). The sequences were automatically analysed on a LI-COR (Lincoln, NE) DNA sequencer 4000L and corrected manually. Sequences of 16S rRNA genes were compared with sequences deposited in publicly accessible databases using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/blast/.

DGGE analysis

Purified DNA from the enrichment cultures or cultured transformants was used as PCR template. A DGGE-suitable 16S rRNA gene amplicon was generated with 35 cycles of 94°C for 30 s, 56°C for 20 s, and 68°C for 40 s. The PCR reaction mixtures were the same as for the total 16S rRNA gene PCR, except for the two primers; F-968-GC and R-1401 (Nubel *et al*., 1996). The PCR products were separated by DGGE (Muyzer *et al*., 1993) by using the Dcode system (Biorad Laboratories, Hercules, CL) using some the modifications (Heilig *et al*., 2002).

GenBank accession numbers

The 16S rRNA gene nucleotide sequences determined in this study have been deposited into the GenBank database under accession numbers DQ342274-DQ342278.

Results

Enrichment and physiological properties

Benzene degradation with chlorate as the electron acceptor was tested in three different anoxic media previously used for (per)chlorate-reducing bacteria: AW-1 medium with sulfate as sulfur source (AW-1-sulfate), AW-1 medium with sulfide as sulfur source (AW-1-sulfide), and GR-1 medium (Rikken *et al*., 1996; Wolterink *et al*., 2002). Benzene was degraded in AW-1-sulfate medium (Fig. 1A), but not in AW-1-sulfide medium. No benzene degradation occurred in GR-1 medium and transfers of the benzene-degrading culture from AW-1-sulfate medium into GR-1 medium did not show degradation of benzene within 100 days of incubation (results not shown). In the absence of chlorate, no benzene degradation was observed in AW-1-sulfate medium, while controls without benzene did not show

chlorate reduction. This indicates that benzene degradation and chlorate reduction were coupled. All further enrichment experiments were done in the AW-1-sulfate medium.

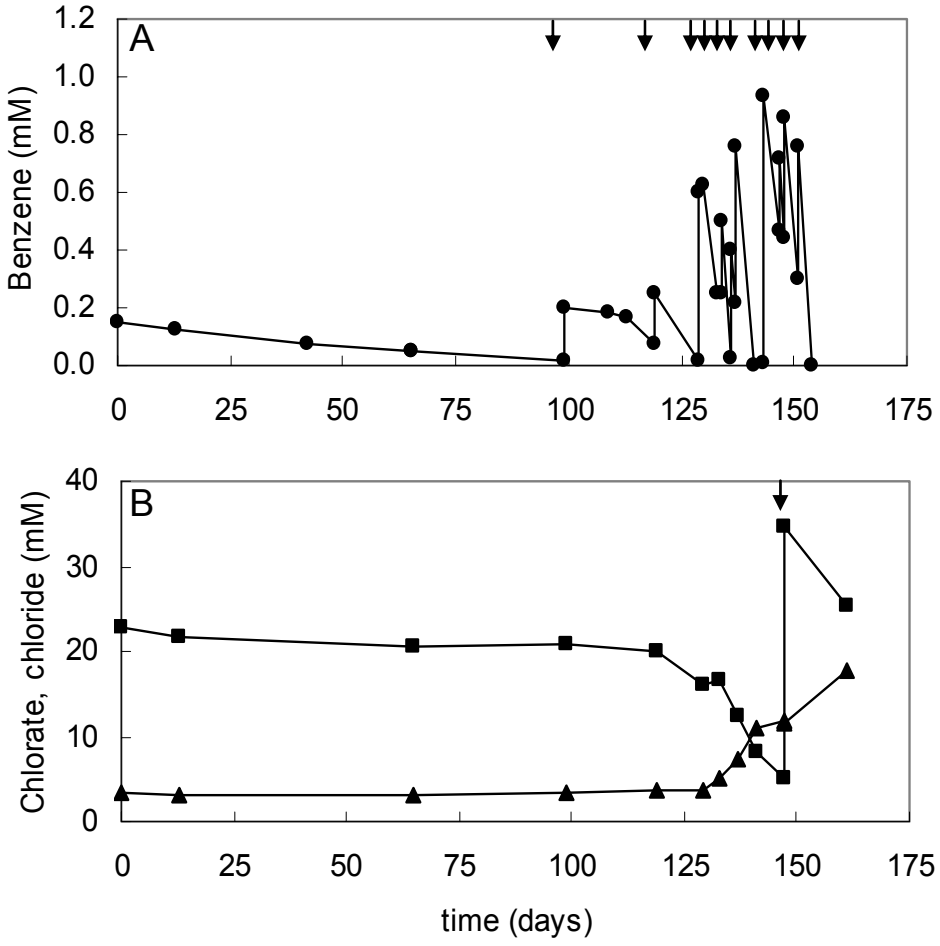

Figure 1. A: Initial degradation of benzene (●) in chlorate reducing AW-1 medium with sodium sulfate (average of two incubations; arrow indicates re-addition of benzene). B: Chlorate (■) and chloride (▲) concentration (average of two incubations; arrow indicates re-addition of chlorate).

The initially added 150 µM benzene was degraded in 100 days (Fig. 1 A). Re-addition of benzene led to a faster degradation, indicating that benzene-degrading bacteria were enriched (Fig. 1A). Chlorate was reduced and chloride was produced (Fig. 1B). The expected stoichiometry of complete oxidation of benzene coupled with chlorate reduction is:

$$C_6H_6 + 5\ ClO_3^- + 3\ H_2O \rightarrow 6\ HCO_3^- + 5\ Cl^- + 6\ H^+ \quad (1)$$

In further mass balance experiments with lower initial amounts of chlorate (3 mM instead of 20 mM; Fig. 2A and B), the ratio of total amount of chlorate reduced with the cumulative amount of benzene degraded was 3.35. The ratio of the amount of chlorate reduced with the amount of chloride formed was 1.15. Benzene degradation coupled to chlorate reduction resulted in growth as indicated by the formation of flocculent biomass. No degradation of benzene or growth were observed in heat-sterilized controls and in controls without inoculum. The yield of the culture was 22.3 ± 9.5 mg protein per mmol of benzene degraded. The benzene degradation rate was about 1.65 mM per day in the first and second transfer of the enrichment culture. Other electron acceptors (oxygen, nitrate and perchlorate) were tested with benzene as the electron donor. Benzene degradation was only observed with oxygen as the electron acceptor. However, further enrichment with oxygen as the electron acceptor resulted in

a loss of the capacity of benzene degradation with chlorate. When chlorite was added to a chlorate-reducing culture fast oxygen production was observed with a Clark-type oxygen electrode, but this was not further quantified. This indicates that the enrichment culture had chlorite dismutase activity during growth with chlorate.

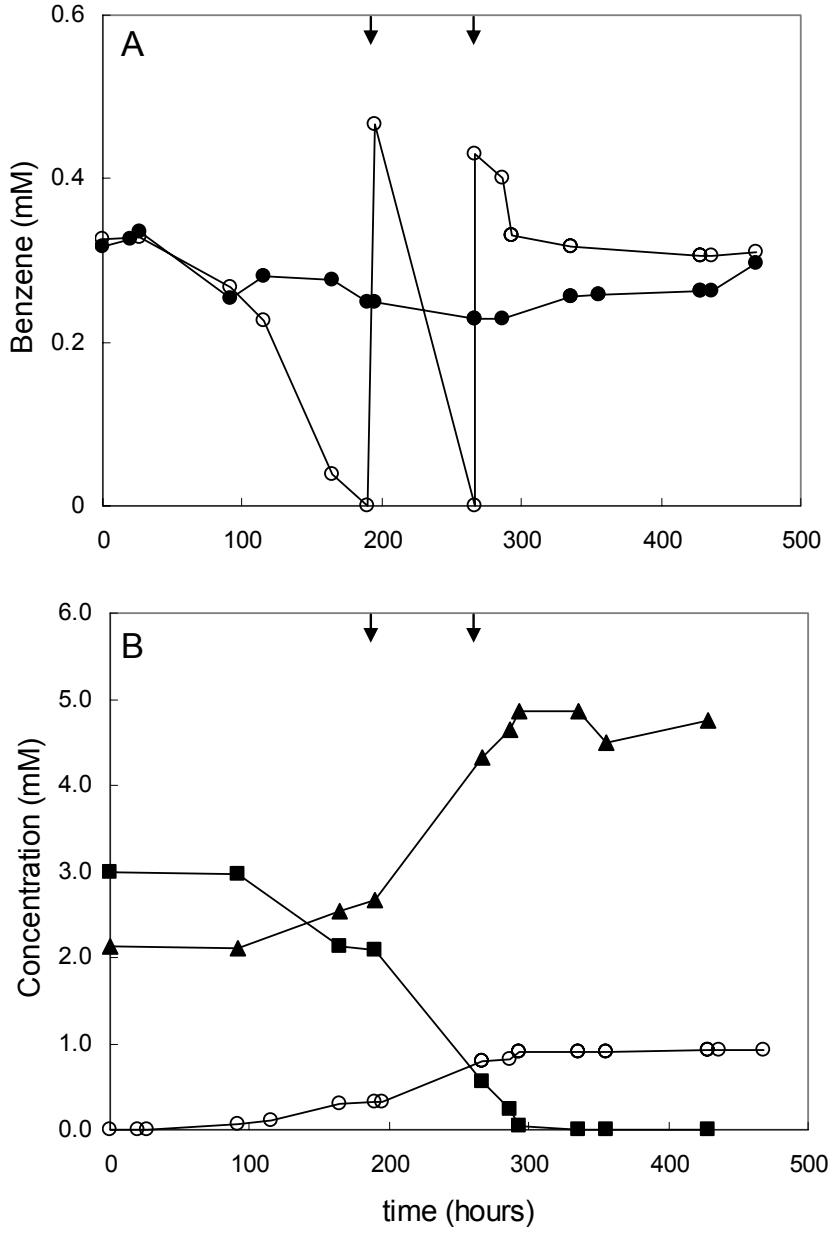

Figure 2. Mass balance of benzene biodegradation with chlorate as the electron acceptor. A: benzene (○) and sterile control (●) (average of two incubations; arrows indicate re-addition of benzene); B: cumulative amount of benzene degraded (○) and chlorate (■) and chloride (▲) measured (average of two incubations; arrow indicates re-addition of benzene).

The possible intermediates phenol and catechol were readily degraded by the enrichment culture within 10 days, both in the absence and presence of benzene (Table 1). The benzene degradation rate in the presence of phenol (0.06 mM day^{-1}) or catechol (0.10 mM day^{-1}) was approximately similar

to the benzene degradation rate without these compounds (0.08 mM day^{-1}). Toluene, another proposed intermediate of anaerobic benzene degradation, was degraded slower than phenol and catechol when added separately or together with benzene. In the presence of toluene, the time to degrade benzene increased from 9 to 16 days. Benzoate was not degraded as a sole substrate, but when incubated with benzene half of the initial amount of benzoate was degraded in 25 days. Benzoate also caused an inhibition of the degradation of benzene.

Table 1. Degradation rates in mM day^{-1} of potential intermediates alone and in the presence of benzene, and degradation rates of benzene alone and in the presence of intermediates. Benzene and intermediates concentration 0.5 mM; except toluene 0.25 mM. Between brackets the time (days) to degrade the compound.

	Degradation rates (mM day^{-1})	
Intermediate (or + benzene)	Intermediate	Benzene
Phenol	0.10 (10)	-
Phenol + benzene	0.05 (10)	0.06 (10)
Catechol	0.04 (10)	-
Catechol + benzene	0.05 (10)	0.10 (13)
Toluene	0.02 (20)	-
Toluene + benzene	0.03 (20)	0.06 (16)
Benzoate	ND [a]	-
Benzoate + benzene	0.06 (25) [b]	0.04 (20)
Benzene	-	0.08 (9)

[a] ND not degraded.

[b] Only half of the initial amount of benzoate was degraded.

When the culture was stored unfed for a longer period (>one month), we had difficulties with subcultivation. At that stage, we decided to add fermented yeast extract (FYE, 0.125 g/L) to the enrichment culture as a nutrient supplement to stimulate growth. The addition of FYE to the enrichment culture indeed stimulated growth and resulted in a decreased lag-phase for benzene degradation (results not shown). Subsequently, the enrichment culture was tested for growth on the following combinations of electron donors and electron acceptors (with the addition of 0.125 g/L FYE): benzene and oxygen, benzene and nitrate, phenol and chlorate, phenol and nitrate, acetate and chlorate. Furthermore, the microbial composition in these batches was analyzed by DGGE. Benzene (0.25 mM) was degraded with oxygen (5% in gas phase) as the electron acceptor within 10 days, but not with nitrate as the electron acceptor within 66 days of incubation. Phenol (0.25 mM) was degraded with chlorate as the electron acceptor within 10 days, which was in accordance with the experiment without FYE (Table 1). With nitrate, on the other hand, phenol was not degraded during 25 days of incubation. Acetate (5 mM) was degraded with chlorate as the electron acceptor within 10 days by the enrichment culture.

Microbial composition

Samples of the different enrichment cultures were taken and stored for 16S rRNA gene analysis. DGGE analysis and clone library analysis derived from the second and seventh transfers indicated that from the first until the seventh transfer changes occurred in the bacterial composition of the enrichment culture (Fig. 3, lanes 1-4). From the seventh transfer until the last two transfers no changes in the DGGE pattern were observed (Fig. 3, lanes 4-6). The time difference between the seventh and thirteenth transfer was about one year (transfers approximately each two months). The culture of the last transfer resulted in (at least) four amplicons on DGGE, suggesting that four different bacterial species were numerically dominant in the culture. However, five different clones were derived

from the amplified and isolated DNA from the second and seventh transfer (Fig. 3). This shows that the part of the 16S rRNA gene amplified for DGGE of two bacteria is not separated on DGGE. The obtained clone sequences were compared with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/blast/). The closest cultured relatives and percentage sequence similarity are shown in the Table 2. One clone, cG10 turned out to be a chimera (Huber *et al*., 2004). Therefore, this clone was initially discarded from the results. Unfortunately, no other clone with a DNA fragment that migrates on the same height on the DGGE gel was available. Therefore, the DGGE part of the sequence of cG10 (436 bp) was used instead of the complete 16S rRNA gene sequence (Table 2).

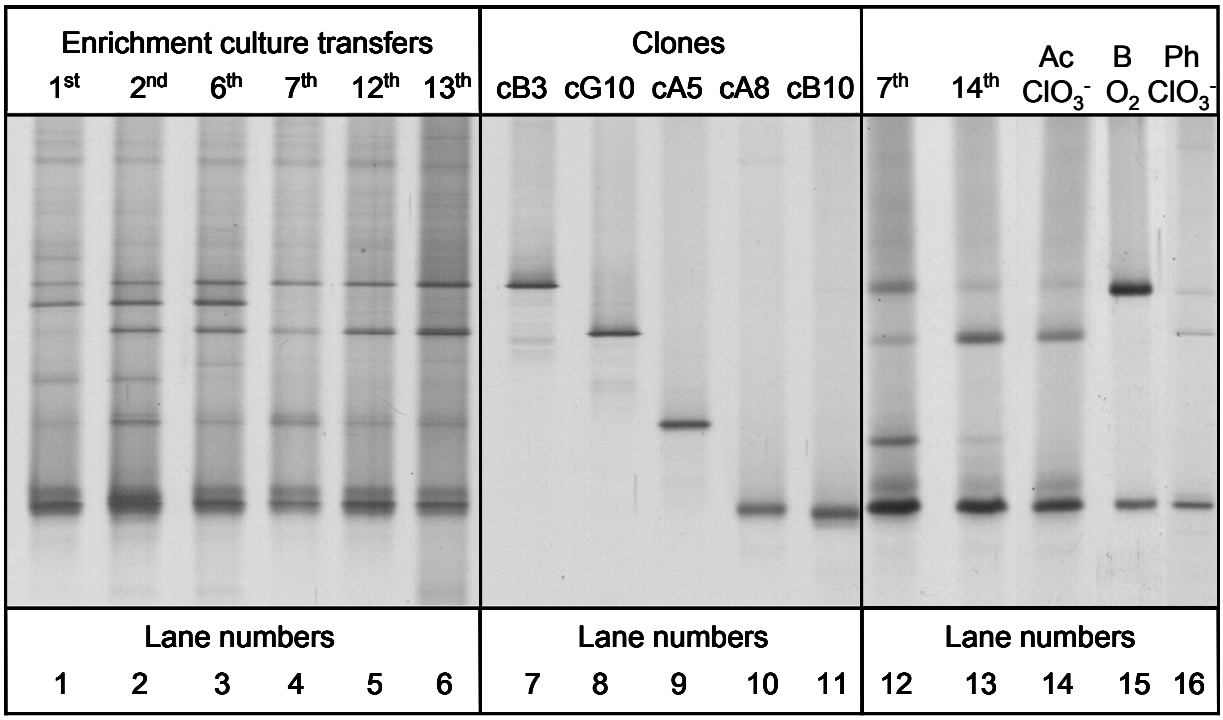

Figure 3. DGGE of PCR-amplified V6-V8 region of 16S rRNA genes from enrichment cultures and clones derived from the second and seventh transfer. Lane 1, first transfer; lane 2, second transfer; lane 3, sixth transfer; lane 4, seventh transfer; lane 5, twelfth transfer; lane 6, thirteenth transfer; lane 7, clone cB3; lane 8, clone cG10; lane 9, clone cA5; lane 10, clone cA8; lane 11, clone cB10; lane 12, seventh transfer; lane 13, fourteenth transfer (with FYE); lane 14, acetate and chlorate culture (with FYE); lane 15, benzene and oxygen culture (with FYE); lane 16, phenol and chlorate culture (with FYE). Lanes 12-16 originate from other DGGE gels than lanes 1-11. Therefore, identical DNA fragments can have small migration pattern differences in lanes 12-16 compared to lanes 1-11.

Table 2. Sequence analysis (NCBI blast results) of the 16S rRNA gene cloned inserts derived from the second and seventh transfer from the benzene-degrading chlorate reducing enrichment culture.

Clone	GenBank accession number	Sequence length base pairs	Closest cultured relative	Percentage sequence similarity (%)	Reference
cB3	DQ342274	1446	*Mesorhizobium* sp. WG	99	(Costa *et al*., 2000)
cG10-part	DQ342275	436	*Stenotrophomonas acidaminiphila*	99	(Assih *et al*., 2002)
cA5	DQ342276	1497	*Zoogloea resiniphila* PIV-3A2w	99	(Probian *et al*., 2003)
cA8	DQ342277	1487	*Alicycliphilus denitrificans* K601 [(a)]	99	(Mechichi *et al*., 2003)
cB10	DQ342278	1487	*Alicycliphilus denitrificans* K601 [(a)]	99	(Mechichi *et al*., 2003)

[a] Clones cA8 and cB10 had 99% 16S rRNA gene sequence similarity with *Acidovorax avenae* isolate C1 (AF508114) and *Alicycliphilus denitrificans* K601 (AJ418042), but only 96% with *Acidovorax avenae* subsp. *avenae* (AF078759), one of the three subspecies of *Acidovorax avenae* (Willems *et al*., 1992). Therefore, we consider *Alicycliphilus denitrificans* K601 as closest cultured relative of clones cA8 and cB10.

Fig. 4 shows a phylogenetic tree of the different clones and their closest cultured relatives. Clone cB3 was closely related to *Mesorhizobium* sp. WG, an acetate-degrading nitrate-reducing bacterium isolated from a denitrifying bioreactor with methane as the electron donor under oxygen-limitation (Costa *et al*., 2000). The DGGE part of clone cG10 showed high similarity with *Stenotrophomonas acidaminiphila*, a strictly aerobic bacterium that was isolated from an anaerobic reactor (Assih *et al*., 2002) and *Stenotrophomonas* sp. BO isolated from the same reactor as the *Mesorhizobium* sp. WG (Costa *et al*., 2000). Clone cA5 had high sequence similarity with *Zoogloea resiniphila* PIV-3A2w, a denitrifying bacterium that anaerobically mineralizes the quaternary carbon containing compound pivilate (2,2-dimethylpropionic acid) (Probian *et al*., 2003). Clones cA8 and cB10 had high sequence similarity with the *Acidovorax avenae* isolate C1, a denitrifying bacterium capable of degrading phenol and reducing nitrate under low-oxygen conditions (Baek *et al*., 2003). Furthermore, both sequences had also high sequence similarity with *Alicycliphilus denitrificans* K601, a cyclohexanol-degrading, nitrate-reducing bacterium (Mechichi *et al*., 2003). Clones cA8 and cB10 were almost identical (only 4 bp difference on 1487 bp) and resembled the thick band on DGGE of the enrichment culture (Fig. 3). Both clones had 99% 16S rRNA gene sequence similarity with *Acidovorax avenae* isolate C1 (AF508114) and *Alicycliphilus denitrificans* K601 (AJ418042), but only 96% with *Acidovorax avenae* subsp. *avenae* (AF078759), one of the three subspecies of *Acidovorax avenae* (Willems *et al*., 1992). Therefore, we consider *Alicycliphilus denitrificans* K601 as closest cultured relative of clones cA8 and cB10 (Table 2).

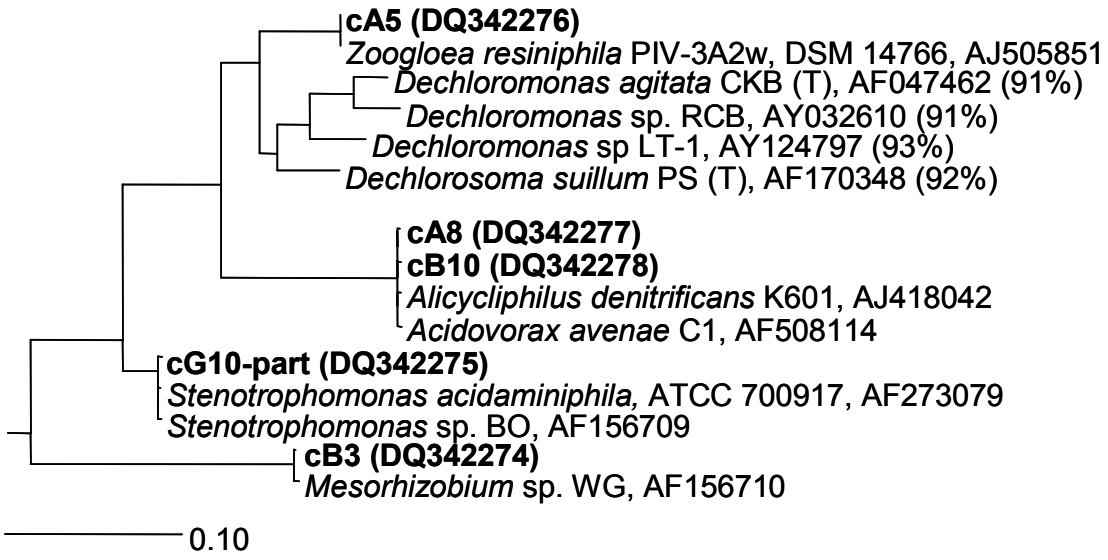

Figure 4. Phylogenetic tree of partial 16S rRNA gene sequences of the five clones obtained from the chlorate enrichment culture (clone cB3, clone cG10-part, clone A5, clone cA8 and clone cB10). Alignment and phylogenetic analysis were performed with the ARB software and the tree was constructed using the neighbour joining method based on *E.coli* positions 101 - 1221, and a 50% conservation filter for β-*Proteobacteria*, as implemented in ARB. The reference bar indicates the branch length that represents 10% estimated sequence divergence. GenBank accession numbers of reference sequences and clones are given. (The percentages between brackets are the sequence similarities of cA5 with (per)chlorate reducing organisms).

Addition of FYE to the enrichment culture resulted in changes in the microbial composition. Based on DGGE analysis, the presence of the bacteria related to *Mesorhizobium* sp.WG (clone cB3) and *Zoogloea resiniphila* PIV-3A2w (clone cA5) decreased when FYE was added to the benzene-degrading chlorate-reducing enrichment culture (Fig. 3, lane 13). When the enrichment culture was grown with acetate and chlorate (Fig. 3, lane 14), no obvious changes in microbial diversity were observed and approximately the same DGGE pattern as in lane 13 was obtained. DGGE analysis of the enrichment culture grown on benzene and oxygen showed that only two bacterial species were present (Fig. 3, lane 15), i.e. the bacteria related to *Alicycliphilus denitrificans* (clone cA8) and *Mesorhizobium* sp.WG (clone cB3). Especially, the latter one dominated this culture based on DGGE results. The phenol and chlorate grown culture (Fig. 3, lane 16) showed three bacterial species, i.e. the bacteria related to *Mesorhizobium* sp.WG (clone cB3), *Stenotrophomonas acidaminiphila* (clone cG10) and *Alicycliphilus denitrificans* (clone cA8). The latter one dominated this culture based on DGGE results.

Discussion

It has been speculated that molecular oxygen formed during (per)chlorate reduction can be used for degradation of anaerobic persistent organic pollutants. Chlorate reduction stimulated benzene degradation in anoxic soil samples and columns (Coates *et al*., 1998; Coates *et al*., 1999a; Tan *et al*., 2006). Additionally, toluene degradation with chlorate in a soil column was enhanced by bioaugmentation with chlorate- and toluene-degrading enrichment cultures (Logan and Wu, 2002). However, in that study no stable enrichment culture was obtained. In this study, on the other hand, a stable enrichment culture was obtained, which coupled benzene degradation to chlorate reduction.

Benzene degradation coupled to chlorate reduction is an energetically favorable process (Table 3). The standard free energy change ($\Delta G^{0'}$) for benzene oxidation with chlorate as the electron acceptor is even more negative than with oxygen or nitrate as the electron acceptor. Mass balance calculations showed that in the enrichment culture the ratio of the amount of chlorate reduced to the amount of benzene degraded was 3.35. The yield of the culture was 22.3 ± 9.5 mg protein per mmol of benzene degraded. To compare the biomass yield with the amount of benzene degraded and chlorate reduced, the f_e (fraction of electrons from benzene used for energy production) and f_s (fraction of electrons from benzene used for cell synthesis) approach was used (Rittmann and McCarty, 2001). The cumulative amount of benzene degraded (0.9 mM) and the amount of chlorate reduced (3 mM) (Fig. 2B) were used to calculate the f_s. This calculation gave an f_s value of 0.33 and gave the following overall balanced equation:

$$C_6H_6 + 3.35\ ClO_3^- + 3.5\ H_2O + 0.5\ NH_4^+ + 2\ CO_2 \rightarrow$$
$$0.5\ <C_5H_7O_2N> + 3.35\ Cl^- + 5.5\ HCO_3^- + 6\ H^+ \quad (2)$$

According to this approach, the biomass ($C_5H_7O_2N$) yield should 56.6 mg biomass per mmol benzene. Assuming that protein comprised 50% of the biomass (Burland and Edwards, 1999), the yield was 28.3 mg protein per mmol benzene, which is in fairly good agreement with the measured yield (22.3 ± 9.5 mg protein per mmol benzene).

Table 3. Stoichiometric equations and standard free energy changes ($\Delta G^{0'}$) for benzene oxidations with various electron acceptors.

Electron acceptor (ox/red)	Stoichiometric equation			$\Delta G^{0'}$ (kJ/mol) [(a)]
ClO_3^-/Cl^-	$C_6H_6 + 5\ ClO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 5\ Cl^- + 6\ H^+$	- 3,813
O_2/H_2O	$C_6H_6 + 7.5\ O_2 + 3\ H_2O$	→	$6\ HCO_3^- + 6\ H^+$	- 3,173
NO_3^-/N_2	$C_6H_6 + 6\ NO_3^-$	→	$6\ HCO_3^- + 3\ N_2$	- 2,978
NO_3^-/NO_2^-	$C_6H_6 + 15\ NO_3^- + 3\ H_2O$	→	$6\ HCO_3^- + 15\ NO_2^- + 6\ H^+$	- 2,061

[a] The data for calculating standard free energy changes (ΔG^0) are from McCarty (1971), Thauer *et al*. (1977) and Stumm and Morgan (1981).

A similar high degradation rate of 1.65 mM benzene per day as we determined for our culture was not reported before for anaerobic cultures. The isolated *Dechloromonas* strain RCB is able to couple benzene degradation with chlorate, nitrate and perchlorate reduction at a much lower rate of around 32 µM per day (Coates *et al*., 2002). Other anaerobic enrichment cultures that degrade benzene with nitrate or sulfate showed degradation rates ranging from 1 to 75 µM per day (Ulrich and Edwards, 2003). The degradation rate determined for our enrichment culture was similar to aerobic benzene degradation rates (Reardon *et al*., 2000).

Several attempts were made to obtain a pure culture of a benzene-degrading chlorate-reducing bacterium, including serial dilutions, soft agar medium, percoll gradient centrifugation, other electron acceptors and electron donors. However, all these attempts using the mineral medium that was used for enrichment (AW-1-sulfate) were unsuccessful (data not shown). In serial dilutions, benzene degradation was observed up to 10^{-4} dilution, while cell counting by phase contrast microscopy suggested that growth until a dilution of 10^{-8} should be possible. Microbial analysis on DGGE showed that several bacterial amplicons were obtained from the enrichment culture. From the seventh transfer onwards no changes were observed in the DGGE profile. The time-span between the seventh and thirteenth transfer covered almost one year. These results show that a stable, highly enriched culture was obtained and this suggested that all the numerically dominant bacteria in the enrichment culture

were involved in benzene degradation with chlorate, directly or indirectly. DGGE analysis of cultures with different combinations of electron donors and acceptors provided some indications about the possible role of the different bacteria in the benzene-degrading chlorate-reducing enrichment culture. Based on our results, not for all the bacteria in the enrichment culture, the role could conclusively be determined. According to DGGE analysis, the benzene-degrading chlorate-reducing enrichment culture was dominated by the bacterium related to *Alicycliphilus denitrificans* (clone cA8), especially when FYE was added (Fig. 3, lane 13). Experiments with different combinations of electron donors and acceptors clearly showed that this bacterium remained in the culture, irrespectively the combinations of electron donors and acceptors (Fig. 3). This bacterium was present in the enrichment culture grown with acetate and chlorate and phenol and chlorate (Fig. 3, lanes 14 and 16) and therefore it is likely that this bacterium is able to use chlorate as the electron acceptor. Moreover, the bacterium was present in the enrichment culture grown with benzene and oxygen (Fig. 3, lane 15). Although these results are not yet direct evidence, they indicated that this bacterium is able to grow with benzene coupled to chlorate reduction. Our recent studies confirmed this.

The bacterium corresponding to clone cA8 is closely related to the phenol-degrading nitrate-reducing bacterium *Acidovorax avenae* isolate C1 (Baek *et al*., 2003) and the cyclohexanol-degrading nitrate-reducing *Alicycliphilus denitrificans* K601 (Mechichi *et al*., 2003). Both bacteria can reduce nitrate in the presence of oxygen. It has not reported whether these bacteria can use chlorate as the electron acceptor and benzene as the electron donor. There are members of the *Acidovorax* genus that have been associated with the aerobic degradation of benzene (Nicholson and Fathepure, 2005; Fahy *et al*., 2006). Moreover, phenol and cyclohexanone (as the oxidation product of cyclohexanol) are possible intermediates of the anaerobic benzene biodegradation pathway (Coates *et al*., 2002).

Clone cB3 is closely related to *Mesorhizobium* sp. WG, an acetate-degrading nitrate-reducing bacterium (Costa *et al*., 2000). The bacterium corresponding to clone cB3 dominated the enrichment culture grown on benzene and oxygen (5% in gas phase) (Fig. 3, lane 15). This suggests that this bacterium is able to degrade benzene with oxygen. *Mesorhizobium* sp. WG is able to grow under microaerophilic conditions and in fact, it grew better under these conditions than under normal aerobic conditions (Costa *et al*., 2000). The *Mesorhizobium* sp. WG like bacterium does not seem to reduce chlorate, because its dominance decreased when the culture was grown with acetate and chlorate (Fig. 3, lane 14). Therefore, this bacterium could be dependent on oxygen provided by chlorate-reducing bacteria in the enrichment culture.

Clone cG10 has high sequence similarity with the strictly aerobic bacterium *Stenotrophomonas acidaminiphila*, isolated from an anaerobic reactor (Assih *et al*., 2002). It also has high sequence similarity with the facultative anaerobe *Stenotrophomonas.* sp. BO, isolated from the same reactor as the *Mesorhizobium* sp. WG. *Stenotrophomonas* sp. BO grows well under microaerophilic conditions (Costa *et al*., 2000). The bacterium corresponding to clone cG10 was present in the enrichment culture grown on acetate and chlorate (Fig. 3, lane 14), but seems absent when grown on benzene and oxygen (Fig. 3, lane 15). Based on these results, the bacterium seems to grow by chlorate reduction, but not by benzene degradation. In our enrichment culture, this bacterium could be dependent on other bacteria to provide electron donors for chlorate reduction.

Clone cA5 has high sequence similarity with *Zoogloea resiniphila* PIV-3A2w. *Zoogloea resiniphila* PIV-3A2w degrades pivilate (2,2-dimethylpropionate) under nitrate-reducing conditions (Probian *et al*., 2003). Due to the quaternary carbon atom of pivilate, this compound is relatively difficult to degrade anaerobically. The function of the bacterium corresponding to clone cA5 is not clear. It seems that this bacterium is not able to degrade benzene aerobically, because it is not present in the benzene and oxygen grown enrichment culture (Fig. 3, lane 15). The 16S rRNA gene sequence of clone cA5 is closest related to known (per)chlorate-reducing bacteria (Fig. 4). However, the *Zoogloea resiniphila* like bacterium seems not able to grow by chlorate reduction, because it seems absent in the

enrichment culture grown on acetate and chlorate (Fig. 3 lane 14) and phenol and chlorate (Fig. 3, lane 16). The presence of this bacterium decreased when FYE was added to the enrichment culture (Fig. 3, lanes 12 and 13). It might be that this bacterium provides an essential growth factor to the other bacteria that was replaced by FYE.
During chlorate reduction, oxygen is produced. Therefore, it is likely that benzene was degraded via an aerobic degradation pathway in our enrichment culture. In addition, the benzene degradation rate in our enrichment culture (1.65 mM day^{-1}) was similar to reported aerobic degradation rates (Reardon *et al*., 2000). The experiments with possible intermediates of benzene biodegradation also provided indications that benzene was degraded via an aerobic pathway in our enrichment culture. Phenol and catechol were readily degraded and did not inhibit benzene degradation. By contrast, benzoate and toluene were only slowly degraded and especially benzoate inhibited benzene degradation. Therefore, an initial conversion of benzene to either phenol or catechol by this enrichment culture is most likely. Phenol and catechol are both intermediates in the aerobic benzene degradation pathway, whereas phenol can be an intermediate in an anaerobic benzene degradation pathway as well (Coates *et al*., 2002; Chakraborty and Coates, 2005; Ulrich *et al*., 2005). Aerobic benzene degradation can be performed by monooxygenases producing phenol or by dioxygenases producing catechol as intermediate (Butler and Mason, 1997; Yerushalmi *et al*., 2001). Phenol can subsequently be converted by a phenol monooxygenase to catechol. Under anaerobic conditions, phenol can be converted to hydroxybenzoate and subsequently to benzoate (Chakraborty and Coates, 2005).

In this study, a stable anoxic enrichment culture was obtained that degraded benzene with chlorate as the electron acceptor. The culture enriched in mineral medium with benzene and chlorate as sole substrates contained at least four different bacterial species. Our results show clearly that other types of bacteria than *Dechloromonas* can be involved in the degradation of benzene with chlorate. Interestingly, our stable enrichment culture degraded benzene much faster than the pure culture of *Dechloromonas* strain RCB. Two benzene-degrading denitrifying *Azoarcus* strains were described recently (Kasai *et al*., 2006). It is not known if these *Azoarcus* strains are able to couple benzene degradation to chlorate reduction. None of the species most related to members in our enrichment culture, has ever been associated with the ability to reduce chlorate or to oxidize benzene, neither anaerobically nor aerobically, indicating new physiological features of these types of bacteria. Our results indicated that the bacterium related to *Alicycliphilus denitrificans* K601 is able to degrade benzene coupled to chlorate reduction. Our recent studies confirmed this. The role of the other bacteria present in the enrichment culture could not be conclusively determined from our results. However, as one of these bacteria seems able to oxidize benzene aerobically, but not able to reduce chlorate and another bacterium seems able to reduce chlorate but not oxidize benzene, cross feeding involving interspecies oxygen transfer is a likely mechanism. This is under present investigation.

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5

Isolation and characterization of *Alicycliphilus denitrificans* strain BC, which grows on benzene with chlorate as the electron acceptor

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Abstract

A bacterium, strain BC, was isolated from a benzene-degrading chlorate-reducing enrichment culture. Strain BC degrades benzene in conjunction with chlorate reduction. Cells of strain BC were short rods, 0.6 µm wide and 1–2 µm long, motile and stained Gram-negative. Strain BC grew on benzene and some other aromatic compounds with oxygen, or in the absence of oxygen with chlorate as the electron acceptor. Strain BC is a denitrifying bacterium, but it is not able to grow on benzene with nitrate. The closest cultured relative was *Alicycliphilus denitrificans* K601 (type strain), a cyclohexanol-degrading nitrate-reducing *Betaproteobacterium*. Chlorate reductase (0.4 U/mg protein) and chlorite dismutase (5.7 U/mg protein) activities were determined in cell extracts of strain BC. Gene sequences encoding known chlorite dismutase (*cld*) were not detected in strain BC by applying the PCR primers reported in previous studies. As physiological and biochemical data indicated oxygenation of benzene during growth with chlorate, a strategy was developed to detect genes of monooxygenase and dioxygenase enzymes potentially involved in benzene degradation in strain BC. Using primer sets designed to amplify distinct evolutionary branches in the catabolic families involved in benzene biodegradation, two oxygenase genes putatively encoding the enzymes performing the initial successive monooxygenations (*BC-BMOa*) and the cleavage of catechol (*BC-C23O*), were detected. Our findings suggest that oxygen formed by dismutation of chlorite can be used to attack organic molecules by means of oxygenases as exemplified with benzene. Thus, aerobic pathways can be employed under conditions where no external oxygen is supplied.

Introduction

Contamination of the groundwater with perchlorate (ClO_4^-) and chlorate (ClO_3^-) has been observed at many places especially throughout the US (Urbansky, 1998). The presence of perchlorate in the environment has primarily been associated with manufacturing, handling and dismantling of munitions, being used as a major component of rocket propellants and explosives. (Per)chlorate contamination poses a significant health threat as toxicological studies have demonstrated (Achenbach *et al*., 2001). Research during the last decade has resulted in the isolation of various (per)chlorate-reducing bacteria and the characterization of the key enzymes in those isolates. Perchlorate and chlorate are ideal electron acceptors for microorganisms due to their high redox potential (ClO_4^-/Cl^- E^0 = 1.287 V; ClO_3^-/Cl^- E^0 = 1.03 V) (Coates and Achenbach, 2004). (Per)chlorate reducing microorganisms reduce (per)chlorate to chloride. Typically, during (per)chlorate reduction the intermediate chlorite is dismutated to molecular oxygen and chloride (Rikken *et al*., 1996; Van Ginkel *et al*., 1996; Logan, 1998). This means that (per)chlorate-reducing microorganisms can produce oxygen of metabolic origin in anaerobic environments. The formation of oxygen during (per)chlorate reduction may result in rapid oxidation of compounds which are slowly degraded under anaerobic conditions (Coates *et al*., 1999a).

An example of such a compound is benzene. Generally, benzene is rapidly degraded by aerobic microorganisms with the appropriate catabolic potential, but in anaerobic environments biodegradation is much slower (Lovley, 2000). Anaerobic biodegradation of benzene under various redox conditions has been described, but only in a few studies the microorganisms involved were identified (Phelps *et al*., 1998; Rooney-Varga *et al*., 1999; Ulrich and Edwards, 2003; Chang *et al*., 2005; Kasai *et al*., 2006). Up to now, only four anaerobic benzene-degrading bacteria have been described; two *Dechloromonas* strains (RCB and JJ) that degrade benzene in conjunction with (per)chlorate (strain RCB only), nitrate or oxygen reduction (Chakraborty *et al*., 2005) and two denitrifying *Azoarcus* strains (DN11 and AN9) (Kasai *et al*., 2006). The optimal physiological conditions for anaerobic benzene-degrading bacteria and the biodegradation pathways are still largely unclear (Chakraborty and Coates,

2005; Ulrich *et al*., 2005; Botton and Parsons, 2007; Musat and Widdel, 2007; Fischer *et al*., 2008; Kunapuli *et al*., 2008).

The relatively high solubility of benzene, toluene, ethylbenzene and xylene (the so-called BTEX compounds), and low solubility of oxygen often results in BTEX contamination in anoxic zones of the environment. Anaerobic bioremediation is attractive when anaerobic conditions prevail at a polluted soil site. The potential use of (per)chlorate-reducing microorganisms for bioremediation (of soils and sediments) has been recognized in previous studies (Coates *et al*., 1999a; Logan and Wu, 2002). It was demonstrated that amendment of (per)chlorate-reducing microorganism and chlorite to an anoxic soil led to complete degradation of ^{14}C-benzene to ^{14}C-carbon dioxide (Coates *et al*., 1999a). Toluene degradation was observed in sand columns inoculated with toluene-degrading and chlorate-reducing enrichment cultures (Logan and Wu, 2002). In another study, addition of chlorate to a soil column polluted with benzene showed removal of benzene in conjunction with chlorate reduction (Tan *et al*., 2006). Recently, we obtained a highly active benzene-degrading chlorate-reducing enrichment culture with mixed material from a wastewater treatment plant and soil samples, and we analyzed which phylogenetic groups of bacteria were present (Weelink *et al*., 2007). Here, we describe the isolation of strain BC from this enrichment. This bacterium is capable of growth on benzene with chlorate as the electron acceptor. Our data suggest that oxygen produced in the dismutation of chlorite is used to degrade benzene, and oxygenase systems potentially involved have been identified.

Materials and Methods

Inoculum, cultivation and isolation procedures

Strain BC was isolated from a stable benzene-degrading chlorate-reducing enrichment culture. Isolation and cultivation of strain BC was performed in strictly anaerobic AW-1-sulfate medium (Weelink *et al*., 2007). Cultivations were done at 30°C, in the dark in an orbital shaker (50 rpm), in 120-ml serum bottles containing 40 ml medium. Soluble electron donors and acceptors were added from sterile, anaerobic stock solutions. Oxygen was added to the gas phase from a sterile 100% oxygen gas stock. Benzene was added from a water-saturated stock solution (20 mM) and was re-added to batches when benzene was depleted. Chlorate was added from a 0.4 M $NaClO_3$ stock solution. Where indicated, fermented yeast extract (FYE; 0.125 g/l) was added to the enrichment as a nutrient supplement to stimulate growth (Holliger *et al*., 1993; Van Doesburg *et al*., 2005; Weelink *et al*., 2007).

For strain isolation, dilution series of the stable enrichment culture were made in AW-1-sulfate medium, with benzene (0.25 mM) and chlorate (10 mM) as energy substrates, 0.125 g/L FYE as nutrient supplement, and 1.2% agar (Agar noble, Difco, Becton Dickinson Microbiology Systems, Sparks, USA) to solidify the medium. Colonies of the highest dilutions were picked and transferred to new agar dilution series. This procedure was repeated four times. Purity was checked by microscopic observation of cultures grown on benzene and easily degradable substrates (e.g. yeast extract + acetate) Furthermore, denaturing gradient gel electrophoresis (DGGE) was used to confirm purity of these cultures. Strain BC was routinely grown with benzene (0.25 or 0.5 mM) and chlorate (10 mM).

The Gram type was determined using Gram staining and electron microscopy as previously described (Plugge *et al*., 2000). Phase contrast micrographs were made with a Leica (Wetzlar, Germany) DMR HC microscope equipped with a Leica DC 250 digital camera. The Leica QWin computer programme was used to digitally make the micrographs.

Physiological studies

All growth parameters of strain BC were determined in either duplicate or triplicate batches in AW-1-sulfate medium (without the addition of fermented yeast extract). When applicable, all electron donors and electron acceptors were added as sodium salts. The growth rate of strain BC was determined by

measuring the increase of OD_{600} and/or cell numbers with time in triplicate batches. Cell numbers were enumerated by phase-contrast microscopy using a Bürker-Türk counting chamber at 1000× magnification. Cell yields were determined by measuring dry-weight of the biomass (in 200 mL cultures). The dry-weight was determined gravimetrically after the cell pellet was dried at 105°C overnight. The pH optimum was determined with acetate (10 mM) and nitrate (10 mM) at 30°C using a pH range of 6.6-9.0 in duplicate batches. Different pH values of the medium were obtained by changing the percentage of CO_2 in the headspace, while the bicarbonate concentration of the medium was kept constant as described before (Wolterink *et al*., 2002). The temperature optimum was determined with acetate (10 mM) and nitrate (10 mM) in the range of 4-55°C in duplicate batches.
To determine the substrate spectrum of strain BC the following electron donors were tested in duplicate batches with nitrate (10 mM) as the electron acceptor: acetate, lactate, pyruvate, succinate, propionate, butyrate, malate, citrate, fumarate, glucose, fructose, xylose, alanine, glycine, glutamate, ethanol, methanol, glycerol (all 10 mM), $Fe(II)Cl_2$ (5 mM), Na_2S (1 mM), H_2 (170 kPa, with the addition of 1 mM acetate) and yeast extract (1 g/l). Late log-phase cells of strain BC grown on acetate (10 mM) and nitrate (10 mM) were used as inoculum (5%) for this experiment. The following electron donors were tested in duplicate batches with either nitrate (10 mM) or oxygen (5% in headspace): benzene (0.25 mM), toluene (0.25 mM), ethylbenzene, *o*-xylene, *m*-xylene, *p*-xylene (all 0.10 mM), monochlorobenzene (0.05 mM), benzoate, phenol, cyclohexanol, *p*-hydroxybenzoate, *o*-cresol, *m*-cresol, *p*-cresol and catechol (all 1 mM). Late log-phase cells of strain BC grown on either acetate (10 mM) and nitrate (10 mM) or benzene (repeated feeds of 0.5 mM) and oxygen (5% in headspace) were used as inoculum (5%) for this experiment. Furthermore, besides benzene the following electron donors were tested with chlorate (10 mM) as the electron acceptor: acetate (10 mM), toluene (0.25 mM), phenol, *o*-cresol, *m*-cresol, *p*-cresol and catechol (all 1 mM). Late log-phase cells of strain BC grown on benzene (repeated feeds of 0.5 mM) and chlorate (10 mM) were used as inoculum (5%) for this experiment. Growth was monitored by visual observation of turbidity and the decrease in nitrate or chlorate concentration, respectively.
The following electron acceptors (10 mM, exceptions in parentheses) were tested in duplicate batches with acetate (10 mM) as the electron donor: oxygen (10% in headspace), perchlorate, chlorate, nitrate, nitrite (5 mM), sulfate, sulfite, thiosulfate, fumarate, manganese(IV)oxide (20 mmol/l), iron(III)pyrophosphate, iron(III)NTA (NTA stands for nitrilotriacetic acid), AQDS (anthraquinone-2,6-disulfonate, 4 mM), bromate (5 and 10 mM), selenate (5 and 10 mM), arsenate (5 and 10 mM). Electron acceptor use was visually monitored and by measuring the acetate concentration and the electron acceptor decrease as well in case the acetate concentration decreased.

Analytical procedures
Benzene was measured by headspace analysis using a gas chromatograph as described previously (Weelink *et al*., 2007). Anions (chlorate, chloride and nitrate) were determined by high-pressure liquid chromatography (HPLC) as described before (Scholten and Stams, 1995). Oxygen in the headspace of batches was measured with a gas chromatograph as described previously (Stams *et al*., 1993). Catechol, benzoate and phenol were analyzed by HPLC equipped with a Chrompack column, Chromspher 5 pesticides (100 mm × 3 mm). The mobile phase consisted of different ratios of 0.1% trifluoro acetic acid (TFA) and 50% acetonitrile + 50% 0.1% TFA at a flow rate of 0.6 ml/min. The detector was a Spectra System UV1000.

Molecular biological techniques
A bead-beating and phenol-chloroform based DNA extraction method was used to extract DNA from pure cultures of strain BC (Van Doesburg *et al*., 2005)]. Amplification with the primers 7f and 1492, purification and sequencing of the 16S rRNA genes was previously described (Van Doesburg *et al*.,

2005). Part of the 16S rRNA gene (424 bp) of strain BC was analyzed and revealed 100% similarity with the 16S rRNA gene sequence of clone cA8 (1487 bp) obtained from the enrichment (Weelink *et al*., 2007). In sequence analysis studies, the 16S rRNA gene sequence fragment of 1487 bp was used. Sequences of 16S rRNA genes were compared with sequences deposited in publicly accessible databases using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al*., 1990; McGinnis and Madden, 2004). DNA from strain BC was used as PCR template for denaturing gradient gel electrophoresis (DGGE) as described previously (Weelink *et al*., 2007). Silver staining and development of the gels were performed according to the method of (Sanguinetti *et al*., 1994). A phylogenetic tree of partial 16S rRNA gene sequences of strain BC and related bacteria was constructed. Alignment and phylogenetic analysis were performed with the ARB software and the tree was constructed using the neighbour joining method based on *E. coli* positions 101 - 1221, and a 50% conservation filter for *Betaproteobacteria*, as implemented in ARB. The GenBank database accession numbers of strain BC and *Alicycliphilus denitrificans* strain K601 are DQ342277 and AJ418042, respectively. Strain BC has been deposited in two different collection of microorganisms, the German Collection of Microorganisms and Cell cultures – DSMZ (Braunschweig, Germany), strain number DSM 18852 and Japanese Collection of Microorganisms - JCM (Riken BioResource Center, Japan), strain number JCM 14587.

To detect chlorite dismutase genes (*cld*) gene sequences, we used the conditions and primers UCD-238F/UCD-646R and DCD-F/DCD-R reported elsewhere (Bender *et al*., 2004). Additionally we tested modified annealing temperatures in a range of +/- 10 degrees. DNA from *Pseudomonas chloritidismutans* isolated in our laboratory was used as a positive control (Wolterink *et al*., 2002).

To detect the benzene and catechol oxygenase genes present in strain BC, we designed the following primers sets targeting three different evolutionary clusters around I.2.A and I.2.B sequence spaces of the Extradiol Dioxygenases (EXDO) Type I Family (Eltis and Bolin, 1996) in a upgraded (2006) database of related sequences (all primer sequences in 5'-3' direction): EXDO-A-F (forward) ATG AAV AAA GGH GTW HTG CGH CCN GG together with reverse primer EXDO-A-R1 GYG GCC ADG CYT CNG GRT TG (expected product size ~430bp) or EXDO-A-R2 ATR TCV AKV GAD GTR TCG STC ATG (expected product size ~730bp); EXDO-B-F (forward) TRA CMG GHG TNH TGC GYC CVG GSC A and EXDO-B-R (reverse) GCC RTG VCG SGT BGG VCC GAT, expected to produce a PCR fragment of ~750bp; EXDO-C-F (forward) CAYTAYCGYGACCGKATYGG with primers EXDO-C-R1: TCR TCA TGB GCY TTR TTG CTG CA (expected product size ~530bp) or EXDO-C-R2 TCG TTS CGR TTD CCS GAV GGR TCG AAG AA (~710bp).

To detect genes encoding the large subunit of the four-component alkene/aromatic monooxygenases and phenol hydroxylases/toluene monooxygenases (ring hydroxylating monooxygenases, RHMO) members (RHMO-TMOPHE) of the soluble diiron monooxygenase family (Leahy *et al*., 2003), the following primers were designed: RHMO-TMOPHE-F (forward): GAY CCB TTY CGY HTR ACC ATG GA and RHMO-TMOPHE-R (reverse): GGC ARC ATG TAR TCC WKC ATC AT. The expected amplification products size is ~701 bp. For the amplification of four-component aromatic monooxygenase large subunits, mainly comprising toluene/benzene monooxygenases (RHMO-T/BMO), the primers used were: RHMO-T/BMO-F (forward): ASR AAC TGC ATR TTG GTR AAR CC and RHMO-T/BMO-R (reverse): GAR TAC GTS MGB RTY CAR CGX GAR AAG GA, annealing from positions 169 to 617 in *P. mendocina* KR1 tmoA coding DNA sequence, and producing an expected PCR product size of 448bp.

Common PCR conditions used on amplifications screening for the presence of the oxygenase genes targeted (described above) were as follows (in a final PCR reaction volume of 50 µl): 1X colorless GoTaq reaction buffer (Promega, Madison, WI, USA), 5 units of GoTaq Polymerase (Promega, Madison, WI, USA), 200 µM of dNTPs (Fermentas), and 10 pmol of each primer (synthesised by Invitrogen GmbH, Karlsruhe, Germany). For thermal cycling, an Eppendorf gradient themocycler was

used as follows: one initial denaturation step at 94 °C for 1 minute, 35 cycles consisting on three steps of 45 seconds at 94°C for, 45 seconds at 50°C, 55°C or 60°C, and 1 minute at 72 °C, followed by one final elongation step at 72°C for 7 minutes. Polymerization reactions were stopped by cooling down the samples at 4°C. Reactions were further analysed on TAE 1X 1% agarose gel electrophoresis to assess presence of PCR products and detection of the expected sizes. For DNA sequencing, the PCR product was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) when a single size was observed. When the PCR products were composed of different sizes, the product size matching the expected fragment size was excised from the agarose gel and purified using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) and this purified size was then subjected to direct sequencing reactions. DNA sequencing was performed according to the manufacturer's instructions using 600 ng of the purified PCR products as DNA templates in two independent sequencing reactions using the same primers used for the original PCR amplification using the BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequence fragments were detected in a DNA capillary sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The corresponding sequence chromatogram reads were assembled using Sequencher software version 4.0 (Genes Codes Corporation, Ann Arbor, MI, USA) and the assembled contigs were exported as text files. The sequence texts were oriented to be on the same direction 5'-3' of the coding DNA sequence.

For each sequence, the conceptual translation of a peptide on one frame not producing stop codons was confirmed using standard and bacterial translation codes with GeneDoc multiple sequence alignment editor software (Nicholas *et al*., 1997). DNA sequences were used on blast searches using tblastx option against the non redundant database, in order to confirm if they belong to the gene family targeted, observed as highest scores retrieved against the known family members expected. Additionally, for each sequence was determined if the conserved protein domain on the family targeted was maintained in the span analysed. The putative protein sequences obtained from strain BC were used to predict a model of protein structure using the resolved crystal structure with higher identity as template, and the modelling software $(PS)^2$: Protein Structure Prediction Server using RAMP model building (Chen *et al*., 2006). These amino acid sequences were also, later on, aligned against the translated multiple sequence alignments of their corresponding families used for primer design using Clustal W using default values (Thompson *et al*., 1994). A block of protein sequence alignments were selected with GeneDoc software (Nicholas *et al*., 1997). This block of sequences was aligned again and Neighbour-Joining trees were calculated together with the generation of bootstrapped values of 1000 trials using the functions implemented inside Clustal W. For minor modifications in sequence alignments or N-J trees presentation, vectorial representations were imported to Openoffice suite 2.0.4 (OpenOffice, URL: http://www.openoffice.org/) from the alignment graphical view available on BioEdit Software BioEdit (BioEdit. URL:http://www.mbio.ncsu.edu/) or from the graphical display of N-J tree files available on MEGA 3.1 software (Kumar *et al*., 2004) selecting rooting on midpoint and arranging taxa for balanced shape. The DNA sequences obtained in this study are available under the EMBL/ GenBank/ DBBJ accession numbers EF596778 (670 bp, putative benzene monooxygenase large subunit (*BC-BMOa*) coding gene of *Alicycliphilus* sp. strain BC) and EF596779 (707 bp, putative catechol 2,3-dioxygenase (*BC-C23O*) coding gene of *Alicycliphilus* sp. strain BC).

Preparation of cell extracts and enzyme activity measurements

For the preparation of cell extract, strain BC was grown in AW-1-sulfate medium (200 mL) with benzene and chlorate and with benzene and oxygen. Cell extracts were prepared as described by (Wolterink *et al*., 2002) with the modification that centrifugation was done at 13,000 rpm for 30 minutes at 4^oC. Chlorate reductase and chlorite dismutase activity were determined in cell extracts of strain

BC. Chlorate reductase was determined spectrophotometrically as described previously by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm and 30°C (Kengen *et al*., 1999). One unit (U) of activity is defined as the amount of enzyme required to reduce 1 µmol of chlorate per min. The chlorite dismutase activity was determined by measuring the oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA) as described previously (Wolterink *et al*., 2002)]. One unit (U) of activity is defined as the amount of enzyme required to convert 1 µmol of chlorite per min. The protein content of the cell extract was determined according to the method of Bradford, with bovine serum albumin (BSA) as standard (Bradford, 1976).

Results

Isolation

A stable enrichment culture that degraded benzene with chlorate as the electron acceptor was obtained previously (Weelink *et al*., 2007). DGGE of 16S rRNA gene fragments followed by cloning and sequencing showed that at least four different bacterial species were numerically dominant in the culture. Initially, isolation of a benzene-degrading chlorate-reducing bacterium from this enrichment was not successful. At a later stage, 0.125 g/L fermented yeast extract was added to the enrichment as a nutrient supplement to stimulate growth. This resulted in a decreased lag-phase for benzene degradation (Weelink *et al*., 2007). Subsequently, a pure culture of a benzene-degrading chlorate-reducing bacterium, strain BC, was successfully isolated by applying serial dilution techniques in agar (1.2%) and liquid medium (Wolterink *et al*., 2002). Benzene (250 µM) and chlorate (10 mM) were added as the electron donor and acceptor, respectively, and fermented yeast extract (FYE, 0.125 g/L) as nutrient supplement. Within 3-4 weeks, colonies appeared in the agar dilutions. The colonies were round, lens-shaped, 0.5-1.0 mm in diameter and brown. Colonies were picked from the highest dilution with a sterile needle and directly transferred to new agar (and liquid) dilution series. This procedure was repeated until a pure culture was obtained. Purity of the culture was confirmed by microscopic observation and DGGE analysis of cultures grown on benzene and easily degradable substrates (e.g. yeast extract + acetate). Identical morphologies and patterns were observed in these cultures by using microscopy and DGGE, respectively. Cells of strain BC were short rods, 0.6 µm wide and 1–2 µm long (Fig. 1) were motile and stained Gram-negative. Flocculated growth of cells of strain BC was observed especially during growth with chlorate as the electron acceptor, and to a lesser extent during growth with nitrate or oxygen as the electron acceptor. Growth of strain BC occurred in a pH range of 6.6 till 9.0, with an optimum at pH 7.3. The optimal temperature for growth was 30-37°C with lower and upper limits of about 15 and 40°C.

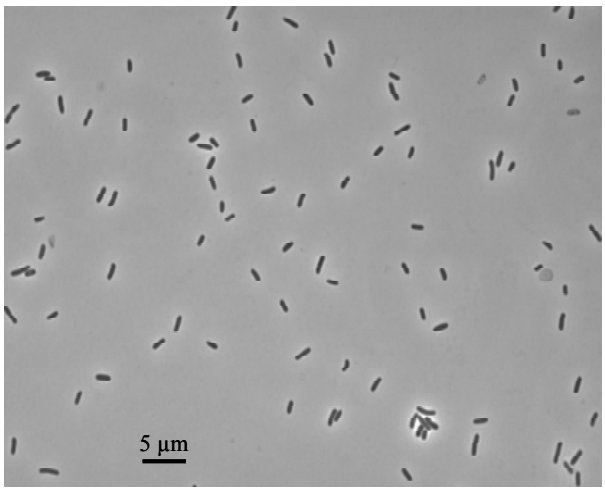

Figure 1. Phase-contrast micrograph of cells of strain BC grown on benzene and chlorate, 1,000× magnification.

Phylogenetic characterization

16S rRNA gene sequence analysis showed that strain BC is closely related to *Acidovorax avenae* isolate C1 and *Alicycliphilus denitrificans* K601 (type strain), with levels of similarity of 99.9 and 99.7%, respectively (Baek *et al*., 2003; Mechichi *et al*., 2003). Strain BC had only 96% 16S rRNA gene sequence similarity with *Acidovorax avenae* subsp. a*venae* (DSM 7227^T) (Willems *et al*., 1992). Therefore, we consider *Alicycliphilus denitrificans* K601 as the closest cultured relative of strain BC and in further physiological and (chemo)taxonomical characterization *Alicycliphilus denitrificans* K601 was included as reference strain. Here, only some key physiological and (chemo)taxonomical characteristics of the two strains will be presented (a more extensive physiological characterization of the two strains is presented in chapter 6).

Strain BC clusters within the family *Comamonadaceae* in the *Beta*proteobacteria (Willems *et al*., 1991) (Fig. 2). This family comprises several genera, including e.g. *Acidovorax*, *Comamonas*, *Delftia*, *Hydrogenophaga*, *Rhodoferax*, *Brachymonas*, *Polaromonas*, *Variovorax*, *Xylophilus* (Wen *et al*., 1999), *Xenophilus* (Blümel *et al*., 2001). Members of this phenotypically heterogeneous family are phylogenetically closely related to strain BC. Species from the genera *Dechloromonas* and *Azospira* (formerly *Dechlorosoma*) of the beta-subclass of the *Proteobacteria* were also included in the phylogenetic tree (Fig. 2), because (per)chlorate-reducing bacteria in the environment are predominantly members of the *Dechloromonas* and *Azospira* genera. The *Dechloromonas* species capable of degrading benzene anaerobically, i.e. *Dechloromonas* spp. strains RCB and JJ, are also included in the phylogenetic tree. They both have a 91% similarity with strain BC based on 16S rRNA gene sequences, while *Azoarcus* spp. strains AN9 and DN11 capable of degrading benzene with nitrate, are more distantly related with only 88% (AN9) and 87% (DN11) 16S rRNA gene sequence similarity with strain BC, respectively.

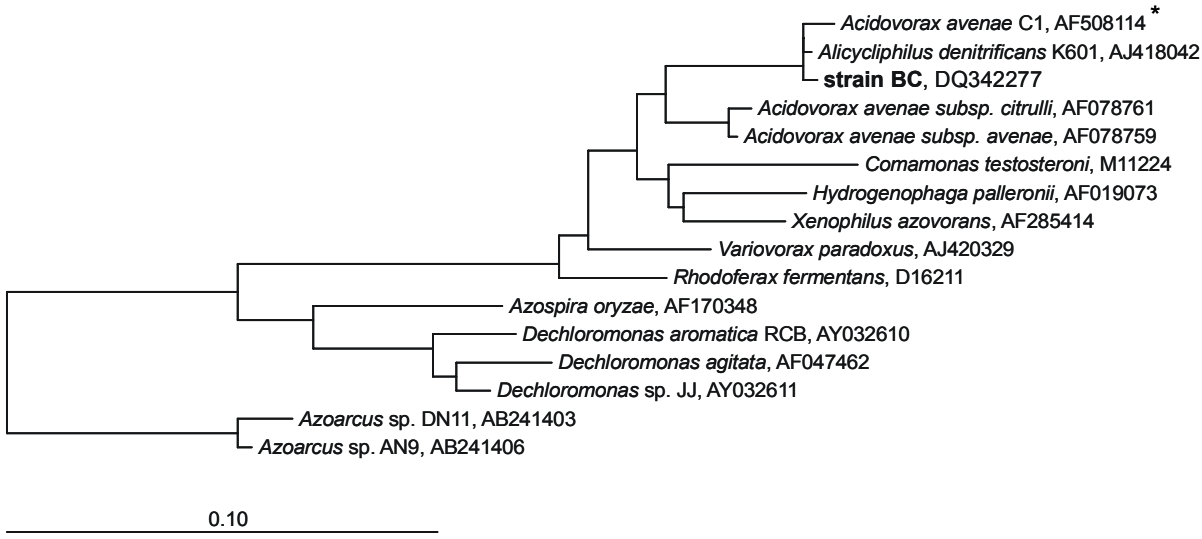

Figure 2. Phylogenetic tree of partial 16S rRNA gene sequences displaying the relationships between strain BC and some other members of the family *Comamonadaceae* in the *Betaproteobacteria*. Alignment and phylogenetic analysis were performed with the ARB software and the tree was constructed using the neighbour joining method based on *E.coli* positions 101-1221, and a 50% conservation filter for *Betaproteobacteria*, as implemented in ARB. The reference bar indicates the branch length that represents 10% estimated sequence divergence. GenBank accession numbers of reference sequences and clones are given. * Means that this bacterium is not officially classified as *Acidovorax avenae* strain. It seems likely that it is misnamed, because it has higher similarity with *Alicycliphilus denitrificans* K601 than with the type strain *Acidovorax avenae* subsp. a*venae* (AF078759).

Benzene degradation with chlorate

Strain BC grows on benzene with chlorate as the electron acceptor as indicated by the increase in cell numbers and the OD_{600} (Fig. 3A and 3B). Cell number and OD_{600} increase coincided with benzene degradation. During chlorate reduction, chloride was produced. During incubation the redox indicator, resazurin, in the medium turned pink, due to the increased redox potential, resulting from chlorate metabolism. When all chlorate was consumed, the medium turned colorless again (after 11 days). No benzene degradation was observed in controls without inoculum or without chlorate; no chlorate reduction was observed in controls without inoculum or without benzene; no growth was observed in controls without benzene or chlorate (results not shown). The specific growth rate (μ) of strain BC on benzene and chlorate was 0.48 day^{-1} (doubling time 1.4 days). In this experiment, two additional spikes of 0.5 mM benzene were supplied to the culture (Fig. 3A). Benzene concentrations up to 1 mM (highest concentration tested) were degraded by strain BC. The expected stoichiometry of complete oxidation of benzene in conjunction with chlorate reduction, without taking into account the production of biomass is:

$$C_6H_6 + 5\ ClO_3^- + 3\ H_2O \rightarrow 6\ HCO_3^- + 5\ Cl^- + 6\ H^+ \quad (1)$$

The experimentally measured ratio of total amount of chlorate reduced and benzene degraded was 2.8. This is lower than the expected ratio of 5 according to reaction 1, but this reaction does not take into account the production of biomass. A fairly good chlorine balance was obtained; 80-90% of the chlorine of chlorate was recovered as chloride, indicating that no or only very low amounts of other chlorine compounds are formed. The growth yield of strain BC (based on dry-weight) determined in two cultures grown on benzene plus chlorate was 14.2-25.8 g biomass per mol of benzene degraded (Table 1). As our strain shows flocculent growth on chlorate, this may have resulted in an underestimation of the growth yield due to difficulties in concentrating the cells in the dry-weight method. To compare the biomass yield with the amount of benzene degraded and chlorate reduced, the f_e (fraction of electrons from benzene used for energy production) and f_s (fraction of electrons from benzene used for cell synthesis) electron equivalent mass balance approach was used as described previously (Rittmann and McCarty, 2001). The cumulative amount of benzene degraded and the amount of chlorate reduced were used to calculate the f_s. This calculation gave an f_s value of about 0.39 (average of 0.34 and 0.43, Table 1), corresponding to the following overall balanced equation:

$$C_6H_6 + 3.05\ ClO_3^- + 2.34\ CO_2 + 0.59\ NH_4^+ + 3.59\ H_2O \rightarrow$$
$$0.59\ <C_5H_7O_2N> + 5.42\ HCO_3^- + 3.05\ Cl^- + 6\ H^+ \quad (2)$$

According to this approach, the biomass ($C_5H_7O_2N$) yield would be 57.6-73.5 g biomass per mol benzene, which is about 3 times higher than the actual measured yield. In active cultures of strain BC with chlorate as acceptor the benzene degradation rate was about 0.35 μmol per h per mg dry biomass.

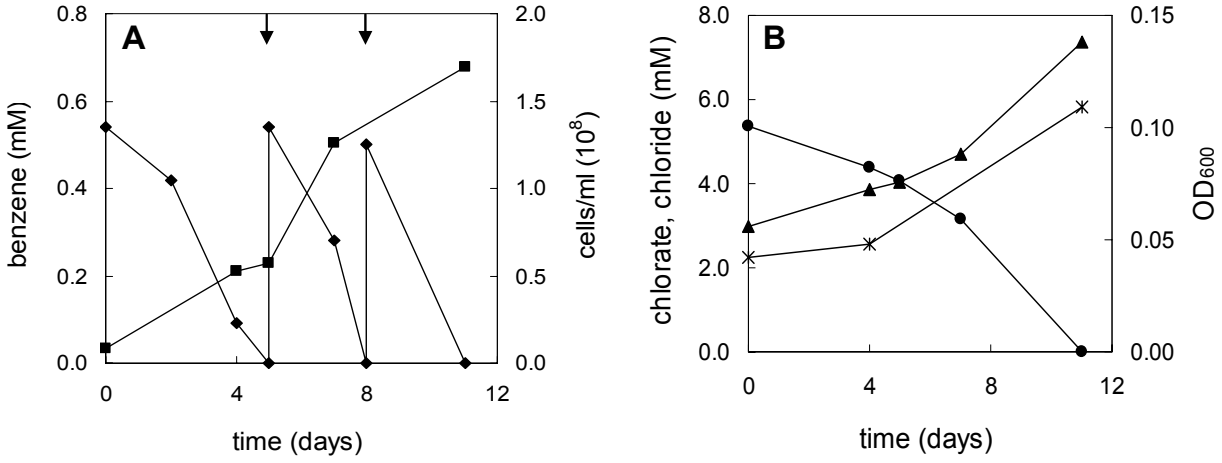

Figure 3. Anaerobic benzene degradation by strain BC with chlorate (5 mM) as the electron acceptor.
A. Benzene (♦) concentration (mM) and cells/ml (■) in time.
B. Chlorate (●), chloride (▲) concentration (mM) and OD_{600} (∗) in time.
Arrows indicate re-addition of benzene.

Table 1. Growth yields based on biomass determination and based on the f_e and f_s approach (Rittmann and McCarty, 2001).

Culture code	Benzene degraded (mM)	Chlorate reduced (mM)	Biomass produced (mg/l)	Yield (g cells/mol benzene)	f_s	Yield based on f_s, theoretical (g cells/mol benzene)
Exp. 1	2.0	6.6	52.2	25.8	0.34	57.6
Exp. 2	3.3	9.4	46.7	14.2	0.43	73.5

Strain BC was also able to grow on benzene with oxygen as the electron acceptor. Different initial concentrations of oxygen were tested, i.e. 2.5, 5 and 20% oxygen in the headspace, corresponding to nominal concentrations of 2, 4 and 16 mmoles oxygen per liter, respectively (volume of the culture and headspace were 40 mL and 80 mL, respectively). Benzene degradation coincided with oxygen consumption and an increase in OD_{600}. Benzene degradation rates in active aerobic cultures were similar to the rates with chlorate and not influenced by the initial oxygen concentration (results not shown). The specific growth rate (μ) of strain BC on benzene and oxygen was 0.40 day^{-1} (doubling time about 1.7 days).
Strain BC was isolated in the presence of FYE, but in further transfers this bacterium grew well on benzene with chlorate in media without FYE. However, difficulties with subcultivation on benzene and chlorate occurred when out-grown cultures were stored unfed for more than 2 days. Benzene degradation ceased when benzene was not re-added within 1-2 days after the benzene was depleted. However, the addition of small amounts of acetate (0.5 mM) resulted in a recovery of the benzene-degrading activity (results not shown). When the cultures were degrading benzene again, benzene degradation could be sustained without addition of acetate.

Electron donors and acceptors

All carboxylic acids tested were used by strain BC as the sole electron donor for growth, i.e. acetate, lactate, pyruvate, succinate, propionate, butyrate, malate, citrate and fumarate (Table 2). With carboxylic acids as electron donors (10 mM), growth started within a few days and all nitrate (10 mM) was consumed within one week. Glycerol and yeast extract were growth substrates as well for strain BC. Glutamate was used by strain BC as the electron donor for nitrate reduction, but the other amino acids tested, i.e. alanine and glycine, were not used. Strain BC did not use sugars as electron donor (fructose, glucose and xylose tested). Within one week strain BC grew on the following aromatic compounds with oxygen or chlorate as the electron acceptor: benzene, toluene, phenol, *o*-, *m*-, *p*-cresol and catechol. Growth on these compounds did not occur with nitrate as the electron acceptor. Strain BC did not degrade cyclohexanol, neither with nitrate nor with oxygen. Growth of strain BC was studied in more detail with acetate (10 mM) as the electron donor and oxygen, chlorate or nitrate as the electron acceptor (Fig. 4). With oxygen as the electron acceptor, strain BC degraded 9 mM acetate within 3 days. The oxygen concentration decreased from 10 to 3 mM (Fig. 4A). Acetate oxidation and oxygen reduction was coupled to growth indicated by the increase in cells and OD_{600} (Fig. 4D). Acetate (9 mM) and chlorate (9 mM) were completely consumed within 4 days with an almost complete recovery of chloride (95%) (Fig. 4B and 4E). Nitrate was also used as the electron acceptor for growth (Fig. 4C and 4F). Both acetate (9 mM) and nitrate (8 mM) were almost completely degraded within 3 days. With chlorate as the electron acceptor the growth yield appeared lower than with oxygen or nitrate as the electron acceptor, i.e. lower values for cells/ml and OD_{600} were measured. Specific growth rates on acetate were about 0.98, 0.56, and 0.95 day^{-1} with oxygen, chlorate and nitrate as the electron acceptor, respectively.

In addition to chlorate and oxygen, strain BC used nitrate and nitrite. Perchlorate, sulfate, sulfite, thiosulfate, fumarate, manganese(IV)oxide, iron(III)pyrophosphate, iron(III)NTA, AQDS (anthraquinone-2,6-disulfonate), bromate, selenate and arsenate were not used as the electron acceptor.

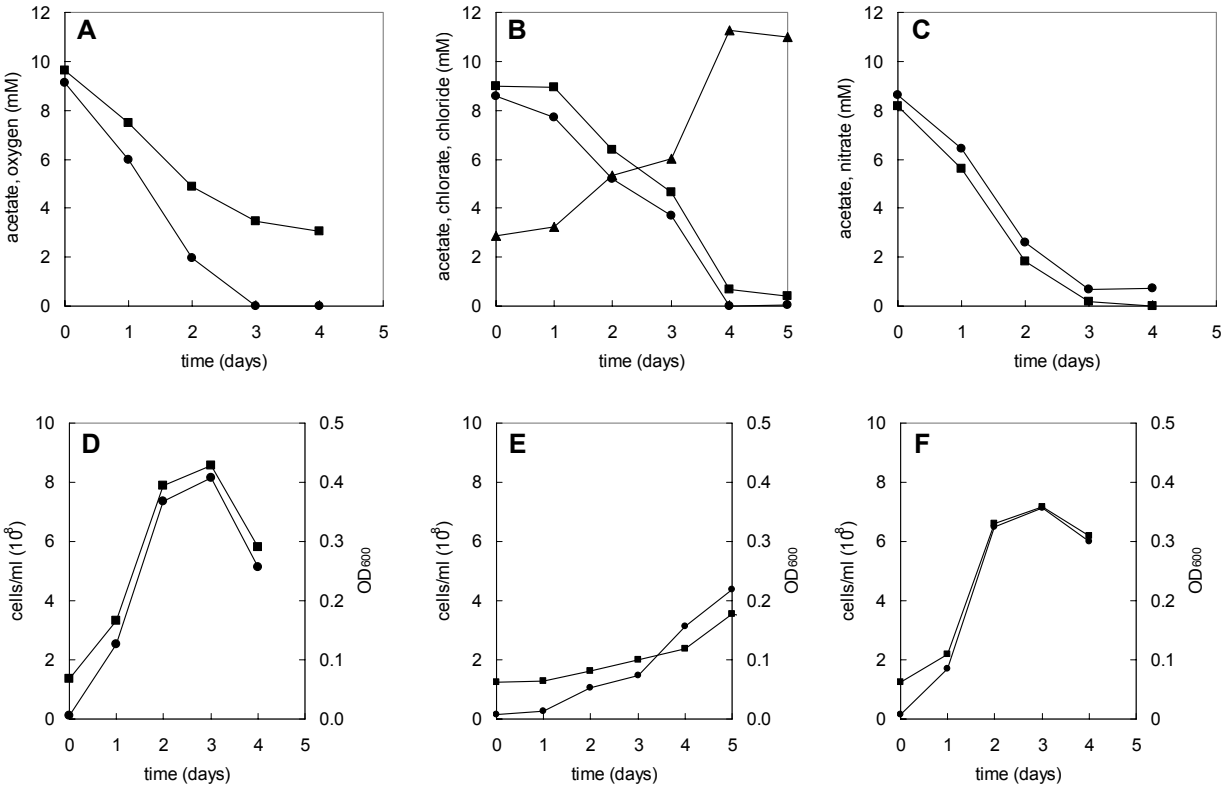

Figure 4. Growth of strain BC on acetate with three different electron acceptors.
The electron acceptors tested were: oxygen (A and D), chlorate (B and E) and nitrate (C and F).
A. Acetate (●) and oxygen (■) consumption
B. Acetate (●) and chlorate (■) consumption, chloride production (▲)
C. Acetate (●) and nitrate (■) consumption
D, E, F. cells/ml (●) and OD_{600} (■) for growth with oxygen, chlorate and nitrate, respectively.

Catechol degradation

Catechol is a central intermediate in several aerobic benzene degradation pathways (Gibson and Subramanian, 1984; Dagley, 1985; Smith, 1990). Therefore, catechol degradation by strain BC (pregrown on benzene and chlorate) was investigated with different electron acceptors. Strain BC degraded catechol with oxygen (1.2 mM) and chlorate (10 mM), but not with nitrate as the electron acceptor. The initial amount of 0.8 mM catechol was degraded within 4 days with chlorate and within 7 days with oxygen as electron acceptor. Controls without inoculum showed chemical catechol conversion, i.e. 0.44, 0.25 and 0.26 mM catechol conversion in 49 days in the presence of oxygen, chlorate and nitrate, respectively; no decrease in oxygen, chlorate or nitrate concentration was observed. The chemical catechol conversion was accompanied by a browning of the solution. It is known that chemical catechol polymerization can occur easily, leading to a browning of the solution (Sanchez-Cortes *et al.*, 2001).

Table 2. Overview of the electron donor use by strain BC. + = growth; - = no growth; n.d. = not determined.

	Concentrations used in this study	Electron acceptors		
		NO_3^-	O_2	ClO_3^-
Carboxylic acids [a]	10 mM	+	+ [b]	+ [b]
Glucose	10 mM	-	n.d.	n.d.
Fructose	10 mM	-	n.d.	n.d.
Xylose	10 mM	-	n.d.	n.d.
Alanine	10 mM	-	n.d.	n.d.
Glycine	10 mM	-	n.d.	n.d.
Glutamate	10 mM	+	n.d.	n.d.
Ethanol	10 mM	-	n.d.	n.d.
Methanol	10 mM	-	n.d.	n.d.
Glycerol	10 mM	+	n.d.	n.d.
Yeast extract	1 g/l	+	n.d.	n.d.
Benzene	0.25 mM	-	+	+
Toluene	0.25 mM	-	+	+
Ethylbenzene	0.25 mM	-	-	n.d.
o-Xylene	0.1 mM	-	-	n.d.
m-Xylene	0.1 mM	-	-	n.d.
p-Xylene	0.1 mM	-	-	n.d.
Benzoate	1 mM	-	-	n.d.
Phenol	1 mM	-	+	+
p-Hydroxybenzoate	1 mM	-	-	n.d.
o-Cresol	1 mM	-	+	+
m-Cresol	1 mM	-	+	+
p-Cresol	1 mM	-	+	+
Monochlorobenzene	0.05 mM	-	-	-
Catechol	1 mM	-	+	+
Cyclohexanol	1 mM	-	-	n.d.
$FeCl_2$	5 mM	-	n.d.	n.d.
Na_2S	1 mM	-	n.d.	n.d.
H_2 [c]	170 kPa	-	n.d.	n.d.

[a] Carboxylic acids tested: acetate, lactate, pyruvate, succinate, propionate, butyrate, malate, citrate and fumarate.

[b] Only acetate was tested, other carboxylic acids were not tested.

[c] Also 1 mM acetate was added for heterotrophic biomass production.

Detection of chlorite dismutase, benzene oxygenase and catechol oxygenase

Chlorite dismutase. Chlorate reductase and chlorite dismutase activity in cell extracts of benzene- and chlorate-grown cells of strain BC were 0.4 and 5.7 U/mg protein, respectively. Similar chlorate reductase activity (0.3 U/mg protein) and higher chlorite dismutase activity (22 U/mg protein) were obtained with cell extracts of strain BC grown on acetate and chlorate. Gene sequences encoding known chlorite dismutase (*cld*) were not detected in DNA extracted from strain BC by applying the PCR primers reported in previous studies (Bender *et al*., 2004). With the same primer sets we were able to detect *cld* genes in *Pseudomonas chloritidismutans* (results not shown). Thus, the gene(s) encoding chlorite dismutase(s) of strain BC is (are) too divergent to the genes possible to detect with the primers used.

Benzene oxygenases and extradiol dioxygenases. As physiological and biochemical data indicated the involvement of oxygenases during growth with benzene and chlorate, we screened for the presence of gene fragments of monooxygenase and dioxygenase enzymes potentially involved in benzene degradation in strain BC. No signal was detected with primers targeting a wide number of toluene/biphenyl/isopropylbenzene dioxygenases that commonly also target benzene dioxygenases of Gram-negative bacteria (Witzig *et al*., 2006). PCR products of the expected size were detected in strain BC genomic DNA with primers sets targeting a group of Extradiol Dioxygenase (EXDO) Type I and a group of aromatic ring-hydroxylating monooxygenases (RHMO). We confirmed the identity of the fragments by DNA sequencing, conceptual translation, and phylogenetic analyses. The translated DNA sequences encoded, in both cases, non-disrupted protein sequence frames that branched as new members of their corresponding groups (Fig. 5). In the case of the RHMO, the protein phylogeny of the large monooxygenase subunits together with the deduced amino acid sequence from strain BC *(BC-BMOa*) (Fig. 5A) is placed in a cluster composed by recently reported members of the three-component aliphatic/aromatic monooxygenases. The closest relatives, when using tblastx searches (available at NCBI web site), are some sequences coming from sludge amplifications of community DNA from nonylphenol treatments (as referenced only in GenBank accession number), groups of peptides of recently described variants able to monooxygenate TCE and benzene (Futamata *et al*., 2001) as well as sequences detected on total DNA from enrichment experiments of soil samples with benzene (Iwai *et al*., 2007). The closest relative with complete or close to complete coding DNA sequence indicate that strain BC sequence is highly similar, as assessed by neighbour-joining tree on the multiple protein sequences alignment, to putative aromatic monooxygenase sequences found in *Comamonas* sp. strain E6 (86% identity), *Dechloromonas aromatica* strain RCB (76% identity) and *Azoarcus* sp. strain BH7 (72% identity) genomes. These bacteria are all betaproteobacterial strains like strain BC, and reported to be able to degrade monoaromatic compounds by diverse mechanisms. For instance, strain E6 is a bacterium with the capability to oxygenate phenol (Watanabe *et al*., 1996).
Strain BC is able to grow on catechol with oxygen and chlorate, but not with nitrate. Thus, we tried to detect genes of enzymes that can degrade catechol. Catechol 2,3-dioxygenases belonging to the three EXDO groups targeted (around I.2.A and I.2.B (Eltis and Bolin, 1996)) are expected to be present in strain BC based on the phylogenetic positioning of the monooxygenase sequence found in strain BC. Due to sequence variability between these clusters, primer sets had to be designed to be able to amplify all the gene members collected, divided in three subgroups, A, B and C (Fig. 5B). As predicted, amplifications with strain BC genomic DNA produced a PCR product with primers targeting the group of catechol 2,3-dioxygenases from strains with similar monooxygenases to the one found in strain BC. In this case, a pattern of different DNA sizes, probably of unspecific products was obtained due to the high degeneracy of the primers, and the intentional overlapping specificity between the sequence groups targeted. A fragment of the expected product size was excised from agarose gel and sequenced. The conceptual translation of catechol 2,3-dioxygenase gene fragment obtained from strain BC (*BC-C23O*) consisted of a single read frame without stop codons and had the conserved domains commonly found in EXDO family as assessed by searches on NCBI repositories. When aligning the putative protein against the whole protein family database, the sequence was located in the middle of two experimentally proved C23O enzymes phylogenetic subgroups and in a new branch of C23O putative proteins identified in genome sequence projects *Delftia* spp., *Azoarcus* spp., *Dechloromonas* spp., and *Pseudomonas* spp. strains (Fig. 5B).

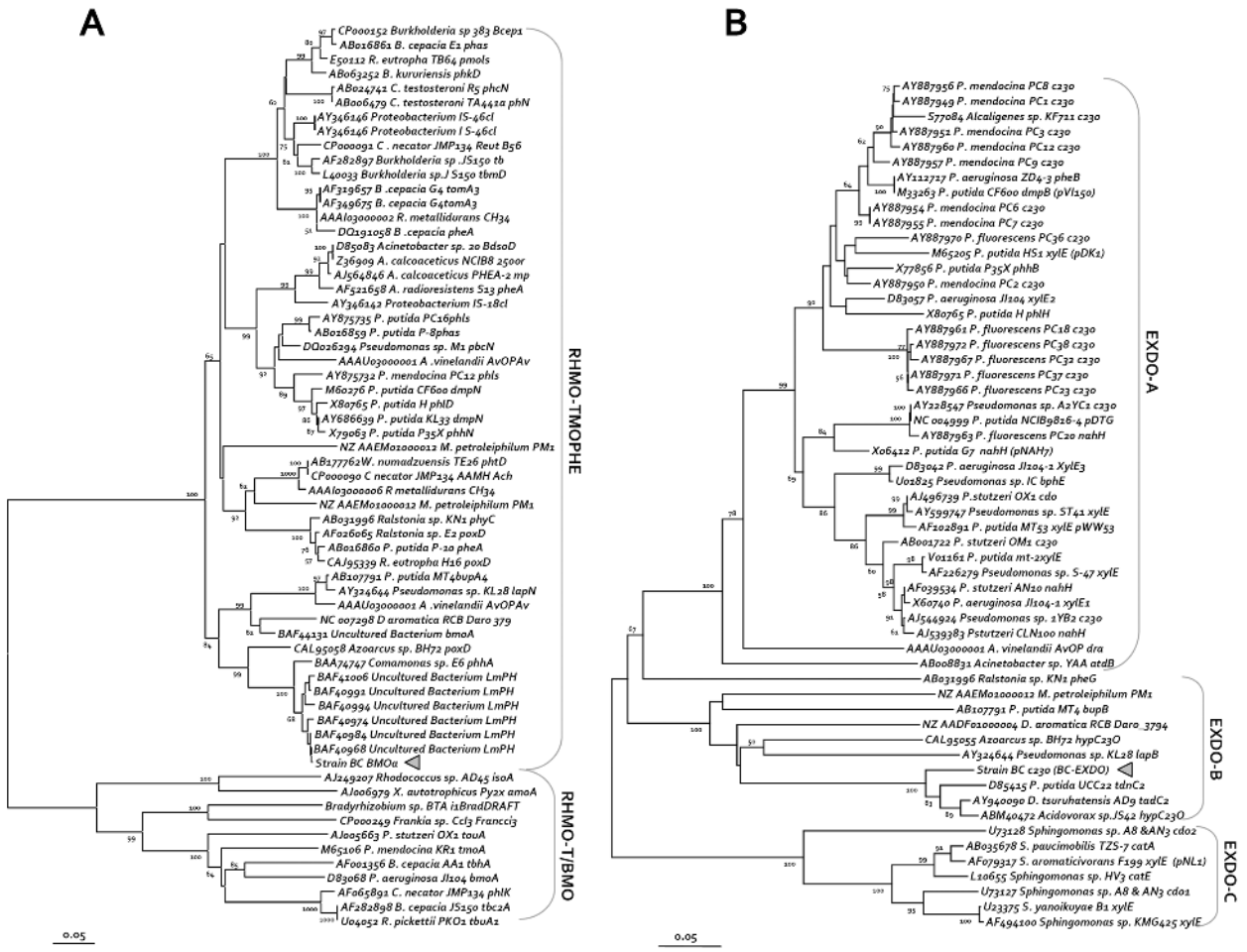

Figure 5. Evolutionary relationships of the benzene monooxygenase and the catechol 2,3-dioxygenase sequences found on strain BC on their corresponding protein family contexts. The deduced amino acid sequences of the same coding DNA sequences used for primers (brackets showing the primer pair names and clusters comprised, see Materials and Methods) designed to find relatives of soluble diiron monooxygenases large subunits or extradiol dioxygenases type I (see main text), were aligned including the putative protein fragments deduced from the DNA sequences obtained from strain BC (indicated by triangles). To generate the Neighbour-Joining trees shown, blocks of (on average) 220 amino acids for phenol hydroxylases and aromatic monooxygenases large subunits (A) or 237 amino acids for catechol 2,3-dioxygenases (B), spanning the length common to the gene members selected, were used (details in Materials and Methods). Bar represents five amino acid changes per 100 amino acids. Bootstrap values above 50% from 1000 neighbor-joining trees are indicated to the left of the nodes. The primers used are written besides the indicated branch targeted. Sequences in the N-J tree were labeled with their corresponding DDBJ/EMBL/GenBank accession number followed by the organism of origin (genus or species), strain designation, and gene abbreviation.

Discussion

We describe here the isolation and characterization of a bacterium, strain BC, that can grow on benzene with chlorate as the electron acceptor. Strain BC is closely related to *Alicycliphilus denitrificans* K601 (type strain), a cyclohexanol-degrading nitrate-reducing *Betaproteobacterium* (99.7% similarity based on 16S rRNA genes). Besides benzene, strain BC grew on some other aromatic compounds with oxygen or in the absence of oxygen with chlorate as the electron acceptor. Strain BC is a denitrifying bacterium, but it is not able to grow on benzene with nitrate.

In batch cultures of strain BC growing on benzene and chlorate, the theoretical biomass yield (Table 1) was about 3 times higher than the actual measured yield. Low biomass production could be due to low or no energy conservation in the chlorate to chlorite step. So far, it is not clear how the respiratory electron transport chain is arranged and how energy is conserved in (per)chlorate-reducing bacteria (Kengen *et al*., 1999; Wolterink *et al*., 2003). Another possibility is, that a major part of the energy is used for maintenance energy instead of biosynthesis. An inefficient biomass synthesis pathway or an inefficient (or incomplete) pathway for benzene degradation due to metabolite toxicity, pathway misrouting or metabolic bottlenecks, is also possible. Although no methods for CO_2 production quantification were executed, it is likely that strain BC degraded benzene (with oxygen or chlorate) completely to carbon dioxide. Namely, strain BC is able to grow with acetate as the electron donor and its oxidation results inevitably in carbon dioxide. Low biomass production was also observed in other studies concerning anaerobic benzene degradation, e.g. only 2.6% for *Dechloromonas* strain RCB when grown on ^{14}C-labelled benzene and chlorate (Chakraborty *et al*., 2005). Low values for incorporation of ^{14}C-labelled benzene into biomass were also observed when strain RCB was grown with nitrate (2%) or oxygen (3%) (Coates *et al*., 2001b).

Cell extracts of strain BC grown on benzene or acetate with chlorate as the electron acceptor showed chlorate reductase (0.3-0.4 U/mg protein) and chlorite dismutase enzyme activity (5.7-22 U/mg protein) respectively. Cell extracts of *P. chloritidismutans* strain AW-1^T had higher chlorate reductase (9.0 U/mg protein) and chlorite dismutase activity (134 U/mg protein), whereas for instance *Azospira oryzae* GR-1 had similar chlorate reductase activity (0.39 U/mg protein) as strain BC, but a higher chlorite dismutase activity (145 U/mg protein) (Van Ginkel *et al*., 1996; Wolterink *et al*., 2002).
During chlorate reduction, oxygen is produced by the dismutation of chlorite. Therefore, it seems likely that benzene is degraded via an aerobic degradation pathway in strain BC. Aerobic bacterial benzene degradation can be initiated by monohydroxylation or dihydroxylation. The first step in the dihydroxylation is the addition of dioxygen to the aromatic nucleus to form *cis*-benzene dihydrodiol, which is further transformed to catechol (Gibson *et al*., 1968). The monohydroxylation is catalyzed by monooxgenases with rather broad substrate specificities: the toluene 4-monooxygenase (T4MO) of *Pseudomonas mendocina* KR1, the toluene 3-monooxygenase (T3MO) of *Ralstonia pickettii* PKO1, and the toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4, all converting benzene to phenol as well as catechol and 1,2,3-trihydroxybenzene in successive hydroxylation reactions (Tao *et al*., 2004). Strain BC degrades phenol and catechol with oxygen and chlorate as the electron acceptor, but not with nitrate. In addition, chlorate reductase and chlorite dismutase activity was demonstrated in cell extracts of strain BC. An aerobic degradation pathway would normally require chlorate reductase, chlorite dismutase and oxygenase enzymes.
While it is obvious that additional experiments are needed to precisely define the activities of the oxygenase systems found in the genome of strain BC, based on the physiological, genetic and biochemical experiments presented in this study, we propose a benzene degradation pathway with chlorate as the electron acceptor in strain BC (Fig. 6). In this pathway, oxygen produced during chlorate reduction is used in oxygenase reactions, i.e. benzene conversion to catechol by two sequential monooxygenase reactions (by benzene monooxygenase, *BC-BMOa*) and catechol conversion to 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase (by catechol-2,3 dioxygenase, *BC-C23O*). Dihydroxylation of benzene to catechol can not be ruled out, but genes encoding benzene dioxygenases were not detected with the primers used in this study. Electrons (reducing equivalents) for chlorate reduction must be derived from intermediates of the aerobic benzene degradation pathway. This pathway could probably explain the experienced difficulties with subcultivation on benzene and chlorate when cultures of strain BC were stored unfed. Oxygen and reducing equivalents are required for benzene degradation by means of oxygenases, while reducing

equivalents are also needed to initiate chlorate reduction. Apparently, in starved cells the lack of reducing equivalents causes problems to initiate the metabolism and growth of strain BC. The addition of an easily degradable substrate, e.g. fermented yeast extract or acetate, results in an onset of growth and benzene degradation. We found that the addition of oxygen was insufficient to initiate benzene degradation, which is another indication for the involvement of (mono)oxygenases.

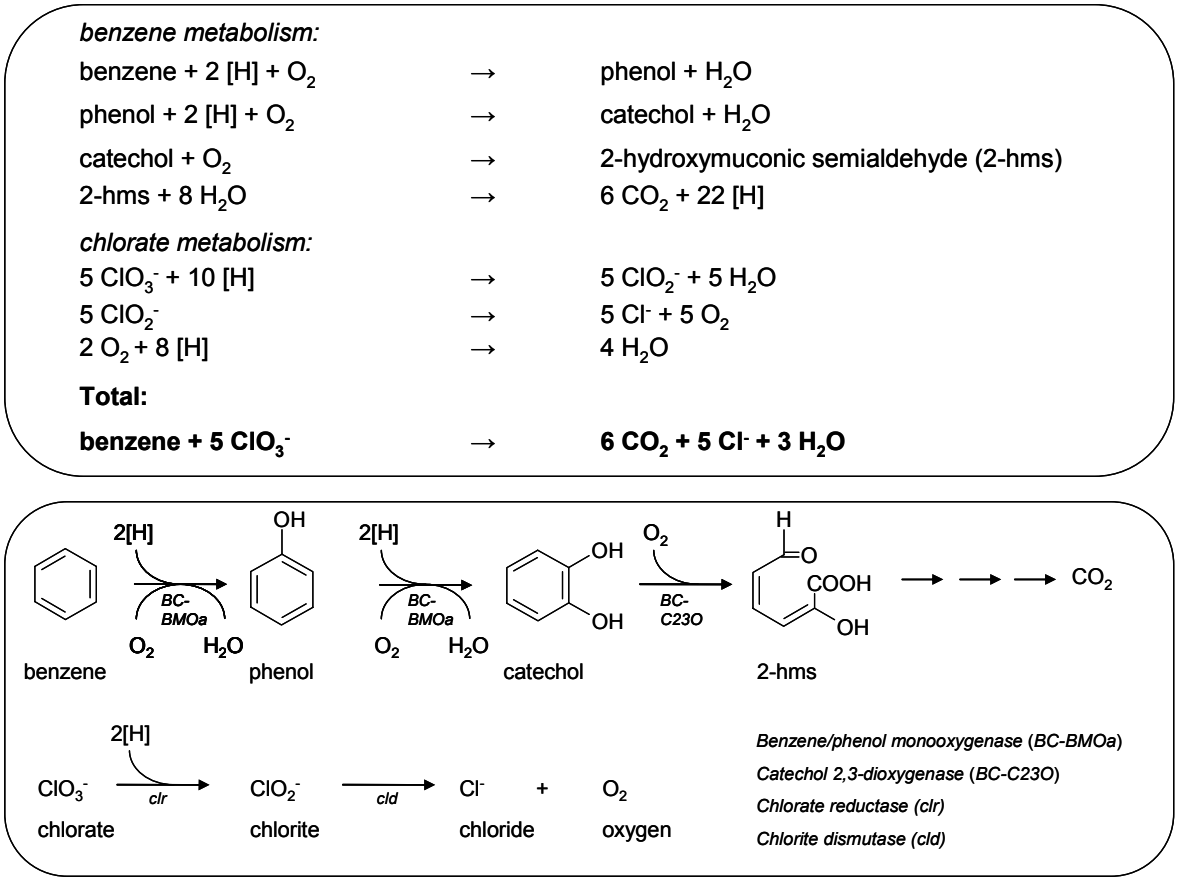

Figure 6. Proposed benzene degradation pathway with chlorate as the electron acceptor in strain BC. Proposed stoichiometric reactions involved in benzene degradation with chlorate as the electron acceptor in strain BC. [H] stands for reducing equivalents. Benzene metabolism involves the hydroxylation of benzene to phenol, phenol hydroxylation to catechol, extradiol (*meta*-) cleavage of catechol to 2-hydroxymuconic semialdehyde and complete oxidation of 2-hydroxymuconic semialdehyde to carbon dioxide and reducing equivalents. Chlorate metabolism involves the reduction of chlorate to chlorite, dismutation of chlorite into chloride and oxygen and subsequent reduction of oxygen to water.

In conclusion, the physiological, genetic and biochemical experiments of this study strongly suggest that oxygen formed in the dismutation of chlorite may not only be used as terminal electron acceptor in strain BC but is also used to attack molecules by means of oxygenases. This demonstrates the existence of aerobic benzene bacterial biodegradation pathways under essentially anaerobic conditions by the concerted action of chlorite dismutases, providing the metabolic oxygen needed by aromatic activating and cleaving oxygenases. Thus, aerobic aromatic degradation pathways can be employed by single organisms under conditions where no external oxygen is supplied.

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6

Physiological characterization of two *Alicycliphilus denitrificans* strains

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To be submitted

Abstract

A bacterium, strain BC, was isolated that degrades benzene in conjunction with chlorate reduction. Phylogenetic analysis demonstrated its relationship with *Alicycliphilus denitrificans* K601^T, a cyclohexanol-degrading denitrifying *beta-Proteobacterium* (16S rRNA genes are 99.7% similar). Strain K601^T is not able to use chlorate as the electron acceptor, while strain BC cannot degrade cyclohexanol.

A benzene-degrading chlorate-reducing bacterium was isolated from a stable benzene-degrading chlorate-reducing enrichment culture (Weelink *et al*., 2007). Cultivations were done at 30°C in 120-ml serum bottles containing 40 ml medium as described previously (Weelink *et al*., 2007). Phylogenetic analysis demonstrated that strain BC was most closely related to *Alicycliphilus denitrificans* K601^T, a cyclohexanol-degrading nitrate-reducing *Betaproteobacterium* (99.7% similarity based on 16S rRNA genes) (Mechichi *et al*., 2003). Physiological and (chemo)taxonomical characteristics of strain BC were compared with those of *Alicycliphilus denitrificans* strain K601^T and the results are described here. The isolation and other (e.g. biochemical) characteristics of strain BC were described elsewhere (Weelink *et al*., 2008).

The GenBank database accession numbers of strain BC and *Alicycliphilus denitrificans* strain K601 are DQ342277 and AJ418042, respectively. *Alicycliphilus denitrificans* strain K601 (DSM 14773) was purchased from the DSMZ, (Braunschweig, Germany). Strain BC was deposited in two different culture collection of microorganisms, at the DSMZ (Braunschweig, Germany), strain number DSM 18852, and the Japanese Collection of Microorganisms - JCM (Riken BioResource Center, Japan), strain number JCM 14587. Cells of strain BC were short rods, 0.6 µm wide and 1–2 µm long, motile and stained Gram-negative. Optimal growth occurs at 30-37°C and pH 7.3. Similar characteristics were reported for *Alicycliphilus denitrificans* K601^T (Mechichi *et al*., 2003).

The G+C content, DNA-DNA hybridization and cellular fatty acids analysis were performed by the DSMZ (Braunschweig, Germany). For cellular fatty acid analysis, strain BC and *Alicycliphilus denitrificans* strain K601 (DSM 14773^T) were grown under identical conditions, i.e. acetate (10 mM) and nitrate (10 mM) in 2000 mL AW-1-sulfate medium at 30°C. Cellular fatty acids of strain BC and *Alicycliphilus denitrificans* K601 showed qualitatively the same fatty acid methyl ester (FAME) pattern (Table 1). The patterns are composed of straight chain saturated and unsaturated FAMEs and substantial amounts of cyclopropane FAMEs. The diagnostic 3-hydroxy decanoic acid (C10:0 3OH) was found in addition. A similarity dendrogram of the fatty acid patterns of the two strains revealed a relatively high Euclidian Distance of 25. This is beyond the value of 11 postulated for strains of the same species (according to MIDI Inc. Del. US report interpretation guide (MIDI, 2001)). The reason of the discrepancy of the DNA/DNA hybridization and the fatty acid results might be explained by the growth conditions of the two strains. In the current experiments both strains had been grown in the same medium and temperature, but the fatty acid pattern of bacterial cells can already be influenced by slightly different culture conditions.

The G+C content of strain BC and *Alicycliphilus dentrificans* was 67.6 and 66 mol%, respectively. DNA-DNA hybridization of strain BC against *Alicycliphilus denitrificans* K601 showed 74.5 $\pm$ 3.5 % similarity. Strain BC belongs to the species *Alicycliphilus denitrificans* when the recommendations of the threshold value of 70% DNA-DNA similarity for the definition of bacterial species are considered (Wayne *et al*., 1987). However, values around 70% have to be regarded as critical and physiological and chemotaxonomical data should also be considered (Rossello-Mora and Amann, 2001; Goris *et al*., 2007).

Table 1. Major cellular fatty acids of strain BC and *Alicycliphilus denitrificans* K601 (DSM 14773^T). In this study both bacteria were grown on acetate (10 mM) and nitrate (10 mM), whereas *Alicycliphilus denitrificans* was grown on R2A medium in the study of Mechichi *et al.,* 2003. n.d. = not detectable; - = no information presented about this fatty acid.

Major cellular fatty acids	Strain BC (this study) (%)	*Alicycliphilus denitrificans* K601^T (this study) (%)	*Alicycliphilus denitrificans* K601^T (Mechichi *et al*. 2003) (%)
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	-

[a] According to Mechichi *et al*. (2003) this fatty acid occurred in small amounts, but no concentration is given.

Therefore, physiological data, i.e. substrates and electron acceptors used for growth, were determined. To determine the substrate spectrum of strain BC different electron donors were tested in duplicate batches with nitrate (10 mM) or oxygen (5% in headspace) as the electron acceptor (Table 2). 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 batches with aromatic compounds as substrate. In these batches late log-phase cells of strain BC grown on benzene (repeated feeds of 0.5 mM) and chlorate (10 mM) were used as inoculum (5%). Growth was monitored by visual observation of turbidity and the decrease in nitrate or chlorate concentration, respectively. The following electron acceptors (10 mM, exceptions between parentheses) were tested for strain BC in duplicate batches with acetate (10 mM) as the electron donor: oxygen (10% in headspace), perchlorate, chlorate, nitrate, nitrite (5 mM), sulfate, sulfite, thiosulfate, fumarate, manganese(IV)oxide (20 mmol/l), iron(III)pyrophosphate, iron(III)NTA (NTA stands for nitrilotriacetic acid), AQDS (anthraquinone-2,6-disulfonate, 4 mM), bromate (5 and 10 mM), selenate (5 and 10 mM), arsenate (5 and 10 mM). Electron acceptor use was visually monitored and by measuring the acetate concentration and the electron acceptor decrease as well in case the acetate concentration decreased. Analytical procedures were used as described previously (Weelink *et al*., 2007).

Strain BC has physiological properties in common with *Alicycliphilus denitrificans* K601 (Table 2). Both bacteria use several carboxylic acids as the electron donor for growth. In addition, both bacteria use similar aromatic compounds as the electron donor for growth, i.e. benzene, phenol, catechol and *o*-, *m*-, *p*-cresol (with oxygen as the electron acceptor). Furthermore, both bacteria use oxygen, nitrate and nitrite as the electron acceptor. However, the two bacteria differ in at least two key physiological features that have led to their isolation. Strain BC uses chlorate as the electron acceptor, *Alicycliphilus denitrificans* strain K601 does not. Strain K601 can degrade cyclohexanol, a property that strain BC lacks. Therefore, based on physiological and chemotaxonomical data and the consideration that the genus until now contains only one validated species, we consider strain BC as an *Alicycliphilus denitrificans* strain.

Table 2. Overview of the electron donor use by strain BC and *Alicycliphilus denitrificans* K601 (DSM 14773^T). Data from strain BC are from this study, whereas data for *Alicycliphilus denitrifcans* K601 are either from Mechichi *et al*. (2003) or this study. + = growth; - = no growth; n.d. = not determined.

	Concentrations used in this study	Strain BC			*Alicycliphilus denitrificans*	
		NO_3^-	O_2	ClO_3^-	NO_3^-	O_2
Carboxylic acids [a]	10 mM	+	+ [b]	+ [b]	+	+
Glucose	10 mM	-	n.d.	n.d.	-	+
Fructose	10 mM	-	n.d.	n.d.	-	+
Xylose	10 mM	-	n.d.	n.d.	-	n.d.
Alanine	10 mM	-	n.d.	n.d.	n.d.	n.d.
Glycine	10 mM	-	n.d.	n.d.	n.d.	n.d.
Glutamate	10 mM	+	n.d.	n.d.	n.d.	n.d.
Ethanol	10 mM	-	n.d.	n.d.	n.d.	n.d.
Methanol	10 mM	-	n.d.	n.d.	n.d.	n.d.
Glycerol	10 mM	+	n.d.	n.d.	n.d.	+
Yeast extract	1 g/l	+	n.d.	n.d.	n.d.	n.d.
Benzene	0.25 mM	-	+	+	- [c]	+ [c]
Toluene	0.25 mM	-	+	+	- [c]	- [c]
Ethylbenzene	0.25 mM	-	-	n.d.	n.d.	n.d.
o-Xylene	0.1 mM	-	-	n.d.	n.d.	n.d.
m-Xylene	0.1 mM	-	-	n.d.	n.d.	n.d.
p-Xylene	0.1 mM	-	-	n.d.	n.d.	n.d.
Benzoate	1 mM	-	-	n.d.	-	-
Phenol	1 mM	-	+	+	-	+ [c]
p-Hydroxybenzoate	1 mM	-	-	n.d.	-	+
o-Cresol	1 mM	-	+	+	-	+
m-Cresol	1 mM	-	+	+	-	+
p-Cresol	1 mM	-	+	+	-	+
Monochlorobenzene	0.05 mM	-	-	-	n.d.	n.d.
Catechol	1 mM	-	+	+	- [c]	+ [c]
Cyclohexanol	1 mM	-	-	n.d.	+	+
$Fe(II)Cl_2$	5 mM	-	n.d.	n.d.	n.d.	n.d.
Na_2S	1 mM	-	n.d.	n.d.	n.d.	n.d.
H_2 [d]	170 kPa	-	n.d.	n.d.	n.d.	n.d.

[a] Carboxylic acids tested: acetate, lactate, pyruvate, succinate, propionate, butyrate, malate, citrate and fumarate.

[b] Only acetate was tested, other carboxylic acids were not tested.

[c] Data from this study, all other data with respect to *Alicycliphilus denitrificans* K601 are from Mechichi *et al*. (2003).

[d] Also 1 mM acetate was added for biomass production.

Acknowledgements

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7

General discussion and summary

Introduction and research goal

Accidental spills, industrial discharges and gasoline leakage from underground storage tanks have resulted in serious pollution of the environment with monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (so-called BTEX) (Fig. 1). High concentrations of BTEX have been detected in soils, sediments and groundwater. The mobility and toxicity of the BTEX compounds are of major concern. Therefore, the cleanup of BTEX pollution, particular benzene, has gained much attention the last decades.

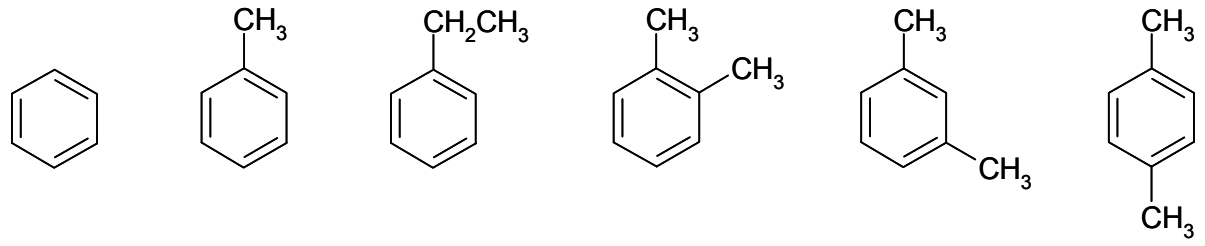


Figure 1. Chemical structures of benzene, toluene, ethylbenzene, *ortho*-xylene, *meta*-xylene and *para*-xylene.

In situ bioremediation of BTEX by using naturally occurring microorganisms or introduced microorganisms is a very attractive option. BTEX compounds are known to be degraded by microorganisms under aerobic and anaerobic conditions. As BTEX compounds are often present in the anaerobic zones of the environment, anaerobic bioremediation is an attractive remediation technique. The bottleneck in the application of anaerobic techniques is the lack of knowledge about the anaerobic biodegradation of benzene. In particular, bacteria involved in anaerobic benzene degradation have not been identified and the anaerobic benzene degradation pathway has still not been elucidated. Thus, due to the lack of available pure cultures it is unclear how benzene is destabilized in the absence of molecular oxygen.

The aim of the research presented in this thesis was to gain more insight in the degradation of benzene and other aromatic hydrocarbons by anaerobic bacteria. The approach applied in this research consisted of using batch incubations inoculated with BTEX-contaminated material (soil or sediment) or BTEX-degrading enrichment cultures obtained in previous studies. In particular, the physiology and phylogeny of the bacteria responsible for the degradation were studied and the results are presented in this thesis.

Anaerobic toluene degradation (Chapter 2 and 3)

In Chapter 2, anaerobic benzene and toluene degradation was studied with different electron acceptors in batch experiments inoculated with aquifer material that was polluted with BTEX-containing leachate from a landfill (Banisveld landfill near Boxtel, The Netherlands). Previous studies at this site showed that the BTEX concentration in the groundwater decreased in downstream direction probably due to microbiological degradation under iron-reducing conditions (Röling *et al*., 2001). Recently, BTX degradation under iron-reducing conditions in microcosms and enrichments incubated with soil and groundwater from the Banisveld landfill was demonstrated (Botton and Parsons, 2006, 2007).

In the batch experiments described in chapter 2, benzene was not degraded during one year of incubation. Toluene degradation, on the other hand, was observed with nitrate, MnO_2 and soluble (Fe(III)NTA) and amorphous (FeOOH) iron(III) as electron acceptors. In transfers and a dilution series, toluene degradation could be sustained with MnO_2 and Fe(III)NTA. A DGGE (Denaturing gradient gel electrophoresis) analysis of amplified 16S rRNA gene fragments showed that in both toluene-

degrading cultures the same bacterial species became dominant. Cloning and sequencing of the 16S rRNA gene fragments revealed that this dominant species is closest related to *Sterolibacterium denitrificans* Chol-1S^T, a cholesterol-oxidizing, denitrifying bacterium of the *Betaproteobacteria* (94.6% similarity). After several serial dilution series in agar (1.2 %) supplemented anaerobic mineral medium, this dominant species was obtained in pure culture (see Chapter 3). This novel bacterial strain, G5G6, is able to grow with toluene as the sole electron donor and carbon source, and amorphous and soluble Fe(III)-species, nitrate and MnO_2 as electron acceptors. Besides toluene, strain G5G6 is able to grow with ethylbenzene, phenol, *p*-cresol, *m*-cresol, benzaldehyde and *p*-hydroxybenzoate. Phylogenetic and physiological characteristics of strain G5G6 and *Sterolibacterium denitrificans* differ considerably. Strain G5G6 possesses the *bssA* gene encoding the alpha subunit of benzylsuccinate synthase, which catalyses the first step in anaerobic toluene degradation (Fig. 2). Its sequence is closest related to that of other *Betaproteobacteria* (72-81% similarity). In addition, strain G5G6 possesses other genes (*bcrA*, *oah*) encoding enzymes involved in anaerobic degradation of mono-aromatic compounds. Based on phylogenetic and physiological data presented, the novel toluene-degrading bacterium strain G5G6 is considered as a hitherto unknown taxon of the *Betaproteobacteria* and the name *Georgfuchsia toluolica* gen. nov. , spec. nov. is proposed.

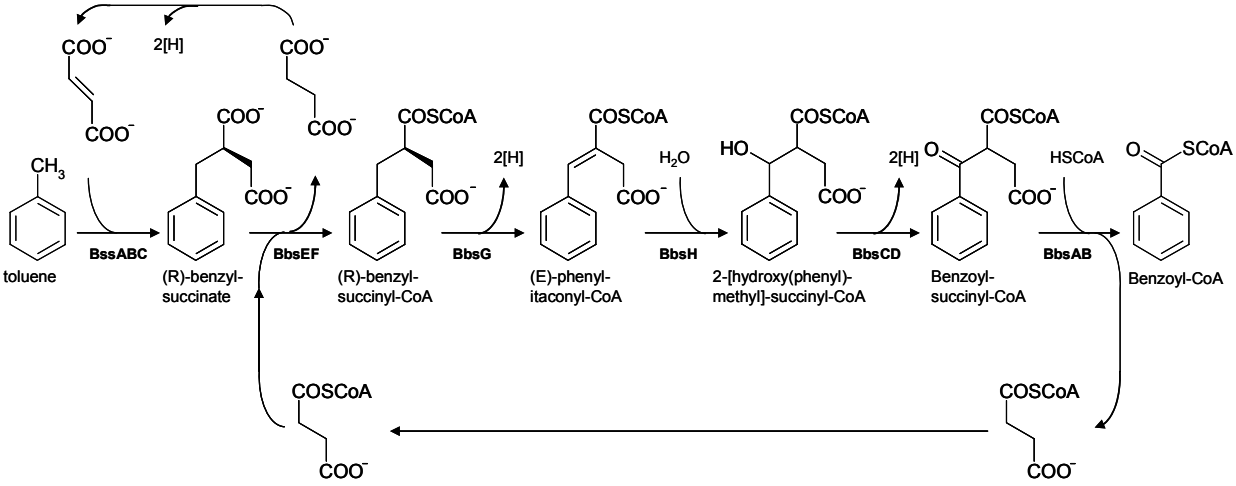


Figure 2. Anaerobic toluene degradation route, according to Kube *et al*. (2004). BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:(R)-benzylsuccinate CoA-transferase; BbsG, (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase.

Strain G5G6 has interesting physiological and phylogenetic characteristics, which will be subject of future research, i.e.:

1. it is the first toluene-oxidizing, iron reducer not belonging to *Geobacteraceae*
2. it is the first the toluene-oxidizing nitrate-reducing bacterium not belonging to the *Azoarcus* and *Thauera* (*Betaproteobacteria)* or to the *Magnetospirillum* genus (*Alphaproteobacteria*).
3. only two other bacteria described so far are able to grow by the degradation of both toluene and ethylbenzene with nitrate i.e. *Azoarcus* sp. EbN1 (recently renamed as *Aromatoleum aromaticum*) and *Dechloromonas aromatica* RCB (Rabus and Widdel, 1995; Chakraborty *et al*., 2005).
4. it only grows with aromatic compounds, not with more common substrates like acetate, hydrogen, lactate, pyruvate, and glucose.
5. it does not use the humic acid analogue AQDS (anthraquinone-2,6-disulfonate) as e-acceptor, while most iron-reducers do show this ability.

Benzene degradation with chlorate (Chapter 4, 5 and 6)

Since benzene and other aromatic hydrocarbons are generally well degraded by aerobic microorganisms, *in situ* bioremediation by creating aerobic conditions in the soil is possible. However, for a number of reasons application of oxygen-dependent processes in the subsurface are technically and financially often not appealing. An alternative bioremediation strategy would be to use the prevailing anaerobic conditions for BTEX degradation or to introduce oxygen in an alternative way, e.g. by *in situ* production. (Per)chlorate reduction is a way to produce molecular oxygen *in situ* under anaerobic conditions, in a light-independent process. This means that (per)chlorate reducing microorganisms can produce oxygen of metabolic origin in anaerobic environments. The formation of oxygen during (per)chlorate reduction may result in rapid oxidation of compounds which are otherwise slowly degraded under anaerobic conditions (Coates *et al*., 1999a). An example of such a compound is benzene.

It has been known for more than 50 years that microorganisms can reduce oxyanions of chlorine such as chlorate and perchlorate. The high redox potential of perchlorate (ClO_4^-/Cl^- E^0 = 1.287 V) and chlorate (ClO_3^-/Cl^- E^0 = 1.03 V) makes them ideal electron acceptors for microbial metabolism (Coates and Achenbach, 2004). In (per)chlorate reduction oxygen is produced (Fig. 3). The potential use of (per)chlorate-reducing microorganisms for bioremediation has been recognized in previous studies (Coates *et al*., 1999a; Logan and Wu, 2002). It was demonstrated that amendment of (per)chlorate reducing bacteria and chlorite to an anoxic soil led to complete degradation of ^{14}C-benzene to ^{14}C-carbon dioxide (Coates *et al*., 1999a). Toluene degradation was observed in sand columns inoculated with toluene-degrading and chlorate-reducing enrichment cultures (Logan and Wu, 2002). In another study, addition of chlorate to a soil column polluted with benzene showed removal of benzene coupled to chlorate reduction (Tan *et al*., 2006).

Benzene degradation coupled to (per)chlorate reduction was studied in the research described in this thesis and the results are presented in Chapter 4, 5 and 6. Early evidence for chlorate-stimulated benzene degradation was obtained at TNO (Gerritse, 2001; Gerritse *et al*., 2001; Langenhoff, 2003). Oxygen production by chlorate reduction to enhance anaerobic biodegradation of benzene was further studied in a Trias-SKB project in laboratory experiments at the Laboratory of Microbiology (WUR) and in field trials at a NAM location (Slochteren, The Netherlands) by TNO Built Environment and Geosciences (Utrecht, The Netherlands). Benzene was chosen because it is a priority pollutant. Moreover, benzene is very persistent under anaerobic conditions, while it is readily degraded aerobically. At the laboratory of Microbiology a benzene-degrading chlorate-reducing enrichment culture was obtained with reactor material from TNO (Tan *et al*., 2004). This TNO-reactor was originally inoculated with mixed material from a wastewater treatment plant and soil samples obtained from a location contaminated with benzene. This stable enrichment culture degraded benzene at rates similar to reported aerobic benzene degradation rates, but 20-1650 times faster than reported for anaerobic benzene degradation (Suarez and Rifai, 1999; Reardon *et al*., 2000; Coates *et al*., 2002; Ulrich and Edwards, 2003).

Molecular analysis of our enrichment culture showed that the culture had a stable composition after the seventh transfer (see Chapter 4). Five bacterial clones were further analyzed. Two clones corresponded to bacteria closely related to *Alicycliphilus denitrificans* K601^T (Mechichi *et al*., 2003). The three other clones corresponded to bacteria closely related to *Zoogloea resiniphila* PIV-3A2w, *Mesorhizobium* sp. WG and *Stenotrophomonas acidaminiphila* (Costa *et al*., 2000; Assih *et al*., 2002; Probian *et al*., 2003). DGGE analysis of cultures grown with different electron donors and acceptors indicated that the bacterium related to *Alicycliphilus denitrificans* K601 is able to degrade benzene coupled to chlorate reduction. The role of the other bacteria could not be conclusively determined. The bacterium related to *Mesorhizobium* sp. WG can be enriched with benzene and oxygen, but not with

acetate and chlorate, while the bacterium related to *Stenotrophomonas acidaminophila* grows with acetate and chlorate, but not with benzene and oxygen. As during chlorate reduction oxygen is produced, an aerobic pathway of benzene degradation is most likely. Furthermore, as one of these bacteria seems able to oxidize benzene aerobically, but not able to reduce chlorate and another bacterium seems able to reduce chlorate but not oxidize benzene, cross feeding involving interspecies oxygen transfer is a likely mechanism. This is presently under investigation at the laboratory of Microbiology (Wageningen University) in a STW-project ('Application of (per)chlorate reduction as a novel concept for bioremediation of polluted anaerobic soils', STW-project nr. 08053).
From the benzene-degrading chlorate-reducing enrichment culture, a bacterium, strain BC, was isolated (see Chapter 5 and 6). This newly isolated bacterium is able to degrade benzene coupled to chlorate reduction. Strain BC grows on benzene and some other aromatic compounds with oxygen, or in the absence of oxygen with chlorate as the electron acceptor. Strain BC is a denitrifying bacterium, but it is not able to grow on benzene with nitrate. The closest cultured relative is *Alicycliphilus denitrificans* K601 (type strain), a cyclohexanol-degrading nitrate-reducing *Betaproteobacterium*. The 16S rRNA gene sequences are 99.7 % similar, while genomic DNA was 74.5 $\pm$ 3.5 % similar as indicated by DNA-DNA hybridization. However, physiological differences are apparent. Strain BC is not able to degrade cyclohexanol, while strain K601^T lacks the ability to reduce chlorate.

During chlorate reduction, oxygen is produced by the dismutation of chlorite. Therefore, it seems likely that benzene is degraded via an aerobic degradation pathway in strain BC. An aerobic degradation pathway would require chlorate reductase, chlorite dismutase and oxygenase enzymes. Chlorate reductase and chlorite dismutase activities were determined in cell extracts of strain BC. Subsequently, we screened the total genomic DNA of strain BC for sequence signatures indicating the presence of genes that can potentially encode the key enzymes, i.e. chlorite dismutases, ring activating oxygenases and ring cleavage oxygenases. Chlorite dismutase activity was detected in cell extracts of strain BC, but we did not obtain any signal using the primers previously reported as designed for the detection of the group of genes assigned to this function (Bender *et al*., 2004). The genetic information available for this group of genes is still very scarce. The genes with experimentally proven activity encoding chlorite dismutases (cld) reported so far are relatively few (Bender *et al*., 2004). The first chlorite dismutase isolated was from *Azospira oryzae* strain GR-1 (Van Ginkel *et al*., 1996). After then, some other chlorite dismutases have been isolated (Coates *et al*., 1999b; Stenklo *et al*., 2001; Bender *et al*., 2002).

The screening of the genomic DNA of strain BC for ring activating oxygenases and ring cleavage oxygenases resulted in the detection of a putative benzene monooxygenase (*BC-BMOa*) gene and a putative catechol 2,3-dioxygenase (*BC-C23O*) gene. The BC-BMOa gene sequence is highly similar to aromatic monooxygenase sequences found in *Comamonas* sp. strain E6 (86% identity), *Dechloromonas aromatica* strain RCB (76% identity) and *Azoarcus* sp. strain BH7 (72% identity) genomes. Like strain BC, these bacteria are all betaproteobacterial strains and reported to be able to degrade monoaromatic compounds by diverse mechanisms. While it is obvious that additional experimental studies are needed to confirm the biodegradation mechanism of the oxygenase systems we have detected (e.g. transcriptional expression, heterologous protein expression, etc), all the sequence features and the physiological data support the hypothesis that these sequences encode functional and active benzene monooxygenases and catechol 2,3-dioxygenases used by strain BC.
Based on physiological, genetic and biochemical experiments, we propose a benzene degradation pathway with chlorate as the electron acceptor in strain BC (Fig. 3). In this pathway, oxygen produced during chlorate reduction is used in oxygenase reactions, i.e. benzene conversion to catechol by two sequential monooxygenase reactions (by benzene monooxygenase, BC-BMOa) and catechol

conversion to 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase (by catechol-2,3 dioxygenase, BC-C23O). Dihydroxylation of benzene to catechol can not be ruled out, but genes encoding benzene dioxygenases were not detected with the primers used in this study. Oxygen formed in the dismutation of chlorite may not only be used as terminal electron acceptor in strain BC but is also used to attack molecules by means of oxygenases. This demonstrates the existence of aerobic benzene bacterial biodegradation pathways under essentially anaerobic conditions by the concerted action of chlorite dismutases, providing the metabolic oxygen needed by aromatic activating and cleaving oxygenases. Thus, it was demonstrated that aerobic pathways can be employed under conditions where no external oxygen is supplied.

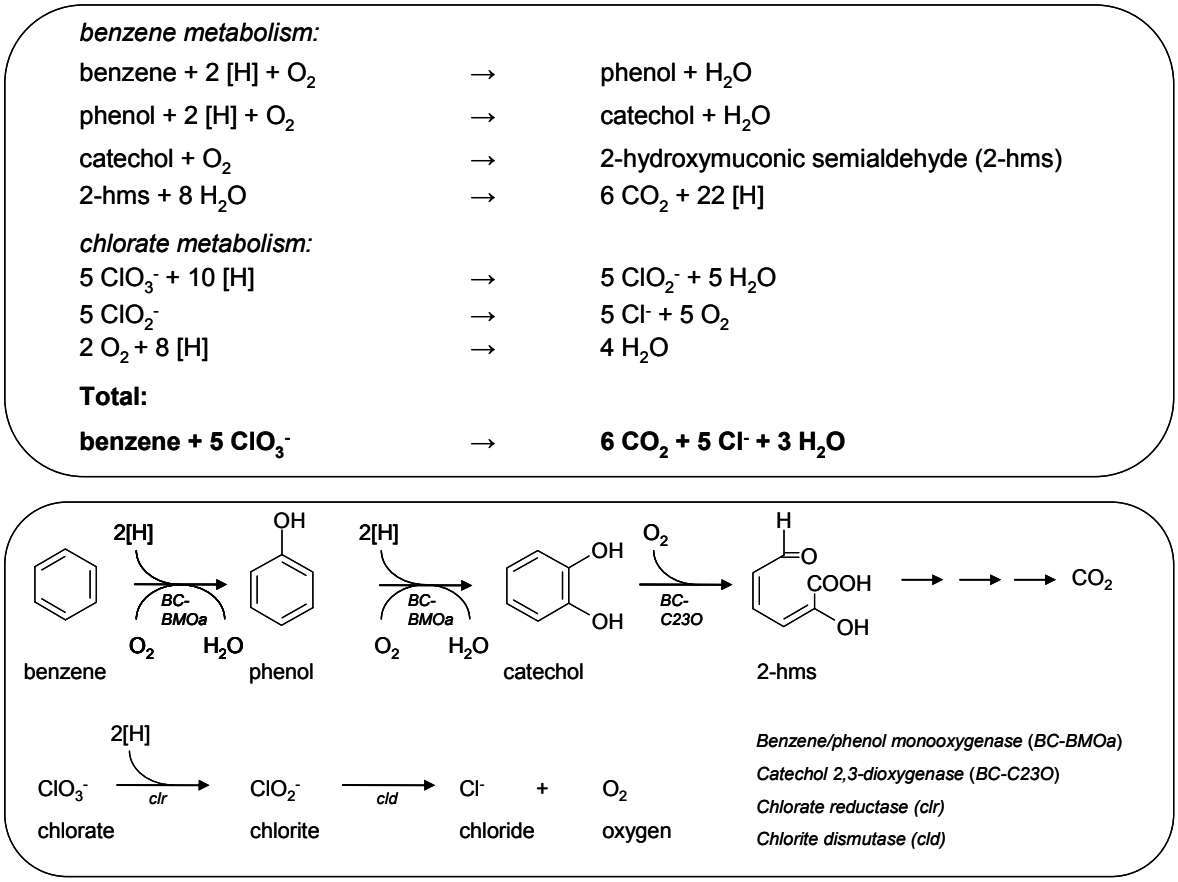


Figure 3. Proposed benzene degradation pathway with chlorate as the electron acceptor in strain BC. A. Proposed stoichiometric reactions involved in benzene degradation with chlorate as the electron acceptor in strain BC. [H] stands for reducing equivalents. Benzene metabolism involves the hydroxylation of benzene to phenol, phenol hydroxylation to catechol, extradiol (*meta*-) cleavage of catechol to 2-hydroxymuconic semialdehyde and complete oxidation of 2-hydroxymuconic semialdehyde to carbon dioxide and reducing equivalents. Chlorate metabolism involves the reduction of chlorate to chlorite, dismutation of chlorite into chloride and oxygen and subsequent reduction of oxygen to water.

Alicycliphilus denitrificans strain BC is able to grow with benzene and chlorate, whereas the closely related *Alicycliphilus denitrificans* K601 (type strain) is not able to reduce chlorate. Recently, a project proposal was submitted to the DOE Joint Genome Institute (JGI) by the laboratory of Microbiology (Wageningen University) entitled 'Unraveling the molecular mechanism of intracellular oxygen transfer in the novel chlorate-reducing bacterium *Alicycliphilus denitrificans* strain BC' (CSP letter of intent ID: CSP_LOI_793831). The project proposal has been accepted and will be incorporated in the

sequencing plans for 2009 (http://www.jgi.doe.gov/sequencing/cspseqplans2009.html). With the available genome sequences, insight into the molecular mechanism of intracellular oxygen transfer and the regulation of chlorate-dependent and chlorate-independent growth can be obtained. Furthermore, indications for interspecies oxygen transfer in the benzene-degrading chlorate-reducing enrichment culture was obtained (Chapter 4). This option would even broaden the degradation possibilities of microbial communities in polluted soils further. Introduction of a chlorate-reducing strain in a polluted soils may lead to growth of non-chlorate-reducing aerobic bacteria, with the capacity to metabolize a broad spectrum of substrates. With the available genome sequence of strain BC, the hypothesized interspecies oxygen transfer and the (potentially) involved genes will be studied in more detail.

It has been estimated that around 270,000 soil sites in the Netherlands are heavily contaminated and about 11,000 of these sites need to be urgently remediated (RIVM, 2008). Contamination with aromatic hydrocarbons is frequently observed at these sites. *In sit*u bioremediation by using naturally occurring microorganisms or introduced microorganisms is often for several reasons (e.g. financial reasons) a very attractive option. (Per)chlorate-enhanced bioremediation may become an important and attractive process for stimulated *in situ* anaerobic soil remediation. The advantage over oxygen injection is the high solubility of (per)chlorate compared with oxygen. Unlike oxygen, which is soluble in the mM-range, (per)chlorate is soluble in the M-range. Furthermore, (per)chlorate can be considered as a slow-oxygen release compound. The advantage of the use of (per)chlorate over other oxygen-releasing chemicals is that oxygen production is biologically controlled. The conversion of (per)chlorate to chlorite requires reducing equivalents that are formed in the oxidation of an organic compound. Without an electron donor (per)chlorate is chemically stable. This is not the case for other oxygen-releasing chemicals, like e.g. percarbonate or hydrogen peroxide.
When (per)chlorate addition to a contaminated aquifer will be applied as bioremediation technique, it has to be taken into account that (per)chlorate itself is a pollutant as well. Perchlorate is a solid rocket propellant, and chlorate is applied as herbicide and defoliant and is formed during paper bleaching (Versteegh *et al*., 1993; Coates and Achenbach, 2004). However, (per)chlorate is present in Chilean nitrate deposits and other mineral deposits. It was found recently that the electric discharge in chloride-containing aerosols may be a major source of naturally occurring perchlorate (Dasgupta *et al*., 2005). Biological (per)chlorate reduction may therefore be a rather ancient process. (Per)chlorate-reducing bacteria are known to use a wide variety of electron donors for growth, which include organic acids (acetate, lactate, etc) as well as inorganic compounds like hydrogen and sulfide (Logan, 1998; Wolterink *et al*., 2002; Coates and Achenbach, 2004). Because (per)chlorate is a pollutant itself, the application of (per)chlorate addition to contaminated aquifers has to be performed in close consultation with the competent authorities. For a field trial, concerning a push-pull experiment with chlorate at the NAM location (Slochteren, The Netherlands) by TNO Built Environment and Geosciences (Utrecht, The Netherlands), permission had been given by the competent authorities. A possible excess of (per)chlorate after the organic pollutants have been degraded, could be reduced by the introduction of easily degradable organic compounds. As a result, no environmentally harmful compounds are left after the bioremediation process. All (per)chlorate is eventually converted to the harmless chloride. In fact, the production of chloride will result in a higher salinity of the groundwater in the aquifer. However, the amount of chloride produced is probably very small with respect to the volume of water in the aquifer and therefore this effect is practically negligible. Concluding, (per)chlorate-enhanced bioremediation may become an important and attractive process for stimulated *in situ* anaerobic soil remediation. (Per)chlorate reduction and subsequent oxygen formation can also be beneficial for other compounds that are anaerobically difficult to degrade but that are readily degraded aerobically. Compounds of interest are naphthalene, monochlorobenzene

(MCB), vinylchloride (Vc), methyl tertiary butylether (MTBE), long and short chain alkanes and a variety of specific compounds like aromatic amines.

Future perspectives

Anaerobic BTEX degradation has been studied since the eighties. Although anaerobic alkylbenzene degradation has been studied in some detail, understanding of the anaerobic degradation of benzene is still in its infancy. This is mostly due to the lack of available pure or enrichment cultures that can degrade benzene anaerobically and the extremely slow growth of the microorganisms in the available enrichment cultures. Apart from the environmental significance of benzene as a contaminant with carcinogenic potential and high toxicity, anaerobic activation of benzene is probably one of the most intriguing reactions in microbial degradation today because the activation of a non-substituted aromatic ring in the absence of molecular oxygen constitutes an unprecedented biochemical reaction (Kunapuli *et al*., 2008). Furthermore, the evaluation of in-situ BTEX biodegradation is essential for the implementation of anaerobic BTEX bioremediation strategies. Traditional methods used to confirm bioremediation in the field included monitoring the contaminant concentrations and electron acceptors, and possible microbial byproducts. However, the challenge is to prove biodegradation in the field, since other processes such as volatilization, dispersion, and sorption can cause contaminant attenuation, and accurate mass balances are difficult to obtain (Mancini *et al*., 2003). In recent years, stable isotope fractionation analysis has gained attention as a tool for characterizing and assessing in-situ biodegradation of organic pollutants in contaminated aquifers. Thus, most studies about anaerobic benzene degradation focus on:

- The identification of the bacteria responsible for anaerobic benzene degradation
- The elucidation of the anaerobic benzene degradation pathway
- The prove and quantification of anaerobic benzene degradation in the field (in particular stable isotope fractionation)

The identification of the bacteria responsible for anaerobic benzene degradation

Only two *Dechloromonas* strains (RCB and JJ) and two *Azoarcus* strains (DN11 and AN9) were isolated, which are capable to degrade benzene anaerobically (Coates *et al*., 2001b; Kasai *et al*., 2006). In addition, several studies with anaerobic benzene-degrading enrichment cultures have resulted in the detection of bacteria potentially involved in anaerobic benzene degradation. Benzene-degrading Fe(III)-reducing sediments and enrichments were dominated by micro-organisms of the family *Geobacteraceae* (Anderson *et al*., 1998; Rooney-Varga *et al*., 1999; Botton *et al*., 2007). However, so far none of the *Geobacter* species tested degrades benzene. In sulfate-reducing and methanogenic enrichment cultures, often bacteria closely related to sulfate-reducing *Deltaproteobacteria*, e.g. in the family *Desulfobacteriaceae*, have been associated with anaerobic benzene degradation. This association was based on the results of culture independent molecular biology techniques (DGGE, clone libraries, real-time PCR, oligonucleotide probes). The *Deltaproteobacteria* detected were related to the sulfate-reducing bacteria able to degrade aromatic compounds, e.g. *m*-xylene, ethylbenzene, phenol, naphthalene and benzoate (Phelps *et al*., 1998; Ulrich and Edwards, 2003; Da Silva and Alvarez, 2007; Musat and Widdel, 2007). Recently, it was suggested that it could be more favorable to use solid support material for the enrichment (and isolation) of anaerobic benzene-degrading microorganisms (Herrmann *et al*., 2008). A solid adsorber (Amberlite XAD-7) was also used as a carrier phase to reduce the actual benzene concentration, for the cultivation of a benzene-degrading iron-reducing enrichment (Kunapuli *et al*., 2008).

In several studies, anaerobic benzene degradation occurred in enrichment cultures, but attempts to isolate the anaerobic benzene-degrading microorganism failed in the majority of these studies. One

possible reason for the difficulty in isolating the bacteria could be that benzene is degraded in syntrophic relationships under anaerobic conditions. Syntrophic benzene degradation was suggested to occur in a methanogenic enrichment (Da Silva and Alvarez, 2007) and iron-reducing enrichments (Botton *et al*., 2007; Kunapuli *et al*., 2007). Using stable isotope probing (SIP) with $^{13}C_6$-benzene, Kunapuli *et al*. (2007) obtained indications that benzene degradation in the enrichment involved a syntrophy, where members of the Clostridia primarily oxidize benzene and partially share electrons from benzene with members of the *Desulfobulbaceae* as syntrophic partners. The precise mode of syntrophic interactions within the consortium remains to be discerned.

The elucidation of the anaerobic benzene degradation pathway

The mechanisms of activation and further degradation of benzene are still unknown. The possible initial steps are hydroxylation, carboxylation and methylation, and subsequent transformation to the central aromatic intermediate benzoyl-CoA, which is further degraded to CO_2 (Fig. 4). Evidence for benzene methylation is very scarce. Sofar, only in a nitrate-reducing and methanogenic enrichment culture evidence for methylation was observed (Ulrich *et al*., 2005). Evidence for benzene hydroxylation was recently found in the anaerobic benzene-degrading *Dechloromonas* strain RCB (Chakraborty and Coates, 2005) and in several enrichment cultures (Ulrich *et al*., 2005; Botton and Parsons, 2007). Recently, it was demonstrated that caution should be taken in interpreting hydroxylated benzene derivatives as metabolic intermediates of anaerobic benzene degradation (Kunapuli *et al*., 2008). Phenol was also identified as an intermediate at high concentration in a benzene-degrading iron-reducing enrichment culture. However, it was clearly shown that phenol was formed abiotically by autoxidation of benzene during the sampling and analysis procedure as a result of exposure to air. The authors suggested that autoxidation during sampling could also be the case in the study involving the anaerobic benzene degradation pathway of *Dechloromona*s strain RCB (Chakraborty and Coates, 2005).

Carboxylation is the most likely initial step in the anaerobic degradation of benzene and some other non-substituted aromatic hydrocarbons, such as naphthalene and phenanthrene (Zhang and Young, 1997; Meckenstock *et al*., 2000). Benzoate has been identified as an intermediate of anaerobic benzene degradation under different electron-accepting conditions (Caldwell and Suflita, 2000; Phelps *et al*., 2001; Chakraborty and Coates, 2005; Ulrich *et al*., 2005; Kunapuli *et al*., 2008). Kunapuli *et al*. (2008) demonstrated that in a benzene-degrading iron-reducing enrichment culture the carboxyl group of benzoate derived from the bicarbonate buffer, indicating a direct carboxylation of benzene. Moreover, indications for carboxylation of benzene were also obtained for the benzene-degrading denitrifying *Azoarcus* strain DN11 and a benzene-degrading sulfate-reducing enrichment (Kasai *et al*., 2007; Musat and Widdel, 2007). Carboxylation is probably the most reasonable activation mechanism, but other biochemical mechanisms for anaerobic benzene degradation under different electron-accepting conditions may exist.

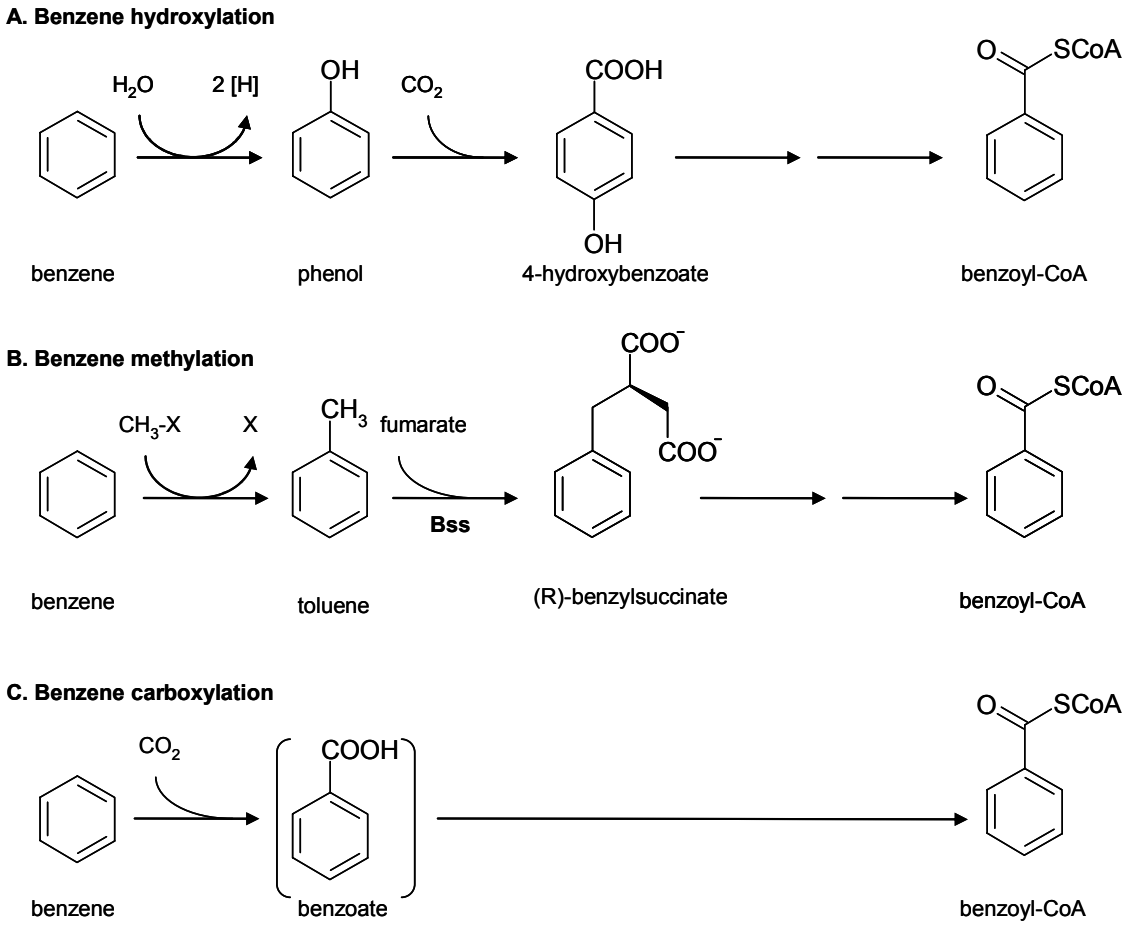


Figure 4. Possible mechanisms of the initial steps of benzene degradation under anaerobic conditions: A, benzene hydroxylation; B, benzene methylation; C, benzene carboxlation.

Stable isotope fractionation

The assessment of biodegradation in contaminated aquifers has become an issue of increasing importance in the recent years. To some extent, this can be related to the acceptance of intrinsic bioremediation or monitored natural attenuation as a means to manage contaminated sites (Meckenstock *et al*., 2004; Fischer *et al*., 2007). Several laboratory and field studies assessing biodegradation of contaminants via stable isotope analysis have been performed recently. These studies demonstrated that stable isotope fractionation can be used to obtain isotope fractionation factors which can be used to quantify the extent of *in situ* biodegradation of individual contaminants. Furthermore, the different isotope enrichment factors can be related to the respective biochemical reactions. Therefore, stable isotope fractionation can be used for a qualitative or even a quantitative assessment of biodegradation of benzene in the environment.

Recently, carbon and hydrogen isotope fractionation factors for aerobic and anaerobic benzene degradation in enrichments and pure cultures were determined in order to decipher the biodegradation pathway (Mancini *et al*., 2003; Fischer *et al*., 2008). Biodegradation of benzene by *Alicycliphilus denitrificans* strain BC under both oxic and chlorate-reducing conditions was also included in the study by Fischer *et al*. (2008) (Table 1). The carbon enrichment factors were higher than reported for dihydroxylation activation of benzene, but corresponded more to reported range of carbon enrichment factors for monohydroxylation. The hydrogen enrichment factors observed for strain BC were in the same range as that observed for the monooxygenase-catalyzed reactions (Fischer *et al*., 2008). The

observed carbon and hydrogen enrichment factors strongly indicate that benzene is attacked by a monooxygenase under aerobic and chlorate-reducing conditions. These results confirmed the results of the molecular biology experiments with strain BC in which also evidence for benzene degradation by monooxygenases was obtained (Chapter 5).

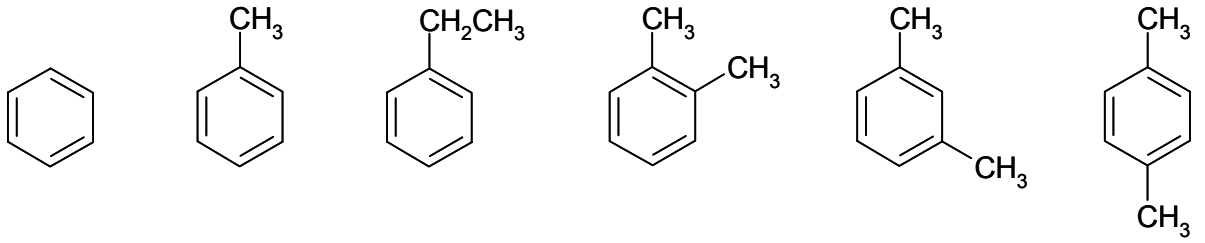
Table 1. Comparison of carbon and hydrogen enrichment factors (ε_C, ε_H) in aerobic and anaerobic benzene degradation.

Culture	Enzymatic pathway	ε_C [‰]	ε_H [‰]	Reference
Burkholderia sp. (aerobic)	unknown	-3.5 ± 0.3	-11 ± 2	(Hunkeler *et al*., 2001)
Acinetobacter sp. (aerobic)	unknown	-1.5 ± 0.1	-13 ± 1	(Hunkeler *et al*., 2001)
Rhodococcus opacus strain B-4	dihydroxylation	-1.3 ± 0.2	No enrichment, ±5 [(a)]	(Fischer *et al*., 2008)
Pseudomonas putida strain ML2	dihydroxylation	-0.7 ± 0.1	No enrichment, ±5 [(a)]	(Fischer *et al*., 2008)
Ralstonia picketii strain PKO1	monohydroxylation	-1.7 ± 0.2	-11 ± 4	(Fischer *et al*., 2008)
Cupriavidus necator ATCC 17697	monohydroxylation	-4.3 ± 0.4	-17 ± 11	(Fischer *et al*., 2008)
Nitrate-reducing enrichment	unknown, C-H bond cleavage expected	-2.2 ± 0.4 -2.4 ± 0.1	-29 ± 4 -35 ± 6	(Mancini *et al*., 2003)
Sulfate-reducing enrichment	unknown, C-H bond cleavage expected	-3.6 ± 0.3	-79 ± 4	(Mancini *et al*., 2003)
Methanogenic enrichment	unknown, C-H bond cleavage expected	-1.9 ± 0.1 -2.1 ± 0.1 -2.0 ± 0.1	-60 ± 3 -59 ± 4 -59 ± 3	(Mancini *et al*., 2003)
Sulfate-reducing mixed culture	unknown	-1.9 ± 0.3	-59 ± 10	(Fischer *et al*., 2008)
Alicycliphilus denitrificans strain BC (aerobic)	unknown	-2.6 ± 0.8	-16 ± 4	(Fischer *et al*., 2008)
Alicycliphilus denitrificans strain BC (chlorate)	unknown	-1.5 ± 0.5	-18 ± 6	(Fischer *et al*., 2008)

[a] Expected range for ε_H given by the uncertainty of hydrogen isotope analysis.

Nederlandse samenvatting

Aromatische koolwaterstoffen, zoals benzeen, tolueen, ethylbenzeen en xyleen (Fig. 1, vaak afgekort als BTEX), zijn een belangrijk bestanddeel van ruwe olie en olie destillaten (met name benzine) en worden daarnaast veel gebruikt in industriële processen. BTEX komen op verschillende plaatsen voor in het milieu als gevolg van menselijke activiteiten, zoals lozingen en lekkages van ondergrondse opslagtanks. Op diverse plaatsen zijn in de bodem, het grondwater en het sediment (waterbodem) hoge concentraties BTEX aangetroffen. BTEX zijn mobiel, met als gevolg verspreiding via het grondwater, en zijn toxisch. Benzeen is in lage concentraties al kankerverwekkend. Daarom is de laatste jaren veel aandacht besteed aan het saneren van BTEX verontreinigingen in de grond en het grondwater.



Figuur 1. Structuurformules van benzeen, tolueen, ethylbenzeen, *ortho*-xyleen, *meta*-xyleen en *para*-xyleen.

Voor de bodemsanering van BTEX verontreiniging is afbraak door micro-organismen (bioremediatie) een interessante optie. Verschillende soorten micro-organismen zijn in staat om BTEX aëroob (in de aanwezigheid van zuurstof) volledig af te breken. In het milieu zijn BTEX echter vaak aanwezig op plaatsen, bijvoorbeeld diep in het grondwater, waar geen zuurstof aanwezig is. Anaërobe bioremediatie door bacteriën zou daarom een aantrekkelijk alternatief zijn voor de sanering van locaties die verontreinigd zijn met BTEX. Er is in de afgelopen tientallen jaren een breed scala aan micro-organismen geïdentificeerd die TEX onder anaërobe omstandigheden kunnen omzetten naar onschadelijke eindprodukten, maar voor benzeen is dit in veel mindere mate het geval. Daarom is de afbraak van benzeen een mogelijk knelpunt onder anaërobe omstandigheden. Er is namelijk nog weinig kennis over de afbraak van benzeen in afwezigheid van zuurstof. Met name de bacteriën betrokken bij de anaërobe benzeenafbraak en de anaërobe afbraakroute zijn nog niet bekend.

In **hoofdstuk 1** is een overzicht gegeven van het onderzoek van de afgelopen decennia naar de aërobe en anaërobe afbraak van BTEX, waarbij de focus op benzeen ligt. De aërobe afbraak van benzeen is de laatste decennia veelvuldig bestudeerd en diverse bacteriën zijn in staat om benzeen aëroob af te breken. Aërobe afbraak vindt plaats met behulp van oxygenases die de aërobe afbraak van benzeen initiëren door het inbouwen van één (mono-oxygenase enzymen) dan wel twee (di-oxygenase enyzmen) zuurstofatomen in de stabiele aromatische ringstructuur van benzeen. Verschillende bacteriën zijn geïsoleerd die tolueenafbraak koppelen aan de reductie van nitraat. De meeste van deze nitraatreducerende bacteriën behoren tot het geslacht *Azoarcus* of *Thauera*. Er zijn ook tolueenafbrekende bacteriën geïsoleerd die sulfaat en ijzer(III) reduceren. IJzerreducerende tolueenafbrekende bacteriën behoren tot het geslacht *Geobacter*. De eerste stap in de anaërobe tolueenafbraakroute is de additie van een fumaraat molecuul aan de methylgroep van tolueen waarbij benzylsuccinaat onstaat; het enzym verantwoordelijk voor deze reactie is benzylsuccinaat synthase (BssABC).

De anaërobe afbraak van benzeen is zowel *in situ* als onder laboratoriumomstandigheden aangetoond onder verschillende redoxomstandigheden. Er zijn tot nu toe vier nitraatreducerende bacteriën beschreven die benzeen anaëroob kunnen afbreken, twee *Dechloromonas* en twee *Azoarcus* stammen. De anaërobe afbraakroute van benzeen is nog niet bekend, maar er zijn globaal drie mogelijkheden beschreven in de literatuur voor de initiële stap: carboxylering (naar benzoaat), hydroxylering (naar fenol) en methylering (naar tolueen). Deze drie afbraakroutes leiden elk tot het centrale aromatische intermediair benzoyl-CoA, dat vervolgens wordt afgebroken tot CO_2.

Eén van de twee anaërobe benzeenafbrekende *Dechloromonas* soorten kan tevens benzeenafbraak koppelen aan de reductie van perchloraat (ClO_4^-) en chloraat (ClO_3^-). (Per)chloraat reductie is een interessant proces omdat hierbij moleculaire zuurstof (O_2) wordt gevormd (Fig. 3, hoofdstuk 7). Deze zuurstof wordt vervolgens gebruikt voor de aërobe afbraak van verbindingen die moeilijk of niet af te breken zijn onder anaërobe omstandigheden, zoals benzeen. Perchloraat wordt vooral geproduceerd als ammoniumperchloraat (NH_4ClO_4), dat bijvoorbeeld gebruikt wordt als raketbrandstof. Met name in de VS worden op diverse plaatsen in het milieu (met name in het grondwater) perchloraat en chloraat aangetroffen en deze stoffen beschouwt men als verontreinigingen. Echter, uit het bovenstaande mag blijken dat ze ook gebruikt kunnen worden als *in situ* zuurstofdonor voor de afbraak van bijvoorbeeld benzeen.

Het doel van het onderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, was om meer inzicht te krijgen in de afbraak van benzeen en andere aromatische koolwaterstoffen door anaërobe bacteriën. De aanpak bestond uit het uitvoeren van batch experimenten geënt met BTEX-verontreinigd materiaal (grond of sediment) of geënt met BTEX-afbrekende culturen die in voorgaande onderzoeken waren verkregen. BTEX afbraak is bestudeerd onder verschillende redoxomstandigheden (bijvoorbeeld met nitraat en ijzer(III)) en daarnaast is ook de benzeenafbraak met chloraat bestudeerd. De nadruk lag hierbij op de fysiologie en de fylogenie van de betrokken bacteriën.

In **hoofdstuk 2** is de afbraak van tolueen en benzeen onder verschillende redoxomstandigheden bestudeerd in batch experimenten met materiaal afkomstig van een ijzerreducerende aquifer, die verontreinigd is door uitloging van BTEX uit een stortplaats (Banisveld stortplaats nabij Boxtel). Benzeen werd niet afgebroken gedurende de duur van het experiment (circa één jaar). Tolueen, daarentegen, werd afgebroken met nitraat, opgelost (Fe(III)NTA)) en amorf (FeOOH) ijzer(III) en mangaan(IV)oxide (MnO_2). In daarop volgende ophopingen en verdunningsseries werd tolueen afgebroken met MnO_2 en Fe(III)NTA. Moleculaire technieken toonden aan dat de Fe(III)- en Mn(IV)-reducerende cultuur werden gedomineerd door een bacterie die meest verwant is aan een bacterie in de subgroep van *Betaproteobacteriën*, namelijk *Sterolibacterium denitrificans* Chol-1S^T (94,6% gelijkenis op basis van 16S rRNA), een cholesterol-afbrekende denitrificerende bacterie. Na diverse verdunningsseries in agar medium werd een reincultuur van deze tolueenafbrekende bacterie verkregen. De isolatie en verdere karakterisering van deze bacterie is beschreven in **hoofdstuk 3**. Deze bacterie, stam G5G6, was in staat om op te tolueen te groeien met nitraat, Fe(III) en MnO_2 als elektronenacceptor. Naast tolueen, was stam G5G6 ook in staat te groeien met ethylbenzeen, fenol, *p*-cresol, *m*-cresol, benzaldehyde and *p*-hydroxybenzoaat. Stam G5G6 beschikt over het *bssA* gen dat codeert voor de alfa subunit van benzylsuccinaat synthase, het enzym dat de eerste stap in de anaërobe tolueenafbraak katalyseert (Fig. 2, hoofdstuk 7).

Stam G5G6 heeft een aantal interessante fysiologische en fylogenetische eigenschapppen, namelijk:

1. Het is de eerste tolueenafbrekende ijzerreduceerder die niet tot de *Geobacteraceae* familie behoort.
2. het is de eerste tolueen afbrekende nitraatreduceerder die niet tot het *Azoarcus* of *Thauera* (*Betaproteobacteriën)* of tot het *Magnetospirillum* geslacht (*Alfaproteobacteria*) behoort.
3. tot nu toe zijn er slechts twee andere bacteriën beschreven die zowel tolueen als ethylbenzeen kunnen afbreken onder anaërobe omstandigheden.
4. stam G5G6 groeit alleen met aromatische stoffen, niet met meer gangbare stoffen zoals acetaat, waterstof, lactaat, pyruvaat en glucose.
5. stam G5G6 kan geen AQDS (anthrachinon 2,6-disulfonaat, een humus-analoge verbinding) gebruiken als elektronenacceptor, terwijl de meeste ijzerreducerende bacteriën dit wel kunnen.

Op basis van de fysiologische en fylogenetische karakteristieken kan de tolueenafbrekende stam G5G6 beschouwd worden als een stam van een tot nu toe onbekend geslacht van de *Betaproteobacteriën*. De voorgestelde naam van het micro-organisme is *Georgfuchsia toluolica*.

In **hoofdstuk 4 en 5** is het onderzoek naar de benzeenafbraak met chloraat als elektronenacceptor beschreven. Op het Laboratorium voor Microbiologie werd een stabiele, benzeenafbrekende, chloraatreducerende ophopingscultuur verkregen met materiaal afkomstig uit een reactor van TNO. Deze reactor was geënt met slib van een waterzuiveringsinstallatie en BTEX verontreinigde grond en er vond benzeenafbraak met chloraat plaats. De snelheid waarmee de afbraak van benzeen in deze stabiele ophopingscultuur plaatsvond, was vergelijkbaar met in literatuur vermelde aërobe afbraaksnelheden. Moleculaire technieken toonden aan dat deze stabiele cultuur uit (tenminste) vijf verschillende soorten bacteriën bestond. Experimenten waarin de cultuur werd gekweekt met andere elektronendonoren en –acceptoren, toonden dat één van de bacterien in de cultuur in staat zou moeten zijn om benzeen af te breken met chloraat. De rol van de andere bacteriën in de stabiele ophopingscultuur kon niet worden vastgesteld, maar wel werd aangetoond dat er zeer waarschijnlijk overdracht van zuurstof tussen de verschillende soorten in de ophopingscultuur plaatsvindt.
Uit de ophopingscultuur is uiteindelijk een bacterie geïsoleerd die benzeen met chloraat kan afbreken. Deze bacterie, stam BC, groeit op benzeen en enkele andere aromatische stoffen als elektronendonor in de aanwezigheid van zuurstof of (zonder zuurstof) met chloraat als elektronenacceptor. Stam BC is een denitrificerende bacterie, maar de stam kan geen benzeen afbreken met nitraat als elektronenacceptor. Stam BC is het meest verwant aan *Alicycliphilus denitrificans* K601^T, een cyclohexanolafbrekende nitraatreducerende *Betaproteobacterie*. De 16S rRNA genen vertonen 99,7% gelijkenis en DNA-DNA hybridisatie resulteerde in 74,5% gelijkenis. Fysiologisch gezien zijn er echter enkele grote verschillen. Bijvoorbeeld, stam BC kan geen cyclohexanol afbreken en stam K601^T kan geen chloraat reduceren. De fysiologische karakterisering van beide *Alicycliphilus* stammen is beschreven in **hoofdstuk 6**. Omdat tijdens chloraatreductie zuurstof wordt gevormd, ligt het voor de hand dat in stam BC benzeen via een aëroob afbraakmechanisme wordt afgebroken. Bij dit mechanisme dienen tenminste de enzymen chloraat reductase, chloriet dismutase en oxygenases aanwezig te zijn. Chloraat reductase en chloriet dismutase activiteit zijn aangetoond in het cel extract van stam BC. Vervolgens is het DNA van stam BC gescreend op de aanwezigheid van genen die coderen voor deze drie typen enzymen. Chloriet dismutase is niet aangetoond, maar dit kan onder andere verklaard worden door het feit dat nog weinig bekend is over deze genen en dat daardoor de gebruikte primers niet voldoende geschikt zijn voor de detectie van deze genen. Wel werden vermeende benzeenmonooxygenase (*BC-BMOa*) en catechol 2,3-dioxygenase (*BC-C23O*) genen aangetroffen in stam BC. Aanvullende experimenten zijn nodig om de exacte functie van deze genen

op te helderen. Echter, de aanwezigheid van deze genen en de fysiologische eigenschappen van stam BC onderbouwen de hypothese van het voorgestelde afbraakmechanisme. Op basis van de resultaten van de fysiologische, biochemische en genetische experimenten, stellen we een benzeenafbraakroute voor in stam BC zoals weergegeven in figuur 3 van hoofdstuk 7. In deze route is zuurstof niet alleen de elektronenacceptor, maar wordt zuurstof ook gebruikt voor de initiële stap in de omzetting van de aromatische ringstructuur door oxygenases. Dit onderzoek heeft aangetoond dat aërobe afbraak van benzeen kan plaats vinden onder, in wezen, anaërobe omstandigheden. Hierbij produceren de chloriet dismutase enzymen de moleculaire zuurstof die de oxygenases nodig hebben voor de afbraak van de aromatische benzeen ring. Dit betekent dat aërobe afbraakprocessen plaats kunnen vinden onder omstandigheden waarbij geen externe zuurstof is toegediend. Dit principe kan worden toegepast in de praktijk bij de bioremediatie van BTEX verontreinigingen.

In **hoofdstuk 7** zijn de bevindingen uit dit proefschrift samengevat en vergeleken met recente inzichten en is een doorkijk naar de toekomst gegeven. Hieruit blijkt dat de toevoeging van (per)chloraat een aantrekkelijke optie zou kunnen zijn voor de gestimuleerde *in situ* sanering van locaties die verontreinigd zijn met benzeen. De bij (per)chloraatreductie geproduceerde zuurstof zou in het algemeen gebruikt kunnen worden voor de afbraak van stoffen die anaëroob moeilijk of niet zijn af te breken maar die aëroob snel worden afgebroken. Voorbeelden van zulke stoffen zijn naftaleen, monochloorbenzeen (MCB), vinylchloride (Vc), methyl-tertiaire-butylether (MTBE), lange en korte alkaanketens en verschillende specifieke verbindingen zoals bijvoorbeeld aromatische aminen.
Het toekomstige onderzoek naar de anaërobe afbraak van benzeen zal zich naar verwachting richten op met name:

- Het identificeren van de bacteriën die betrokken zijn bij de anaërobe benzeenafbraak. De focus zal wellicht meer verschuiven naar het onderzoek naar ophopingsculturen en de interacties in deze culturen, omdat het verkrijgen van reinculturen die benzeen anaëroob afbreken erg moeilijk is gebleken en vooralsnog waarschijnlijk geen doorbraak op dit terrein is te verwachten.
- Het ophelderen van de afbraakroute van benzeen onder anaërobe omstandigheden. Vooralsnog lijkt carboxylering de best onderbouwde anaërobe afbraakroute voor benzeen te zijn. Echter, andere afbraakroutes zijn, wellicht afhankelijk van de redoxomstandigheden, mogelijk.
- Het aantonen en kwantificeren van anaërobe benzeenafbraak in het veld (met name door stabiele isotopen fractionering). Stabiele isotopenfractionering kan ook opheldering geven over de manier waarop benzeen wordt afgebroken. Bij deze methode wordt gebruik gemaakt van isotopen van koolstof (^{12}C and ^{13}C) en waterstof (^{1}H and ^{2}H). De reactiesnelheid van de lichte en zware isotopen zijn verschillend in bepaalde (afbraak)reacties, met het gevolg dat in de resterende benzeenfractie relatief meer van het zware isotoop (^{13}C en ^{2}H) aanwezig is. Bovendien is recentelijk aangetoond dat verhouding $^{12}C/^{13}C$ en $^{1}H/^{2}H$ informatie kan geven over het benzeenafbraakmechanisme (bijvoorbeeld mono- of dioxygenase of anaëroob). Uit experimenten met stam BC, gegroeid met zuurstof en chloraat, bleek dat dat de koolstof en waterstof fractionering bij beide groeicondities overeen kwamen met gerapporteerde waarden voor mono-oxygenase reacties. Deze resultaten sluiten aan bij de moleculaire biologische experimenten (hoofdstuk 5), waarbij ook bewijs voor mono-oxygenases was verkregen.

De hierboven genoemde thema's waren de afgelopen decennia ook al onderwerp van onderzoek, maar uit onderzoek beschreven in dit proefschrift en onderzoek elders blijkt dat er nog steeds vele onduidelijkheden en leemtes zijn.

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Curriculum vitae

Sander Andreas Bernardus Weelink werd geboren op 19 februari 1979 te Doetinchem. In 1997 behaalde hij zijn VWO-diploma aan het Marianum te Groenlo. Vervolgens werd in dat zelfde jaar begonnen met de studie Milieuhygiëne aan de Landbouwuniversiteit in Wageningen. Binnen deze studie werd de specialisatie Milieutechnologie gevolgd.

Tijdens zijn studie deed hij twee afstudeervakken. Het eerste afstudeervak, bij de leerstoelgroep Milieutechnologie, was gericht op de biologische reductie van stikstofoxiden, afkomstig van rookgassen, in ijzer-EDTA oplossingen. Het tweede afstudeervak, bij het Laboratorium voor Microbiologie, was gericht op de reductie van geoxideerde metalen door halorespirerende microorganismen. Vervolgens deed hij een stage bij TNO, Milieu, Energie en Procesinnovatie (TNO-MEP, afdeling Milieubiotechnologie) te Apeldoorn. Hier deed hij onderzoek naar de ontwikkeling van een anaërobe membraanbioreactor voor de zuivering van afvalwater. In augustus 2002 behaalde hij *cum laude* zijn doctoraal diploma.

Na zijn studie werd begonnen met promotieonderzoek bij het Laboratorium voor Microbiologie, bij de werkgroep Microbiële Fysiologie (november 2002-november 2006). De resultaten van dit onderzoek staan beschreven in dit proefschrift. Sinds 1 februari 2007 is hij werkzaam bij advies- en ingenieursbureau Tauw in Deventer, bij de afdeling Bedrijven Bodem. Daar houdt hij zich voornamelijk bezig met *in situ* saneringstechnieken, natuurlijke afbraak en het opstellen van saneringsonderzoeken en –plannen.

Dankwoord

Mijn proefschrift is dan eindelijk af! Bijna zes jaar na het begin van mijn promotieonderzoek is het nagenoeg af, echter ik had gehoopt dat het wat sneller af zou zijn. Bij de start van mijn promotieonderzoek had ik me voorgenomen om na vier jaar al een groot gedeelte van het proefschrift op papier te hebben staan. Na vier jaar onderzoek waren de resultaten er wel (ik had vele data verzameld achter mijn favoriete, stokoude GC436), maar van het schrijven was nog niet zo veel terecht gekomen. De afgelopen twee jaar heb ik in mijn vrije tijd aan het proefschrift gewerkt. Hierbij kwam ik er gaandeweg achter dat het lastig was de vaart erin te houden. Tegen het einde van 2007 begon ik te twijfelen of het proefschrift ooit af zou komen en ik was op een bepaald punt beland waarbij de gedachte 'nu of nooit' door mijn hoofd spookte. Vanaf dat moment heb ik meer tijd vrijgemaakt voor het schrijven van het proefschrift en dit heeft uiteindelijk geresulteerd in het boekje dat nu voor je ligt. Nu is het moment gekomen om terug te kijken en mensen te bedanken die op een of andere manier hebben bijgedragen aan de totstandkoming van dit proefschrift.

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Sander
Oktober 2008

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

CERTIFICATE

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

Sander Weelink

Born on: *19 February 1979* at: *Doetinchem, The Netherlands*

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

Place: *Wageningen* Date: *18 November 2008*

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. R. Leemans	Dr. A. van Dommelen

The SENSE Research School declares that Mr. Sander Weelink has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 36 ECTS, including the following activities:

SENSE PhD courses:

- **Environmental Research in Context**
- **Research Context Activity: "Evaluation of the WIMEK/SENSE research school activities by PhD students of the Laboratory of Microbiology, Wageningen University, 2003-2006"**
- **Euro Summerschool: Trends in Bioremediation of Soils and Sediments**
- **Environmental Biotechnology**

Other Phd and MSc courses:

- **Selected applications of Isotope methods in marine geochemistry and biogeochemistry**
- **Techniques for writing and presenting Scientific Paper**
- **Centa course Scientific writing**
- **Course: Organising and supervising MSc-thesis**
- **Course: Safe handling of radioactive materials and sources**
- **Course: Emergency team member (Dutch: BHV)**

Research and Management Skills:

- **Detailed elaboration research set-up, work plan and time schedule of the PhD project**

Management Skills:

- **Member of the General Board of the research institute (Laboratory of Microbiology).**

Oral Presentations:

- **Soil & Water 2004, 2 – 3 June 2004, Zeist, The Netherlands**
- **Soil & Water 2005, 1 – 2 June 2005, Zeist, The Netherlands**
- **SENSE Summersymposium, 23 June 2005, Ede, The Netherlands**
- **Presentation during visit to laboratories of Marburg and Konstanz, May 2006, Marburg, Konstanz, Germany**
- **SENSE Summersymposium, 23 June 2006, Amsterdam, The Netherlands**
- **PhD-Post Doc seminars at the Laboratory of Microbiology, yearly presentation, 2002-2006, Wageningen, The Netherlands**
- **Workgroup meetings, workgroup Microbial Physiology at the Laboratory of Microbiology, about 2 times a year a presentation, 2002-2006, Wageningen, The Netherlands**
- **Colloquium at Laboratory of Microbiology, 17January 2007, Wageningen, The Netherlands**

Mr. J. Feenstra
SENSE Coordinator PhD Education and Research

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