Anaerobic degradation of limonene and *p*-xylene in freshwater enrichment cultures

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2009

Anaerobic degradation of limonene and *p*-xylene in fresh-water enrichment cultures

Dissertation

zur

Erlangung des Grades eines

Doktors der Naturwissenschaften

Dr. rer. nat.

Fachbereich Biologie/Chemie

der Universität Bremen

vorgelegt von

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Bremen, 2009

Die Untersuchungen zur vorliegenden Doktorarbeit wurden am Max-Planck-Institut für Marine Mikrobiologie in Bremen durchgeführt.

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Aceasta carte este dedicata parintilor mei care m-au sustinut cu dragoste si incredere, de-a lungul acestei „calatorii“ in necunoscut.

Contents

List of abbreviations

BssA	α-subunit of benzylsuccinate synthase
bssA	gene encoding the α-subunit of benzylsuccinate synthase
BTEX	benzene, toluene, ethylbenzene, xylenes
CARD-FISH	catalyzed reporter deposition - fluorescence *in situ* hybridization
DAPI	(4',6-diamidino) 2-phenylindol
$\Delta G^{0'}$	delta G (free energy) under standard conditions (25°C, 1 atm, pH 7.00)
FISH	fluorescent *in situ* hybridization
GC	gas chromatography
PAH	polyaromatic hydrocarbons
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SR	sulfate reduction
SRB	sulfate reducing bacteria
TEA	terminal electron acceptor

Summary

The anaerobic degradation of hydrocarbons has been intensively explored in the last decade yielding insights into new physiological capabilities and biochemical pathways. However, for a few hydrocarbons, e.g. *p*-xylene, it proved to be more difficult to enrich microorganisms and to isolate pure strains. Thermodynamically, the mineralization of hydrocarbons is least favorable under anoxic conditions and especially under methanogenic conditions. In my thesis, two enrichment cultures were characterized, a methanogenic freshwater enrichment culture grown on limonene, the most abundant monoterpene in nature, and a denitrifying freshwater enrichment culture grown on *p*-xylene.

The methanogenic enrichment culture consumed limonene with proportional formation of methane. The full cycle 16S rRNA gene approach revealed the presence of *Archaea* related to *Methanosaeta* and *Methanoculleus* and *Bacteria* related to *Syntrophobacteraceae, Bacteroidetes,* and the Candidate Division OP3. This candidate phylum lies within the *Planctomycetes, Chlamydiae, Verrucomicrobia, Lentisphaerae* superphylum and has no member in culture. Hence nucleic acid probes were developed to target the OP3-phylotype. The probe detected very small spherical cells, which lived alone or attached to larger cells and represented 18% of the total DAPI-stained population. Thus they may play an important role in limonene degradation. The other *Bacteria* were mainly *Deltaproteobacteria* (13%), and only 1% were *Bacteroidetes*. Together with a new EUB-338 probe specific for the OP3 cells, the bacterial 338 probe mixture detected 40% of the total cells whereas the *Archaea* probe detected 33%. The presence of several phylotypes suggests that more then one syntrophic bacterium and one methanogenic archaeon are involved in limonene degradation to methane gas and carbon dioxide.

Syntrophic bacteria were isolated using fumarate and lactate as organic substrates for fermentation. All seven strains belonged phylogenetically to *Deltaproteobacteria*, with 11% 16S rRNA gene dissimilarity to *Desulfoarculus baarsii*. Initial co-culture experiments with *Methanosarcina mazei* showed growth on lactate or fumarate in the presence of limonene. However, their role in limonene degradation could not demonstrated.

Denitrifying enrichment cultures were established with a freshwater sediment mix and *p*-xylene as sole electron donor and carbon source. Several batch transfers and liquid dilution-to-extinction series enriched a curved rod morphotype, 0.5 $\times$ 2 µm in size. This dominant morphotype (96% of all cells) was as well identified as the dominant phylotype (91-95%) using the 16S rRNA full cycle approach. The organism affiliated phylogenetically with *Betaproteobacteria* and not with other anaerobic hydrocarbon degrading microorganisms from the *Azoarcus-Thauera* clade. Their closest relative was a group of steroid-

degraders: *Denitratisoma oestradiolicum, Sterolibacterium denitrificans* and strain 72Chol. This enrichment cultures coupled complete mineralization of *p*-xylene to denitrification of nitrate to dinitrogen gas. By gas chromatography mass spectrometric analysis of metabolites found in cell extracts, (4-methylbenzyl) succinate and (4-methylbenzyl) itaconate were identified, supporting an activation mechanism by addition to fumarate. A gene fragment for a benzylsuccinyl synthase could be sequenced, and revealed amino acid similarities with TutD from the well known toluene degrading denitrifier, *Thauera aromatica*.

This study has revealed that anaerobic hydrocarbon degradation involves a broad diversity of microorganism, even outside the phylum *Proteobacteria*. My thesis established the participation of novel organisms to anaerobic hydrocarbon degradation and revealed for the first time the morphology of Candidate phylum OP3 cells.

Introductory remarks on anaerobic degradation

Deep sediment layers of lakes, rivers and soils, as well aquifers and underground waters, host organisms capable to degrade organic matter. In these habitats oxygen is limiting, so anaerobic life is the only alternative. Today, two types are known, fermentation and respiration of alternative electron acceptors. Nitrate, metals (Fe^{3+} or Mn^{4+}), sulfate or carbon dioxide are the major electron acceptors respired by microorganisms.

In my thesis I will focus on two different anaerobic respiratory processes, having as terminal electron acceptors 1) nitrate and 2) carbon dioxide. In both cases the microorganisms gained energy by degradation of hydrocarbons.

Hydrocarbons are an ubiquitous class of natural compounds that consist solely of hydrogen and carbon atoms. There are two major categories of hydrocarbons, aliphatic and aromatic. Aliphatic hydrocarbons are saturated (alkanes) and unsaturated (alkenes and alkynes), linear (e.g. *n*-alkanes), branched or cyclic, whereas aromatic hydrocarbons are characterized by the presence of one or more benzene rings (mono- or polycyclic) which can be substituted by aliphatic alkyl groups (e.g. alkylbenzenes). Some hydrocarbons are produced by plants (Cheesbrough and Kolattukudy, 1984; Singer et al., 2003), animals (Hylemon and Harder, 1999), insects (Page et al., 1997), and microorganisms (Birch and Bachofen, 1988; Harder and Foss, 1999). Saturated aliphatic and aromatic hydrocarbons are also formed at very high temperatures and pressures, after deposition and sedimentation of dead organic matter. These are the main constituents of oil and natural gas (Tissot and Welte, 1984). Besides, numerous hydrocarbons are extensively produced, or refined in industry for different purposes. Because hydrocarbons are widespread in the environment, the microbes degrading them are expected to be as ubiquitous (Spormann and Widdel, 2000).

In the last three decades, anaerobic microbes capable of hydrocarbon degradation were studied extensively (Widdel et al., 2006) especially the ones mediating „useful“ processes. Bioremediation of petroleum polluted environments or obtaining novel industrial biocatalysts are some examples. Beyond the applications of anaerobic hydrocarbon degradation, the ultimate goal is to identify unusual enzymes and reactions catalyzed by microbes, to discover how interactions are established between organisms and between organisms and their environment, and what role anaerobic hydrocarbon degraders play in the global carbon cycle.

Part I

Anaerobic aliphatic hydrocarbon degradation

1 Anaerobic degradation of saturated aliphatic hydrocarbons

Saturated aliphatic hydrocarbons or alkanes are nonreactive hydrocarbons which contain exclusively apolar σ-bonds. They are constituents of petroleum and natural gas and enter the biosphere from gas seeps and underground oil plumes, or are produced by microorganisms and plants (Tissot and Welte, 1984; Cheesbrough and Kolattukudy, 1984; Birch and Bachofen, 1988). In the present world, oil refineries, chemical industry and human activities are supplementary sources of alkanes in the environment.

Denitrifying bacteria degrading alkanes had been isolated from ditch sediments (Ehrenreich et al., 2000) whereas mesophilic sulfate reducing bacteria were isolated from oil fields, oil waste-waters (strain Hxd3, *Desulfoglaeba alkanexedens*), petroleum contaminated estuary sediments (strain AK-01), marine sediments (*Desulfatibacillum aliphaticivorans*), and deep sea hydrocarbon seeps (strain BuS5) (Aeckersberg et al., 1991, 1998; So and Young, 1999; Cravo-Laureau et al., 2004a; Davidova et al., 2006; Kniemeyer et al., 2007). Cold adapted alkane degrading organisms have not been described yet. However, the potential for alkane degradation was observed at low temperatures in sulfate reducing enrichment cultures growing with ethane at 12°C (Kniemeyer et al., 2007). One thermophilic isolate, had been purified from Guyamas basin sediments and degrades C_6-C_{16} *n*-alkanes while growing optimally at 60°C (Rueter et al., 1994). Thermophilic propane degrading bacteria were also enriched from Guyamas basin sediments (Kniemeyer et al., 2007). So far there are no studies on *n*-alkane degradation under iron reducing conditions, and only few studies showed *n*-alkane degradation under methanogenic conditions (Zengler et al., 1999; Anderson and Lovely, 2000). Branched and cyclic alkanes had been less studied although it was shown they can be utilized under sulfate reducing or denitrifying conditions in enrichments from contaminated aquifers (Bregnard et al., 1997; Rios-Hernandez et al., 2001).

Phylogeny

All isolated, described anaerobic microbes (Table 1.1) capable to degrade alkanes are grouped within the phylum *Proteobacteria* (Widdel and Rabus, 2001; Widdel et al., 2006; Wentzel et al., 2007). Denitrifiers that utilize alkanes belong to two different *Proteobacteria* classes, *Betaproteobacteria* and *Gammaproteobacteria* (Ehrenreich et al., 2000). Whereas, sulfate-reducing bacteria degrading alkanes, group within *Deltaproteobacteria* (Aeckersberg et al., 1991, 1998; So and Young, 1999; Cravo-Laureau et al., 2004a; Davidova et al.,

2006; Kniemeyer et al., 2007) and are related to *Desulfosarcina - Desulfococcus* group or are classified as members of two novel genus, *Desulfatibacillum* (Cravo-Laureau et al., 2004a) and *Desulfoglaeba* (Davidova et al., 2006).

Table 1.1: Isolated microorganisms capable of alkane utilization under anaerobic conditions.

PHYLUM	ORGANISM	ALKANE	RESPIRATION	REFERENCE
β-*Proteobacteria*	*Azoarcus* sp. strain HxN1	C_6- C_8	Nitrate	(Ehrenreich et al., 2000)
β-*Proteobacteria*	Strain OcN1	C_8- C_{12}	Nitrate	(Ehrenreich et al., 2000)
γ-*Proteobacteria*	Strain HdN1	C_{14}- C_{20}	Nitrate	(Ehrenreich et al., 2000)
γ-*Proteobacteria*	*Marinibacter* sp. BC-36, 38, 42	C_{18}	Nitrate	(Bonin et al., 2004)
γ-*Proteobacteria*	*Pseudomonas balearica* sp. BerOC6	C_{15}- C_{18}	Nitrate	(Grossi et al., 2008)
δ-*Proteobacteria*	Strain BuS5	C_3- C_4	sulfate	(Kniemeyer et al., 2007)
δ-*Proteobacteria*	*Desulfoglaeba alkanexedens* Lake	C_6- C_{10}	sulfate	(Davidova et al., 2006)
δ-*Proteobacteria*	*Desulfoglaeba alkanexedens* ALDC	C_6- C_{12}	sulfate	(Davidova et al., 2006)
δ-*Proteobacteria*	Strain TD3	C_6- C_{15}	sulfate	(Rueter et al., 1994)
δ-*Proteobacteria*	Strain Hxd3	C_{12}- C_{20}	sulfate	(Aeckersberg et al., 1991)
δ-*Proteobacteria*	Strain Pnd3	C_{14}- C_{17}	sulfate	(Aeckersberg et al., 1998)
δ-*Proteobacteria*	Strain AK-01	C_{13}- C_{18}	sulfate	(So and Young, 1999)
δ-*Proteobacteria*	*Desulfatibacillum aliphaticivorans*	C_{13}- C_{18}	sulfate	(Cravo-Laureau et al., 2004a)

Mechanism of activation

Two mechanisms for alkane activation under anaerobic conditions have been proposed (Figure 1.1): (1) homolytic C-H bond cleavage followed by addition to fumarate and (2) carboxylation (Spormann and Widdel, 2000; Widdel and Rabus, 2001; Widdel et al., 2006; Wentzel et al., 2007).

(1) The suggested addition to fumarate is supported by: i) identification of alkylsuccinates in cell extracts of enrichment cultures and pure cultures (Kropp et al., 2000; Rabus et al., 2001; Rios-Hernandez et al., 2001; Callaghan et al., 2006), ii) the presence of C-even fatty acids or C-odd-alkane fed cultures and C-odd or methyl branched fatty acids in C-even-alkane fed cultures, iii) the detection of deuterium labeled (methylalkyl) succinate after incubation with labeled alkane (Callaghan et al., 2006), iv) the detection of an organic radical by electron paramagnetic resonance (EPR) spectroscopy (Rabus et al., 2001) which suggests the existence of a radical enzyme, and v) the sequencing of genes, potentially encoding the subunits of (methylalkyl) succinate-synthase of cells grown on an *n*-alkane (Grundmann et al., 2008). An alternative addition mechanism to fumarate was observed by metabolite analysis, and fatty acid profiling of propane growing cultures (Kniemeyer et al., 2007). In these cultures the expected (isopropyl) succinate was not found, but (*n*-propyl) succinate was identified suggesting an activation both by addition of the secondary and terminal carbon of propane to fumarate. This mechanism could be especially useful for ethane activation, that consists only of terminal carbon atoms (Kniemeyer et al., 2007).

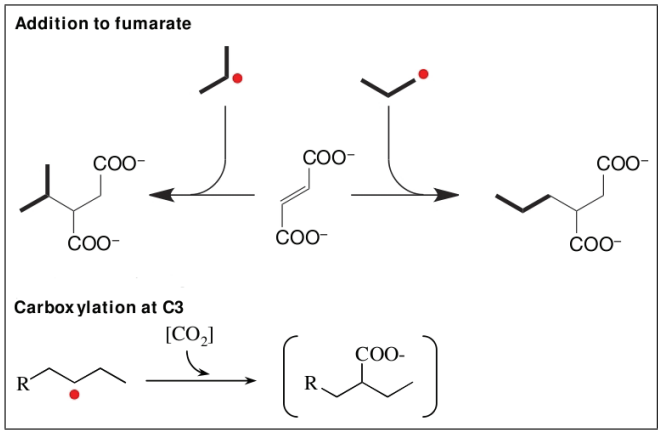

Figure 1.1: Activation of *n*-alkanes by addition to fumarate at the secondary and primary carbon atom and carboxylation to the third carbon atom (modified from Kniemeyer et al. (2007); Grossi et al. (2008)). With red are specified the carbon atoms where the alkane is activated.

(2) The other activation mechanism for *n*-alkanes is carboxylation at the tertiary carbon atom (So et al., 2003) as suggested for the sulfate reducing strain Hxd3 (Aeckersberg et al., 1991, 1998) . This organism when grown on C-odd or C-even alkanes has a C-even or C-odd fatty acid composition which results from alkane carboxyl addition at C_3, subsequently followed by loss of two subterminal carbon atoms (So et al., 2003).

2 Anaerobic degradation of unsaturated aliphatic hydrocarbons

Unsaturated aliphatic hydrocarbons are characterized by the presence of double (alkenes) or triple (alkynes) bounds. A particular category of alkenes are isoprenoids or aliphatic terpenes, derived from isoprene units with or without other substituents (e.g. hydroxyl groups). According to the number of C_5- units they are subdivided into hemiterpenes (C_5, e.g. isoprene), monoterpenes (C_{10}, e.g. limonene), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}, e.g. squalene), tetraterpenes (C_{40}, e.g. β-carotene) and other polyterpenes (>C_{45}, e.g. gutta-percha). Mono- and sesquiterpenes are the chief constituents of plant essential oils while the other are constituents of resins, waxes, balsams and rubber. Mono- and sesquiterpenes are produced as secondary metabolites by plants, where they are used as repellents, attractants, or to maintain integrity against stress caused by dehydration (Kesselmeier and Staudt, 1999). Alkenes are ubiquitous, naturally occurring as they are synthesized not only by plants but also by microorganisms, insects and larger organisms.

Aliphatic hydrocarbons with triple bounds are not common in nature. The Earth's atmosphere has only trace amounts of acetylene (0.00004 ppm), whereas on other planets from our Solar System (Jovian planets and Titan) acetylene is in appreciable amounts (up to 4 ppm), where it is probably formed by photolysis of methane (Oremland and Voytek, 2008). Such an atmospheric composition of acetylene was predicted for the Archean times of our planet. Therefore, acetylene is considered a potential key player in the bioenergetics and evolution of Earth's first anaerobic ecosystem, and there is a lot of interest in its biodegradation (Oremland and Voytek, 2008).

In general, unsaturated bonds are more prone to attack than the simple sigma bonds in alkanes or aromatic bonds. Both alkenes and alkynes could be relatively easily degraded chemically as well as biologically. As a result they are rarely found in petroleum and condensates, where their origin is related to abiogenic alteration of *n*-alkanes rather then to the preservation of the original molecules (Curiale and Frolov, 1998).

Microorganisms capable of unsaturated aliphatic hydrocarbon degradation under anaerobic conditions, were isolated from petroleum polluted marine sediments (Aeckersberg et al., 1998; Cravo-Laureau et al., 2004a,b), brackish or freshwater anoxic mud (Schink, 1985b; So and Young, 1999; Cravo-Laureau et al., 2007), oil production plants wastewaters (Aeckersberg et al., 1991), activated sludge and forest ditches (Foss et al., 1998; Foss and Harder, 1998). Until now there are no proofs of unsaturated aliphatic hydrocarbon

degradation in iron reducing conditions. However, under methanogenic conditions, it had been shown that both alkenes and alkynes can be degraded (Schink, 1985a,b; Harder and Foss, 1999).

Table 2.1: Isolated microorganisms capable of alkene utilization under anaerobic conditions. All alkenes are 1-ene when not specified. (M) stands for cyclic isoprenoic alkenes (monoterpenes), with one or two double bonds (dienes) at different positions within the cycle or on the alkyl chain. (T) is for the triterpene squalene with six double bounds.

PHYLUM	ORGANISM	ALKENE	RESPIRATION	REFERENCE
β-*Proteobacteria*	*Thauera terpenica*	C_{10}(M)	Nitrate	(Foss and Harder, 1998)
β-*Proteobacteria*	*Castellaniella defragrans*	C_{10}(M)	Nitrate	(Foss et al., 1998)
γ-*Proteobacteria*	*Pseudomonas balearica* BerOC6	C_{17}	Nitrate	(Grossi et al., 2008)
γ-*Proteobacteria*	*Marinobacter* sp. 2sq31	C_{30}(T)	Nitrate	(Rontani et al., 2002)
δ-*Proteobacteria*	Strain Hxd3	C_{14}, C_{16}, C_{18}	sulfate	(Aeckersberg et al., 1991)
δ-*Proteobacteria*	Strain Pnd3	C_{14}, C_{16}, C_{18}	sulfate	(Aeckersberg et al., 1998)
δ-*Proteobacteria*	Strain AK-01	C_{15}, C_{16}	sulfate	(So and Young, 1999)
δ-*Proteobacteria*	*Desulfatibacillum aliphaticivorans*	C_7- C_{23}	sulfate	(Cravo-Laureau et al., 2004a)
δ-*Proteobacteria*	*Desulfatibacillum alkenivorans*	C_8- C_{23}	sulfate	(Cravo-Laureau et al., 2004b)
δ-*Proteobacteria*	*Desulfatiferula olefinovorans*	C_{14}- C_{23}	sulfate	(Cravo-Laureau et al., 2007)

Phylogeny

Denitrifying, sulfate reducing (Table 2.1) or fermenting (Schink, 1985b) isolates capable of unsaturated aliphatic hydrocarbon degradation are all clustered in the same *Proteobacteria* phylum as other hydrocarbon degrading microorganisms. Denitrifying strains, isolated on *n*-alkenes and aliphatic monoterpenes (e.g. α-terpinene, limonene) belong to the same *Proteobacteria* classes as *n*-alkane degrading anaerobes, namely *Betaproteobacteria* and *Gammaproteobacteria*. The only described denitrifier degrading *n*-alkenes was associated with *Pseudomonas* (Grossi et al., 2008), whereas denitrifying alkenoic monoterpene degraders grouped with other anaerobic hydrocarbon degrading microorganisms from the genus *Thauera* (Foss and Harder, 1998) and the newly described genus *Castellaniella* (Foss and Harder, 1998; Kampfer et al., 2006). Sulfate reducing isolates are all members of *Deltaproteobacteria* and group within the *Desulfosarcina-Desulfococcus* group (Aeckersberg et al., 1991, 1998; So and Young, 1999), and two new genera *Desulfatibacillum* (Cravo-Laureau et al., 2004b,a) and *Desulfatiferula (Cravo-Laureau et al., 2007)*. The only alkyne degrading strain isolated until now is the acetylene utilizer, *Pelobacter acetylenicus* (Schink, 1985b) which belongs to a genus within *Deltaproteobacteria*, that comprises of strictly anaerobic Gram negative bacteria which could act as important syntrophic oxidants of primary aliphatic alcohols in sediments and sludges (Schink, 2006).

Mechanism of activation

In a first study on anaerobic degradation of unsaturated hydrocarbons, Schink (1985a) demonstrated mineralization of hexadecene under methanogenic conditions and speculated on the activation by addition of hydroxyl-groups at the double bound (Schink, 1985a). Recent studies brought evidence for this pathway, by recovery of [deuterated]-fatty acids and -alcohols from cultures grown on labeled hexadecene and pentadecene (So et al., 2003; Grossi et al., 2007, 2008). The attack seems to be initiated at the double bound by a hydroxylase, resulting in the corresponding alcohols, and as well by the addition of undefined organic carbon units (methyl or ethyl-groups) at subterminal carbon atoms of the carbon chain. In the case of the multiple alkenoic terpene, squalene, the attack had been proposed to occur, as well, by hydration of double bounds to symmetric tertiary diols as seen in *Marinobacter* strain 2sq31 under denitrifying conditions (Rontani et al., 2002).

For cyclic alkenoic monoterpenes it was previously suggested that (e.g. α-pinene, limonene, 2-carene) the attack is initiated at the sp^2-hybridized C_1 atom. Whereas if the reaction occurred at an isolated sp^2 C atom, the resulting carbocation could not be stabilized. A rearrangements of π-bonds so the C_1 atom becomes sp^2 hybridized was observed in *C. defragrans* grown on a monoterpene containing an sp^3 hybridized C_1atom (Heyen and Harder, 1998). It was suggested (Hylemon and Harder, 1999) that monoterpenes are transformed into an initial ionic compound which stays intracellular for use in metabolism. Later it had been shown that geranic acid is formed during growth of *C. defragrans* on different cyclic alkenoic monoterpenes (Heyen and Harder, 2000). Today, there is no other information on the ring opening or the initial attack of cyclic monoterpenes. The understanding of alkene metabolism in anaerobic microorganism is still a field of active research, and novel enzymes and reactions are to be discovered.

Acetylene is the only unsaturated aliphatic hydrocarbon whose activation was studied under fermenting conditions in *Pelobacter acetylenicus* (Schink, 1985b). The hydrating reaction of acetylene to acetaldehyde is catalyzed by a monomeric tungsten-[4Fe-4S] protein, acetylene-hydratase (Meckenstock et al., 1999; Einsle et al., 2005; Seiffert et al., 2007). The activity of this enzyme is extremely oxygen sensitive and is irreversibly lost upon exposure to air with the degradation of the [4Fe-4S] cluster (Meckenstock et al., 1999). The tungsten center binds a water molecule that is activated by an aspartate residue. The active water molecule will further attack acetylene, however it is yet unknown if the active water molecule will act as an electrophile or nucleophile during the attack (Seiffert et al., 2007).

3 Anaerobic limonene degradation: results and discussions

3.1 Scope of the study

Reason: Decayed plant material is transported by rain, runoff and wind into rivers and lakes, especially during autumn foliage, when large amounts of alkenoic hydrocarbons, like monoterpenes are deposited and sedimented. Deeper sediment layers are a favorable habitat of anaerobic microbes, and when all the electron acceptors are consumed only methanogenic communities can degrade the remaining organic matter. Previous studies showed that alkenoic monoterpenes, such as 2-carene and α-pinene, can be degraded under methanogenic conditions. Interestingly, when cultures were grown on other two monoterpenes, α- phellandrene and sabinene, these two monoterpenes were aromatized to *p*-cymene (Harder and Foss, 1999; Foss and Harder, 1998). Limonene, the most widespread monoterpene, can be degraded under denitrifying conditions (Foss et al., 1998; Foss and Harder, 1998). However, there is more to learn about its biomineralization under anaerobic conditions since the biology involved in its degradation is yet not understood.

Scope: This work focused on a methanogenic enrichment culture degrading limonene with the following aims:

1. to demonstrate limonene degradation under methanogenic conditions.
2. to identify and quantify the members of the microbial community in methanogenic limonene degrading enrichment using a molecular approach (Amann et al., 1995).
3. to isolate microorganisms that are potentially involved in syntrophic limonene degradation, and determine if limonene could have a toxic effect on their growth.
4. to clarify if the new isolates play a role in limonene degradation in the presence of a methanogenic partner.

(see Part II - 7.1)

3.2 Degradation of limonene under methanogenic conditions

Earlier studies suggested that monoterpenes undergo non-biological catagenic processes in anoxic environments rather than biological transformations (Tissot and Welte, 1984). However, latest research did show biodegradation of alkenoic terpenes as well as of other alkenes under methanogenic conditions (Harder and Foss, 1999; Schink, 1985a). Regardless of the abundance of limonene in nature, its biodegradation under methanogenic conditions was never studied.

This study was initiated by incubation of enriched cultures (Harder and Foss, 1999) in the absence of an electron acceptor with 5% limonene (vol/vol in HMN). Cultures with a volume of 300 ml accumulated more then three liters of gas in circa two years. After 14 transfers, we monitored enrichments for circa 7 months, to demonstrate limonene utilization under methanogenic conditions. Cultures were incubated with different concentration of limonene (2% or 5% in HMN) in the presence or absence of additional acetate (2 mM).

After circa 3 months of lag phase the optical density increased and reached its maximum during the 6^{th} month of incubation (Figure 3.1A). The consumption of limonene (Figure 3.1B) ranged between 22% and 48% from the total limonene added (Table 10.2). Cultures without acetate showed a larger consumption of limonene ranging between 22% and 48% of the total limonene added. In sterile controls the limonene amount did not decrease significantly (Figure 3.1B). All enrichment cultures released methane gas (Figure 3.1C) which corresponded to the amount of limonene consumed (45% to 75% methane recovery) (Table 10.2). We added acetate and cysteine since these are required growth factors or stimulatory nutrients for different groups of methanogens (Whitman et al., 2006). However, some microorganisms could use these compounds as organic energy sources (Whitman et al., 2006). Our data (Table 10.2) support the stoichiometry: $C_{10}H_{16}$ + 6 H_2O →7 CH_4+ 3 CO_2

In cultures incubated with 2% limonene in HMN (v/v) and without additional acetate, we observed that acetate was transitory formed (0.6 mM to 1.75 mM) (Figure 3.1A). In these cultures without acetate methane production started later (Figure 3.1C) compared to limonene degrading cultures supplemented with acetate. It is possible that substrate fermentation is decoupled from methanogenesis. Therefore in the absence of acetate, acetate-dependent methanogens would not survive. The addition and accumulation of acetate was reported to inhibit syntrophic degradation of fatty acids and benzoate (Ahring and Westermann, 1987; Fukuzaki et al., 1990; Warikoo et al., 1996). It is possible that larger amounts of acetate could have an „end-metabolite“ inhibitory effect on the syntrophic microbial community, and finally on the limonene degradation process itself. However, such inhibitory effect was not observed in cultures incubated on limonene (5% v/v in HMN) in the presence of acetate (2 mM).

RELEASING REACTION - ACETATE AND HYDROGEN AS TRANSFER METABOLITES
Releasing acetate and hydrogen:
$C_{10}H_{16}$ + 10 $H_2O \rightarrow$ 5 $C_2H_4O_2$ + 8 H_2 {$\Delta G^{0\prime}$= + 156 kJoule (mol limonene)$^{-1}$}
CONSUMING REACTIONS
Consuming acetate:
$C_2H_4O_2 \rightarrow CH_4$ + CO_2 {$\Delta G^{0\prime}$= - 49 kJoule (mol methane)$^{-1}$}
Consuming hydrogen:
4 H_2+ $CO_2 \rightarrow CH_4$+ 2 H_2O {$\Delta G^{0\prime}$= - 131 kJoule (mol methane)$^{-1}$}
RELEASING REACTION - FORMATE AND HYDROGEN AS TRANSFER METABOLITES
Releasing formate and hydrogen:
$C_{10}H_{16}$ + 20 $H_2O \rightarrow$ 10 CH_2O_2 + 18 H_2 {$\Delta G^{0\prime}$= + 949 kJoule (mol limonene)$^{-1}$}
CONSUMING REACTIONS
Consuming formate:
4 $CH_2O_2 \rightarrow$ CH_4 + 3 CO_2 + 2 H_2O {$\Delta G^{0\prime}$= - 284 kJoule (mol methane)$^{-1}$}
Consuming hydrogen:
4 H_2+ $CO_2 \rightarrow CH_4$+ 2 H_2O {$\Delta G^{0\prime}$= - 131 kJoule (mol methane)$^{-1}$}
TOTAL LIMONENE DEGRADATION
$C_{10}H_{16}$ + 6 $H_2O \rightarrow$ 7 CH_4 + 3 CO_2 {$\Delta G^{0\prime}$= - 348 kJoule (mol limonene)$^{-1}$}

Limonene degradation in the absence of an electron acceptor, is less exergonic when compared to aerobic or other anaerobic respiratory processes. Consequently, microorganisms adapt to the exploitation of minimal energy spans, by establishing mutualistic interactions - syntrophy. In such microbial co-operative interaction the carbon and electron flow follows a rather simple pattern. Polymers are hydrolyzed into oligo- and monomers by extracellular enzymes produced by primary fermenting bacteria (Schink and Stams, 2006). These organisms ferment the monomers furthermore to fatty acids, alcohols, succinate, lactate etc. Secondary fermenters or syntrophic bacteria convert the short chain fatty acids and alcohols to acetate, formate, hydrogen and carbon dioxide. Methanogens act as scavengers of reducing equivalents, which otherwise would inhibit further degradation of the initial substrate (Schink and Stams, 2006; Stams et al., 2006).

A similar microbial food-chain could degrade limonene (Figure 3.2). Fermenting bacteria could degrade limonene to different fatty acids and alcohols and then break them down to smaller molecules. Syntrophic bacteria could utilize smaller fatty acids as substrates and their catabolic end-products would feed the methanogenic community (Figure 3.2). Acetate build-up and consumption in cultures incubated with limonene, suggest acetate exchange between syntrophs and methanogens. Similarly, acetate was regarded as a likely intermediate for alkane degradation under methanogenic conditions (Dolfing et al., 2008).

Table 3.1: Limonene consumption and methane production in enrichment cultures with limonene in the presence or absence of traces of acetate (2mM). Control cultures (Ctrl) showed how much of the methane produced is related to the endogenous carbon sources carried with the inocula, or to the traces of acetate added to the media.

Culture name	Inocula (% v/v)	Initial limonene (mmol)	Final limonene (mmol)	Final methane (mmol)	Methane (mmol) from:			Methane recovery (%)***
					Limonene (T)	Acetate (T)	Cysteine (T)	
165A	10	10	7.8	12.3	15.4	0.6	0.3	75
165B	10	10	6.3	12.0	25.9	0.6	0.3	45
165Ctrl	10	-	-	0.9	-	0.6	0.3	97
164A	**1.3	4.6	2.6	9.7	14.0	-	0.3	65
164B	**1.3	4.6	2.4	8.2	15.4	-	0.3	50
164Ctrl	**1.3	-	-	0.1	-	-	0.3	-

* The theoretical methane formed from limonene was calculated according to equation 1. Whereas the theoretical methane formed from acetate and cysteine was calculated from the equations: i) $C_2H_4O_2 \rightarrow CH_4 + CO_2$ and ii) $4\ C_3H_7NO_2S + H_2O \rightarrow 5\ CH_4 + 7\ CO_2 + 4\ NH_3 + 4\ H_2S$, respectively.

**The inocula was 20 $\times$ concentrated cell suspension added to the media in the mentioned amount.

***The methane recovered is the percentage of methane produced by cultures in relation to the theoretical value ($\Sigma\ CH_4$ from limonene acetate and cysteine).

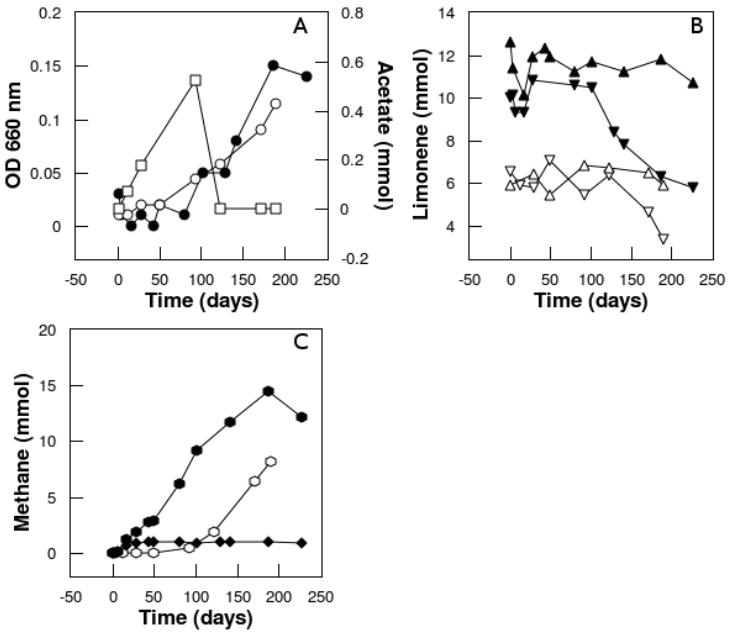

Figure 3.1: Limonene degradation in two enrichment cultures incubated in the presence (filled symbols) or absence (opened symbols) of acetate (2mM) under methanogenic conditions. Panel A shows the optical density increase in the two enrichment cultures, growing with 5% (filled circle) and 2% (opened circles) limonene in HMN. There is a transient accumulation of acetate in enrichment cultures incubated only with limonene (empty squares). Panel B shows the consumption of limonene in the same enrichment cultures, with 5% (filled down-triangles) and 2% (empty down-triangles) limonene in HMN versus incubated controls without inocula (corresponding filled and empty up-triangles). In panel C is shown the methane production in enrichment cultures (corresponding filled and empty hexagons) versus an inoculated control with acetate 2 mM (filled diamonds).

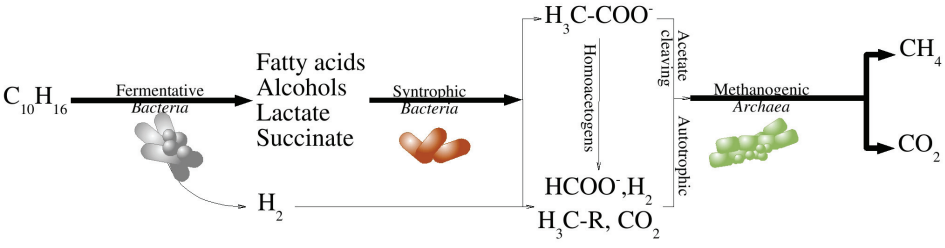

Figure 3.2: Model of microbial interactions in methanogenic enrichment cultures thriving on limonene. At least two groups of *Bacteria*, primary fermenters and secondary fermenters (syntrophs), are involved in limonene degradation to acetate, formate, hydrogen and carbon dioxide. These small substrates could be used by methanogenic *Archaea* which produce carbon dioxide and methane gas. Acetate could be as well utilized by homoacetogens.

3.3 Microbial community composition

Few studies showed the biodegradation of unsaturated hydrocarbons under methanogenic conditions (Schink, 1985b,a; Harder and Foss, 1999), and solely one microorganism was isolated in the absence of alternative electron acceptors, the acetylene fermenting *Pelobacter acetylenicus* (Schink, 1985b, 2006). The scope of this study was to identify phylogenetically what microorganisms play a role in limonene degradation and determine their abundance. For this purpose, we used the 16S rRNA approach (Amann et al., 1995). We established clone libraries for *Bacteria* and *Archaea* 16S rRNA genes. ARDRA analysis was performed on 327 Bacteria clones and 141 *Archaea* clones. The representative clones for each ARDRA pattern were sequenced. We partially sequenced 141 *Archaea* clones and 130 *Bacteria* clones. Phylogenetic analysis were performed with 34 *Bacteria* and 28 *Archaea* representative 16S rRNA almost full length sequences. 16S rRNA gene sequences with more then 98.5% identity were regarded as one OTU or one phylotype. The quantitative analysis of the enrichment cultures, was done by CARD-FISH. This *in situ* hybridization method was preferred to the more usual mono-labeled FISH, to surpass: i) lower cell metabolic activities; or ii) different cell wall composition, which such a complex community might have.

Employing this culture-independent approach, we revealed the presence of unusual phylotypes spanning through the super-kingdoms of *Bacteria* and *Archaea*. *Bacteria* 16S rRNA gene sequences were represented by 11 OTUs (Table 2) related to: (i) *Bacteroidetes*, (ii) *Deltaproteobacteria,* (iii) Candidate Division OP3 and as well (iv) *Firmicutes* (Figure 3.3). *Archaea* 16S rRNA gene sequences were represented by 10 *Euryarchaeota* OTUs, related to microorganisms from the orders (v) *Methanomicrobiales* and (vi) *Methanosarcinalles* (Figure 3.4).

(i) Bacteroidetes. Five *Bacteroidetes* OTUs showed 12% to 16% sequence identity differences to their next cultured relative, *Prolixibacter bellariivorans*. The other two Bacteroidetes OTUs had 16% to 18% difference from their next in culture relative, *Alistipes putredinis.* All seven phylotypes showed large differences in between each-other (4% to 20%). Genus *Prolixibacter* is represented by facultative anaerobes, which ferment sugars by mixed-acid fermentation (Holmes et al., 2007). Whereas genus *Alistipes,* is represented by strictly anaerobic microorganisms, which thrive on complex substrates and have as main metabolic product, succinate (Rautio et al., 2003; Song et al., 2006). Considering the large phylogenetic differences, the phylotypes retrieved from limonene degrading enrichments likely belong to new genera, within family *Rikenellaceae.*

A *Bacteroidetes* probe, CF-319a matched *in silico* all retrieved phylotypes. However this probe matched only 1% of the DAPI stained cells in the enrichment cultures. *Bacteroidetes* are usually involved in breakdown and fermentation of complex organic material, however their low numbers during mid exponential growth could not explain the turnover of limonene.

(ii) Deltaproteobacteria. Two *Deltaproteobacteria* OTUs were identified, at 10% distance from each-other and 8% distance from the non-syntrophic *Syntrophobacter* sp. strain

TsuA1 (Figure 3.3). This *Syntrophobacter* strain is a sulfate reducer which grows on adipate and is able to utilize C_1- C_{12} straight fatty acids, C_2- C_{10} straight chain primary alcohols, 2-, 3- hexadionate, pyruvate, and lactate (Tanaka et al., 2000). Our *Syntrophobacter*-related phylotypes, were only far related to this sulfate-reducer, and showed even larger differences to syntrophic microorganisms of the family *Syntrophobacteraceae*. This implies that they belong to novel genera within the class *Deltaproteobacteria*. *Deltaproteobacteria,* including *Syntrophobacter*-related microorganisms were targeted by probe Delta-495a and made up for 13% of the entire microbial population. Most likely they play an important role in the break down fatty acids which are released by fermenting bacteria.

***(iii)* Candidate Division OP3.** Interestingly, one OTU represented by five sequences, was very far from anything cultured until now, with 24.2% difference to the nearest in culture relative, *Opitus* sp. VeSm13, from phylum *Verucomicrobia*. This phylotype showed higher 16S rRNA gene identity (16.7% different) to members of the uncultured phylum Candidate Division OP3. A newly designed probe, OP3-565, was used to target specifically OP3-related microorganisms, and very small and round shaped cells, found either alone or attached to larger cells that were not OP3-related (Figure 3.5), made up for 18% of the entire microbial community (Table 10.4).To double check the presence and quantity of Candidate Division OP3 member, we used probe Pla-46, a *Planctomycete* specific probe*,* which matched *in silico* the Candidate Division OP3 phylotype. When applied, this probe stained the same morphotype like OP3-565, accounting for 13% of the total detected cells.

Within Candidate Division OP3 there is no isolate available, so their metabolic capabilities are unknown. However, numerous 16S rRNA sequences of Candidate Division OP3 were recovered solely from anoxic habitats like: i) anoxic sediments of Yellowstone Hot Spring (Hugenholtz et al., 1998), ii) anoxic water body of Cariaco basin (Madrid et al., 2001), iii) groundwater of a gold mine (Lin et al., 2006) or of a pristine coastal aquifer (Lopez-Archilla et al., 2007) and iv) deep subsurface of the Antarctic continental shelf (Bowman and McCuaig, 2003) etc. Besides, OP3 sequences were found in two anaerobic and mesophilic chemostats thriving on propionate or butyrate (Shigematsu et al., 2006; Tang et al., 2007) which implies that OP3 members are anaerobes that might be involved in short chain fatty acid degradation.

(iv) Firmicutes. Our *Bacteria* clone library revealed also the presence of one *Firmicutes* phylotype. This sequence showed 96.3% similarity to *Syntrophomonas cellicola,* which grows syntrophically with *Methanobacterium formicium* on $C_4$to C_9 fatty acids (Wu et al., 2006). *S. cellicola* degrades C-even fatty acids to acetate whereas the C-odd fatty acids are broken down to propionate. Genus *Syntrophomonas* is generally involved in fatty acid degradation to shorter fatty acids (Schink and Stams, 2006). Hence *Syntrophomonas*-like organisms could play an intermediate role in limonene degradation between the „primary“ fermenters and the „secondary fermenters“ - syntrophs.

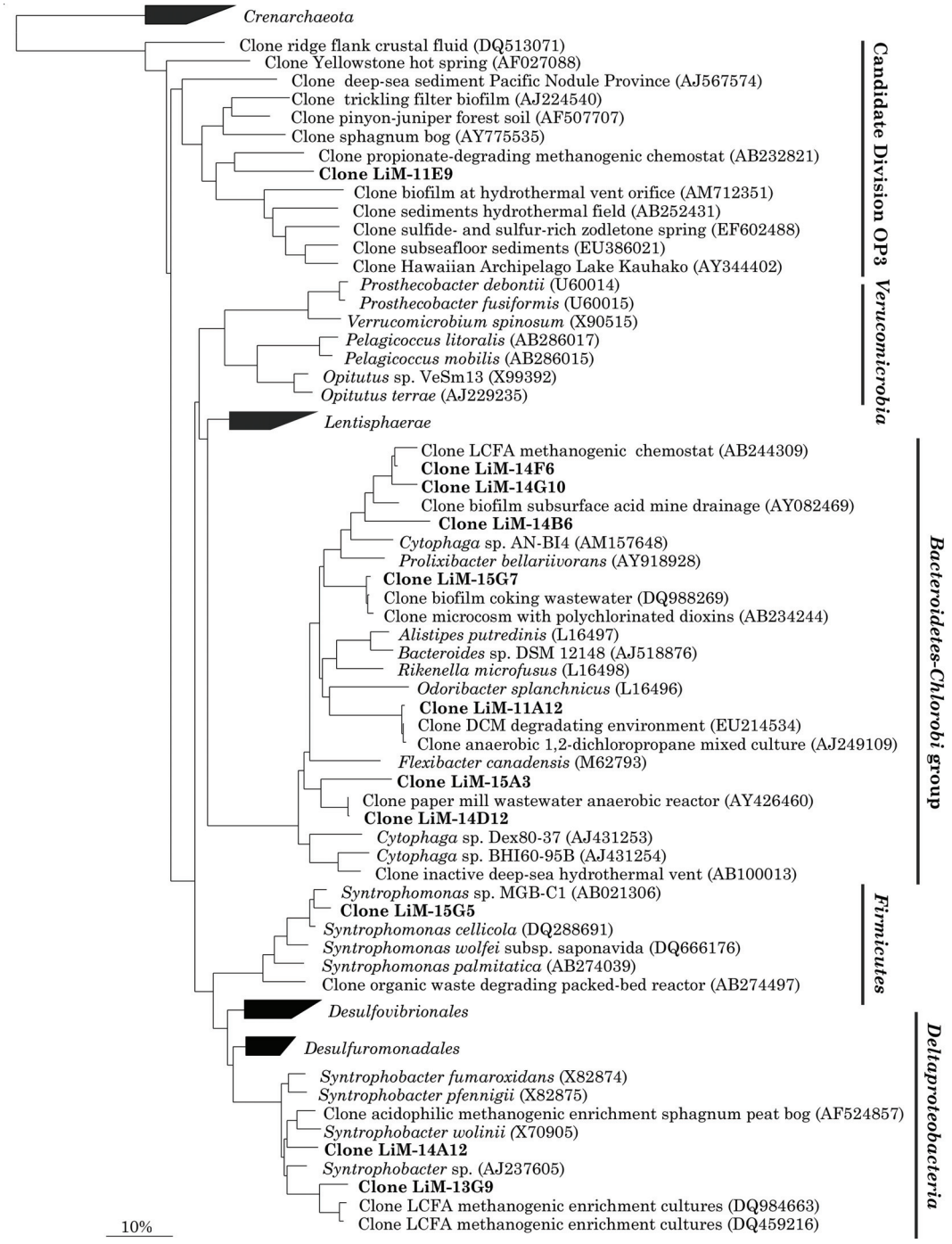

Figure 3.3: *M*aximum parsimony tree of *Bacteria* 16S rRNA gene sequences retrieved from limonene degrading methanogenic enrichment cultures. The representative *Bacteria* sequences obtained in this study are emphasized in bold letters. The accession number of reference sequences is shown in parenthesis. *Crenarchaeota* sequences were used as out-group. The scale bar corresponds to 10 substitutions per 100 nucleotides.

(v) Methanomicrobiales. Two Archaea phylotypes recovered from limonene degrading enrichments, had as closest in culture relative, *Methanoculleus palmolei* (88% to 92%) a highly irregular cocci, isolated from an anaerobic bioreactor treating wastewater of a palm oil mill (Zellner et al., 1998). *M. palmolei*, like most members of genus *Methanoculleus,* has complex nutritional requirements such as potassium and tungsten ions as growth promoters, or acetate as organic mineral supplement (Zellner et al., 1998; Whitman et al., 2006). In limonene degrading enrichments in the absence of supplementary acetate, we observed a longer period until methane formation started. Methane was formed likely after the bacterial community broke down limonene to acetate.

(vi) Methanosarcinalles. The order *Methanosarcinalles* was represented by 8 OTUs from the *Archaea* clone library, which were very similar or identical to the 16S rRNA gene of *Methanosaeta* sp. strain AMPB Zg and *Methanosaeta concilii. Methanosaeta concilii* was isolated from a pear waste digestor (Patel and Sprott, 1990) whereas *Methanosaeta* sp. AMPB-Zg was isolated from freshwater sediment (Scholten and Stams, 2000). Both were described as sheathed rods that aggregate into bundles. We identified such filaments with a *Methanosaetaceae* specific probe, MX-825, that targeted *in silico* all the *Methanosaeta* phylotypes from our *Archaea* clone library. However, during *in situ* hybridization experiments, MX-825 stained only 1% of the total cells from a limonene degrading enrichment. This probe stained *Methanosaeta*-like filaments heterogeneously as showed in a recent hybridization study on *M. concilii* (Kubota et al., 2008). Genus *Methanosaeta* comprises obligate anaerobes which use as sole energy source acetate, converting it into equimolar amounts of methane and carbon dioxide. The presence of *Methanosaeta* in cultures incubated with limonene in the absence of acetate, is an indirect proof for acetate release during limonene degradation under methanogenic conditions.

Kingdom specific probing. The *Eubacteria* general probe, Eub-338 (I), matched *in silico* most sequences from the *Bacteria* clone library, with the exception of Candidate Division OP3 phylotype. This phylotype did not match *in silico* any other published *Eubacteria* probes (Eub-338 II and III). Therefore we designed a new *Eubacteria* probe, Eub-338 (VI), which perfectly paired positions 338-355 on the 16S rRNA gene of Candidate Division OP3 sequences. These two probes when applied in equimolar amount, targeted 40% from the total DAPI stained cells. The *Archaea* probe, Arch-915, matched cells within *Methanosaeta*-like filaments and rods with flat ends, making up for 33% of the entire microbial community detected by DAPI. As control probe probe Non-338 was used, and gave one signal for each 300 DAPI stained cells. Circa 26% of the DAPI stained cells were not detected with HRP-labeled general probes (Figure 10.4). The reason could be that the general Arch-915 did not target homogeneously the *Methanosaeta*-like filaments but rather scarce cells through the filaments (Figure 10.4C and D), hence underestimating the abundance of the *Archaea* population. When mono-labeled FISH was applied, the *Archaea* abundance did not differ, but then OP3-phylotypes could not be detected.

The reasons why kingdom specific probes accounted only for 73% of the total DAPI stained cells, could be: i) an uneven permeabilization treatment of the different cell types

prior to hybridization, ii) dead cells or empty sheath stretches within *Methanosaeta* filaments, and iii) the existence of other unknown phylas in our enrichments which are not targeted by known phylogenetic probes and primers.

Nevertheless, these methanogenic enrichments thriving on limonene displayed a complex syntrophic community spread through the *Bacteria* and *Archaea* kingdoms. Limonene is most likely degraded by *Bacteria* to acetate, and then is further on metabolized by methanogenic *Archaea,* to methane and carbon dioxide. We identified unusual *Bacteria* phylotypes, which could be representatives of novel genus within *Rickenelaceae*, and *Syntrophobacteraceae.* We visualized and quantified members of the hitherto uncultured Candidate Division OP3, whose small round cells often attach to the surface of other bacteria.

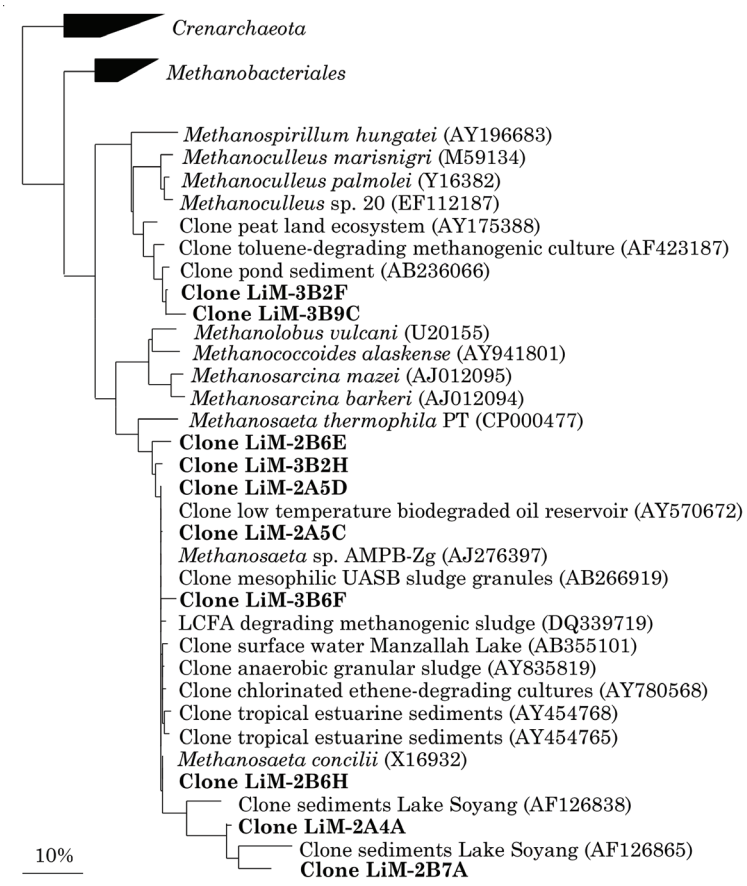

Figure 3.4: *M*aximum parsimony tree of *Archaea* 16S rRNA gene sequences retrieved from limonene degrading methanogenic enrichment cultures. The representative *Archaea* sequences obtained in this study are emphasized in bold letters. The accession number of reference sequences is shown in parenthesis. *Crenarchaeota* sequences were used as out-group. The scale bar corresponds to 10 substitutions per 100 nucleotides.

Table 3.2: Relative abundance of different groups of *Bacteria* and *Archaea* in methanogenic enrichment cultures thriving on limonene as quantified by CARD-FISH.

PROBE	TARGET	KINGDOM SPECIFIC (%)*	GROUP SPECIFIC (%)*
Eub-338 (I & VI)	*Eubacteria*	40	-
Non-338	Nonsense	0	-
Arch-915	*Archaea*	33	-
Delta-495a	δ-*Proteobacteria*	-	12
CF-319a	*Bacteroidetes*	-	1
Pla-46	*Planctomycetes*	-	**13
OP3-565	Candidate OP3	-	18
MX-825	*Methanosaeta*	-	1
Total recovery	-	73	32

*Numbers show the percentages of cells that hybridized to a probe versus DAPI-stained cells in the same visual field.

**This probe was used to target Candidate Division OP3 , since *in silico* it fully matched our OP3 sequences.The 13% Pla-46 targeted cells were excluded from the total recovery.

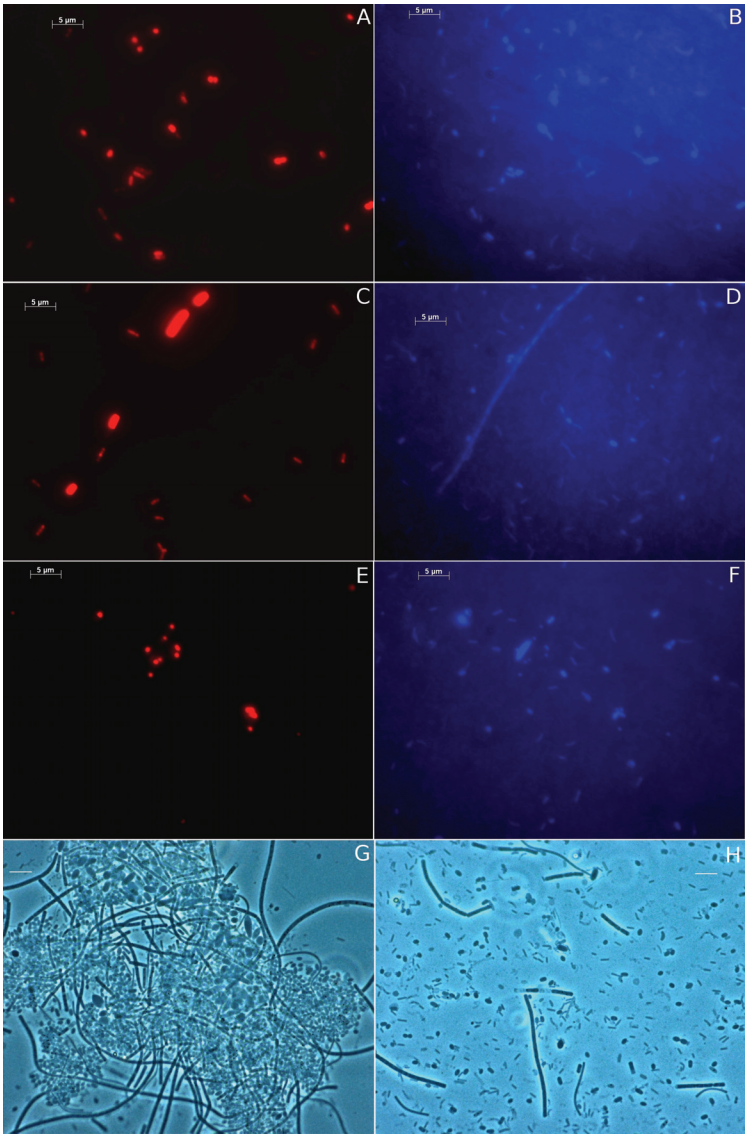

Figure 3.5: Microscopic images of samples from methanogenic enrichment cultures thriving on limonene, as visualized by epifluorescence microscopy (A-F) and phase contrast microscopy (G and H). The left panels represent samples stained by different HRP labeled probes. The red signals are given by the HRP catalyzed deposition of Alexa-594 tyramides. To the right are the same samples visualized by a DNA stain, DAPI. Panel A shows cells stained by the general Eubacteria probes, Eub-338 (I and VI). The same microscopic field is shown to the left, in panel B, with cells only stained by DAPI. In panel C are shown Arch-915 stained cells. The corresponding microscopic field visualized with DAPI is shown to the righ, in panel D. Panel E, depicts cells stained with the newly designed probe, OP3-565, specific for the Candidate Division OP3 phylotype from the *Bacteria* clone library. To the right is the same microscopic field visualized by DAPI. The two phase contrast microscopic images, show an aggregate (panel G) and the usual morphotypes (panel H) encountered in limonene degrading enrichment cultures. The scale bar is 5 µm for all images.

3.4 Isolation of novel *Deltaproteobacteria*

Syntrophic microorganisms capable of fatty acid and alcohol degradation are difficult to isolate, because they depend on the methanogenic partners for the removal of excess reducing equivalents. The use of such substrates that are more oxidized then the initial one is a well described procedure for isolation of syntrophic microorganisms (Schink, 1985a; Beaty and McInerney, 1987; Wallrabenstein et al., 1994, 1995a). For a better understanding of the relationships established between members of limonene degradation enrichments, we attempted isolation of syntrophic bacteria. Fumarate and lactate were used as substrates for microbial growth under fermenting conditions. We purified the strains by dilutions in solid agar.

Yellow-white colonies were obtained using the roll tube technique (Hungate, 1969). We selected 40 colonies, for transfer in freshwater methanogenic liquid media. Transfers took more then 6 months for growth and only 8 colonies developed into stable liquid cultures. From these, three fermented lactate (SynL-5-9-c1, SynL-5-9-c2 and SynL-65) and the other five fermented fumarate (SynF-5-17-c1, SynF-5-17-c2, SynF-5-17-c3, SynF-5-18-c1). Lactate isolated strains were represented by large vibrio shaped cells with an average size of 0.9 μm $\times$ 2.5 μm. The fumarate isolated strains were less curved and slightly smaller with an average size of 0.8 μm $\times$ 2.4 μm.

The phylogenetic position was determined by 16S rRNA sequence analysis (Figure 3.6). We obtained sequences of each isolate and compared them using the ARB software. All isolates were the representatives of a single phylotype with more then 99% 16S rRNA gene identity between each other. Their closest relative (91.2%) was a sequence retrieved from 4-methyl-benzoate degrading methanogenic enrichment (Wu et al., 2001). Their closest cultured relative, was *Desulfoarculus baarsii* (11% difference), a sulfate reducer that oxidizes formate, and does not oxidize lactate (Widdel, 1980, 1981). Our isolates are far related to members of genus *Syntrophobacter*, and even the nearest neighbor, *Syntrophobacter phennigii* (Wallrabenstein et al., 1995b), showed only 85% 16S rRNA gene identity.

To determine if these isolates are represented in limonene degrading enrichment cultures, DGGE was performed on isolates and DNA extracted from an enrichment culture. The amplified 16S rDNA gene of all isolates migrated to the same position in denaturing gel (Figure 3.7). Two of the fumarate isolates showed a second band at a higher position on the gel. This could be due to: i) impurity of cultures, so different DGGE patterns emerged; ii) each strain has two different 16S rRNA operons, which could result from an insertion or a higher G+C content in one of the 16S operons. We sequenced the dominant DGGE band that was common for all the isolated strains, and analyzed its relationships to the 16S rRNA genes of isolates and observed 100% identity (Figure 3.7). Clone libraries did not provide information on this phylotype, however a weak band was present at the same position with the isolates, in a limonene degrading enrichment culture, implying that these organisms are present in enrichment cultures.

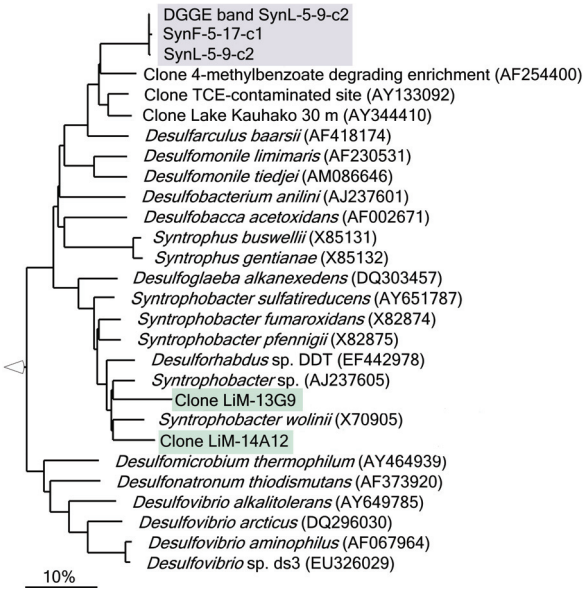

Figure 3.6: Maximum parsimony phylogenetic tree of 16S rRNA gene sequences of microorganisms isolated from limonene degrading methanogenic enrichment cultures (gray). The closest related clones from limonene degrading enrichments are emphasized in green. The tree was rooted using *Chlorobi* sequences as an out-group. The scale bar represents 10 substitutions per 100 bp .

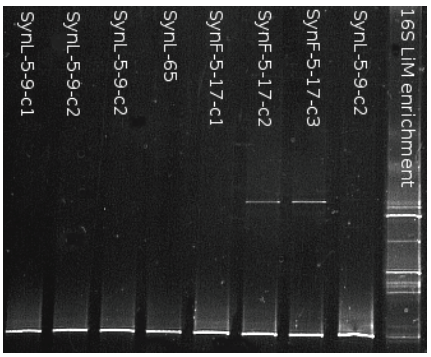

Figure 3.7: DGGE patterns of newly isolated strains and extracted 16S rDNA of an enrichment thriving on limonene. The front of the gel is not shown.

To understand the physiology of these strains their fermentation profile was analyzed. Three strains fermented lactate to acetate, and traces of propionate and butyrate. Whereas fumarate was fermented by four strains to acetate and succinate. The closest in-culture relative at the phylogenetic level is incapable to utilize lactate even under sulfate reducing conditions. Lactate fermentation to acetate was observed for other organisms in pure culture (Plugge et al., 2002) or co-culture with a hydrogen and/or formate utilizing methanogen (Wallrabenstein et al., 1995b). Growth of our isolates in co-cultures with an acetotrophic and formate-hydrogen utilizing methanogen, *Methanosarcina mazei*, in the presence of limonene likely occurred at the expense of substrates carried with the inocula. We suggest that these newly isolated *Deltaproteobacteria* do not play the role of primary fermenters in limonene degradation, but of secondary fermenters (syntrophs), likely being involved in short chain fatty acid or alcohol degradation. A better understanding of the physiology of these strains, could offer more insights on the mechanism of limonene methanogenic degradation.

3.5 Conclusions and outlook

Our results show that limonene is converted to methane and carbon dioxide by a microbial community represented by five phylas. Microbial communities like the one studied here, may contribute to the disappearance of monoterpenes from buried biomass in deep biosphere, underground waters, deep sea vents where alkene degradations is mostly attributed to geochemical processes (Tissot and Welte, 1984). A better understanding of alkene degradation under methanogenic conditions could uncover the significance of microbial mediated monoterpene degradation for diagenesis.

Differences in Bacterial communities could be seen between methanogenic enrichments thriving on the saturated aliphatic hydrocarbon, hexadecane (Zengler et al., 1999) the aromatic hydrocarbon, toluene (Ficker et al., 1999) and the alkenoic monoterpene, limonene (this study). The alkane (hexadecane) *Eubacterial* community was represented by *Syntrophus* and *Desulfovibrio* phylotypes (Zengler et al., 1999). The *Eubacterial* community in the monoaromatic (toluene) degrading enrichment was represented mostly by *Desulfotomaculum*, and a phylotype which did not group with any known genus *(Ficker et al., 1999)*. Whereas in our enrichments which degrade alkenoic monoterpenes, we determined that most *Eubacteria* were members of *Syntrophobacteraceae* and Candidate Division OP3. This could be the result of very different microbes involved in the primary and secondary steps of degradation of alkanes, alkenes or aromatics. Unsurprisingly, the methanogenic community was overall similar in all three enrichments, regardless of the electron donor. Similar metabolic end products are likely produced by *Eubacteria* (acetate, formate, hydrogen) which are further utilized by acetotrophic (*Methanosaeta*) and hydrogenotrophic (*Methanoculleus* and *Methanospirillum*) *Archaea.*

For the first time members of Candidate Division OP3 were visualized and quantified in this study. The small round shaped cells found in limonene enrichments as single cells

or clustered around other larger cells have unknown function. Monitoring the variation in OP3-like cells during different growth stages of the culture, could provide information on the right time for different separation approaches: i) filtration, ii) flow-cytometry and iii) Percol gradient. The fraction where these cells dominate could be used for metagenomic studies, which could provide insights into their genetic potential. The inferred functions from metagenomic studies could be the ground basis for establishing different isolation strategies.

Isolation of lactate and fumarate fermenting *Deltaproteobacteria* from limonene degrading enrichments, revealed the existence of a different *Deltaproteobacteria* phylotype compared to the *Deltaproteobacteria* phylotypes uncovered by the molecular approach. These microorganisms might be involved in the degradation of short chain fatty acids, and alcohols, playing the role of „secondary fermenters“ or „syntrophs“. Hence the combination of cultivation-dependent and cultivation-independent methods, helped to better understand the composition and possible functioning of limonene degrading enrichment cultures.

We suggest further cultivation attempts of „primary fermenting“ microorganisms by utilizing large fatty acids or alcohols (e.g. geraniol), in the presence or absence of an electron acceptor (sulfate). The fatty acids or alcohols to be used could be potential immediate products of limonene hydrolysis, or products expected to be next in the degradation pathway.

Growth on limonene under sulfate reducing conditions, could be followed by isolation of microorganisms, approach that could reveal the identity of limonene degrading microorganisms from enrichments. Afterward, their ability to degrade limonene could be demonstrated in co-culture experiments with „primary fermenting“ microorganism and the readily isolated „secondary fermenting“ *Deltaproteobacteria* grown on limonene under methanogenic conditions. Moreover, purification of methanogenic *Archaea* could be attempted with hydrogen and carbon dioxide, formate or acetate as electron donors. This would provide the basis for tri-culture experiments, with a „primary fermenting“, „secondary fermenting“ and a specific methanogenic *Archaea* metabolic type. A culture-dependent approach could answer some of the „How many metabolic-types are needed to degrade limonene?“; „How fast is it done without other competing microorganisms?“; and „Which step is the limiting one?“.

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Part II

Anaerobic aromatic hydrocarbon degradation

5 Monoaromatic hydrocarbon degradation

All aromatic hydrocarbons have as common structural unit the benzene ring, which has six carbon atoms connected by simple σ-bounds and non-localized double bonds, that form a shared π-electron cloud. This explains the stability of the aromatic ring structure, which becomes more labile at the addition of side chains.

Alike saturated aliphatic hydrocarbons, aromatic hydrocarbons are formed by long-term geochemical reactions of buried biomass (Tissot and Welte, 1984) or by plants and insects, where they function as secondary metabolites. However, petroleum and fossil fuels are the main natural source of aromatic hydrocarbons in the environment. The most toxic and soluble fraction of petroleum consists of benzene, toluene, ethylbenzene, and xylenes (BTEX). Our industrialized world produces large amounts of such monoaromatic hydrocarbons, e.g. 565 $\times 10^3$ tones alkylbenzenes (Association of petrochemicals producers in Europe, A.P.P.E.) are produced annually from crude oil. The extensive use of aromatic hydrocarbons in industry led to increased accidental accumulation which is disruptive for most life forms in the contaminated environments (Exxon Valdez Oil Spill Trustee Council, 2009). Bioremediation of oil contaminated areas, provided evidence that most of these hydrocarbons could be removed by aerobic processes whereas anaerobic hydrocarbon degradation was „negligible“ (Atlas, 1981). Later it has been attested that extensive anaerobic zones develop when soils and sediments are contaminated with hydrocarbons (Chakraborty and Coates, 2004). On the other hand, the most soluble oil fraction, BTEX (Shiu and Ma, 2000), could easily get transported from a localized spill area, penetrating deep sediment layers and protruding in underground waters where oxygen is less available.

Initial observations on anaerobic aromatic hydrocarbon degradation were limited to enrichment cultures and in situ experiments (few examples Kuhn et al., 1985; Grbìc-Galìc and Vogel, 1987; Kuhn et al., 1988; Edwards and Grbic-Galic, 1994; Ball and Reinhard, 1996). Presently, anaerobic microorganisms capable of anaerobic aromatic hydrocarbon degradation have been isolated and characterized (see Table 5.1). In general, the sources of isolation for anaerobic alkylbenzene degraders, vary from contaminated to uncontaminated sites (see Fries et al., 1994). Further studies on pure cultures helped elucidating pathways and genes that encode for key enzymes in catabolism.

Table 5.1: Isolated microorganisms capable of anaerobic degradation of benzene (Bz), toluene (Tol), ethylbenzene (Eb), *o*- (oX) and *m*- xylene (mX). Some of the strains are capable of utilizing more then one hydrocarbon, however this is not listed for simplicity purposes. Furthermore, the list comprises phylotypes retrieved from methanogenic or other TEA enrichments, with special emphasis on benzene were only few pure cultures are available.

PHYLUM	ORGANISM (STRAIN / RELATIVE)	HC	ELECTRON ACCEPTOR	REFERENCE
β- *Proteobacteria*	*Alicycliphylus denitrificans* (BC)	Bz	Chlorate	(Weelink et al., 2008)
β- *Proteobacteria*	*Dechloromonas aromatica* (RCB and JJ)	Bz	Nitrate	(Coates et al., 2001)
β- *Proteobacteria*	*Azoarcus* sp. strains DN11 and AN09	Bz	Nitrate	(Kasai et al., 2006)
β- *Proteobacteria*	Clone CartN1 (*Sterolibacterium*)	Bz	Nitrate	(Ulrich and Edwards, 2003)
δ- *Proteobacteria*	Clone BznS295 (*Desulfobacterium anilini*)	Bz	sulfate	(Musat and Widdel, 2007)
δ- *Proteobacteria*	Clone OR-M1 (*Desulfosporosinus*) and OR-M2 (*D. anilini*)	Bz	Bicarbonate	(Ulrich and Edwards, 2003)
α- *Proteobacteria*	*Blastochlosis sulfoviridis* (ToP1)	Tol	Anox. phototr.	(Zengler et al., 1999)
β- *Proteobacteria*	*Thauera aromatica* (T1)	Tol	Nitrate	(Evans et al., 1991)
β- *Proteobacteria*	*Thauera aromatica* (K172)	Tol	Nitrate	(Anders et al., 1995)
β- *Proteobacteria*	*Azoarcus* sp. (T)	Tol	Nitrate	(Dolfing et al., 1990)
β- *Proteobacteria*	Azoarcus tolulyticus (Tol-4, Td-1, 2, 15)	Tol	Nitrate	(Fries et al., 1994; Zhou et al., 1995)
β- *Proteobacteria*	Azoarcus toluvorans (Td-17 and Td-21)	Tol	Nitrate	(Fries et al., 1994)
β- *Proteobacteria*	*Azoarcus* sp. (ToN1)	Tol	Nitrate	(Rabus and Widdel, 1995)
β- *Proteobacteria*	Azoarcus sp. (T2, T4, T6, T10)	Tol	Nitrate	(Hess et al., 1997)
β- *Proteobacteria*	Azoarcus toluclasticus (MF7, MF23, MF63^T, MF441)	Tol	Nitrate	(Song et al., 1999)
α- *Proteobacteria*	*Magnetospirillum* sp. (TS-6, BM-1232, PM-1331 and 2411)	Tol	Nitrate	(Shinoda et al., 2005)
δ- *Proteobacteria*	*Geobacter metallireducens* (GS-15)	Tol	Iron	(Lovley and Lonergan, 1990)
δ- *Proteobacteria*	*Geobacter grbiciae* (TACP-2T and TACP-5)	Tol	Iron	(Coates et al., 2001a)
δ- *Proteobacteria*	*Desulfobacula toluolica* (Tol2)	Tol	sulfate	(Rabus et al., 1993)
δ- *Proteobacteria*	Strain TRM1	Tol	sulfate	(Meckenstock, 1999)
δ- *Proteobacteria*	Strain PRTOL1	Tol	sulfate	(Beller et al., 1996)
Firmicutes	Clone Eub-1 (*Desulfotomaculum*)	Tol	Bicarbonate	(Ficker et al., 1999)
Unknown	Clone Eub-1	Tol	Bicarbonate	(Ficker et al., 1999)
β- *Proteobacteria*	*Aromatoleum aromaticum* (EbN1)	Eb	Nitrate	(Rabus and Widdel, 1995)
β- *Proteobacteria*	*Azoarcus* sp. (EB1)	Eb	Nitrate	(Ball et al., 1996)
δ- *Proteobacteria*	Strain EbS7	Eb	sulfate	(Kniemeyer et al., 2003)
δ- *Proteobacteria*	*Desulfosarcina ovata* (oXyS1)	*o*X	sulfate	(Harms et al., 1999; Kuever et al., 2005)
Firmicutes	*Desulfotomaculum* sp. (OX39)	*o*X	sulfate	(Morasch et al., 2004)
β- *Proteobacteria*	*Azoarcus* sp. (mXyN1)	*m*X	Nitrate	(Rabus and Widdel, 1995)
β- *Proteobacteria*	*Azoarcus* sp. (M3-M7, M9, M12)	*m*X	Nitrate	(Hess et al., 1997)
δ- *Proteobacteria*	Strain mXyS1	*m*X	sulfate	(Harms et al., 1999)
Firmicutes	*Desulfotomaculum* sp. (OX39)	*m*X	sulfate	(Morasch et al., 2004)
δ- *Proteobacteria*	DGGE band (*D. ovata* oXyS1)	pX	sulfate	(Nakagawa et al., 2008)

Phylogeny

The majority of described anaerobic BTEX degraders group within phylum *Proteobacteria* (Table 5.1), together with other alkylbenzene degraders (e.g pCyN1 and 2, or PbN1) (Rabus and Widdel, 1995b; Harms et al., 1999a; Widdel and Rabus, 2001; Widdel et al., 2006) and with aliphatic hydrocarbon degraders (see Chapter 1 and 2).

Nitrate and chlorate reducing. Most nitrate and chlorate respiring aromatic degraders (Table 5.1) are *Betaproteobacteria,* from the genera *Azoarcus* (Dolfing et al., 1990; Fries et al., 1994; Rabus and Widdel, 1995; Ball et al., 1996; Hess et al., 1997; Song et al., 1999; Kasai et al., 2006)*, Thauera* (Evans et al., 1991; Anders et al., 1995) and *Dechloromonas* (Coates et al., 2001). Cell hybridization experiments confirmed that *Betaproteobacteria* predominate in diesel fuel contaminated laboratory aquifer columns (Hess et al., 1997), whereas the *Azoarcus-Thauera* group predominate in denitrifying enrichment cultures grown on crude oil (Rabus et al., 1999). Other anaerobic aromatic degraders have been isolated such as the benzene degrading, *Alicycliphylus denitrificans* strain BC (Weelink et al., 2008) which respires chlorate; a denitrifying toluene degrading *Alphaproteobacteria* from genus *Magnetospirillum* (Shinoda et al., 2005). Whereas in an enrichment grown under denitrifying conditions with benzene, *Sterolibacterium*-related microorganisms dominate (Ulrich and Edwards, 2003). Thus, aromatic hydrocarbon degradation under nitrate and perchlorate reducing conditions seems not to be restricted to the well described *Azoarcus-Thauera* and *Dechloromonas* genera. It is of importance to mention that perchlorate could release oxygen, hence the degradation of BTEX under perchlorate reducing conditions should not be considered as an usual anaerobic process.

Iron (III) reducing. The only described iron(III)-reducing organisms capable of toluene utilization belong to genus *Geobacter* (Lovley and Lonergan, 1990; Lovley et al., 1993; Coates et al., 2001a). Moreover, *Geobacteraceae* phylotypes were found in iron (III)-reducing enrichment cultures grow on benzene, toluene and xylenes (Botton and Parsons, 2007). The same study provided *bssA* phylogeny data which suggest that an *Azoarcus-Thauera*-related microorganism might play the role of a hydrocarbon utilizer. And they proposed a syntrophic cooperation between iron(III)-reducing microorganisms and the hydrocarbon degraders (Botton and Parsons, 2007).

Sulfate reducing. Almost all SRB capable of monoaromatic hydrocarbon degradation belong to *Deltaproteobacteria* within genus *Desulfobacula* and *Desulfosarcina.* A few strains form a clade of hydrocarbon degrading *Deltaproteobacteria* which includes strains capable of *m*-xylene (Harms et al., 1999), ethylbenzene (Kniemeyer et al., 2003) and naphthalene degradation (Galushko et al., 1999). In enrichment cultures capable of benzene degradation, the dominant members were related to this clade of aromatic hydrocarbon degraders and to *Desulfobacterium anilini* (Musat and Widdel, 2007). The only isolated SRB able to degrade aromatic hydrocarbons that is not a *Deltaproteobacteria,* is the Gram-positive, *Desulfotomaculum* sp. OX39 (Morasch et al., 2004), capable of *o*-xylene, *o*-ethyltoluene and toluene degradation.

Methanogenic. Although aromatic hydrocarbon degradation under methanogenic conditions was first proved more then two decades ago in enrichment cultures (Grbìc-Galìc and Vogel, 1987), and lately *in situ* in anaerobic groundwaters (Reinhard et al., 2005), so far no isolates had been obtained and only two studies looked at the phylogeny of microorganisms in mixed cultures (Ulrich and Edwards, 2003; Ficker et al., 1999). Benzene degrading enrichment cultures were dominated by two phylotypes: one *D. anilini*-related phylotype which had as nearest uncultured relative a sequence from a SR marine enrichment degrading benzene (Phelps et al., 1998), and one related to *Desulfosporosinus* sp. a microorganism that utilizes lactate, pyruvate, ethanol and certain fatty acids (Ulrich and Edwards, 2003). Another methanogenic culture analyzed by a molecular approach was a toluene-degrading methanogenic enrichment which had two dominant phylotypes, one related to *Desulfotomaculum* sp. whereas the second one did not group closely to known microorganisms (Ficker et al., 1999).

Mechanism of activation

The anaerobic attack of BTEX compounds differs depending on the absence or presence and type of the alkyl chain, therefore they are discussed individually below.

Benzene. Benzene loss was observed under chlorate-, nitrate-, iron(III)-, sulfate-reducing and methanogenic conditions (Foght, 2008). Although a benzene degrading microorganisms, *D. aromatica* strain RCB, has been fully sequenced, yet the genes encoding enzymes involved in anaerobic hydrocarbon degradation were not found, on the other hand different types of monooxygenases genes were present (Foght, 2008). This suggests that *D. aromatica* either grew with traces of oxygen from the culture media or unknown enzymes encoded by hypothetical genes in the genome of *Dechloromonas* strain RCB are capable of benzene activation under anaerobic conditions.

Presently, the anaerobic activation mechanism of benzene is still under considerable debate with at least three pathways regarded as possible: i) hydroxylation to phenol in methanogenic enrichments (Vogel and Grbìc-Galìc, 1986; Grbìc-Galìc and Vogel, 1987; Ulrich et al., 2005) ii) methylation to toluene (Coates et al., 2002; Ulrich et al., 2005), and iii) carboxylation to benzoic acid (Caldwell and Suflita, 2000; Kunapuli et al., 2008) which could result from more then one enzymatic reaction (Foght, 2008). Lately it has been shown that phenol is formed abiotically by exposure to air during the manipulation of samples (Kunapuli et al., 2008). Hence, benzene activation by hydroxylation should be considered with care.

Toluene and xylenes. The activation of methyl-substituted aromatics takes place by addition to fumarate of carbon atom from the methyl group adjacent to the ring (Figure 5.1). Initially, (alkylbenzyl)succinic acids were regarded as dead-end metabolites (Evans et al., 1992; Frazer et al., 1993). Later they were identified as intermediates in toluene degradation as demonstrated under nitrate- (Biegert et al., 1996; Beller and Spormann, 1997; Rabus and Heider, 1998), sulfate- (Rabus and Heider, 1998; Beller and Spormann, 1998), iron(III)-reducing (Kane et al., 2002; Botton and Parsons, 2007), phototrophic (Zen-

gler et al., 1999) and methanogenic (Beller and Edwards, 2000) conditions (reviewed by Spormann and Widdel, 2000; Boll et al., 2002; Chakraborty and Coates, 2004; Heider, 2007). The products of such an activation reaction were identified for *o- and m*-xylene (Krieger et al., 1999) under nitrate- and sulfate-reducing conditions (Seyfried et al., 1994; Beller and Spormann, 1997,b; Krieger et al., 1999; Morasch et al., 2004) and for *m*-xylene under iron(III)-reducing conditions (Botton and Parsons, 2007). On the other hand, for *p*-xylene such intermediate was detected in sulfate-reducing enrichment cultures, (Morasch and Meckenstock, 2005) but not in denitrifying and iron(III)-reducing cultures. This is likely because of a reduced number of *p*-xylene degrading enrichment cultures available for analysis. For reasons yet unknown, *p*-xylene seems recalcitrant to anaerobic degradation, therefore no anaerobic isolates had been isolated yet.

The fumarate addition reaction is catalyzed by benzylsuccinate synthase a heterohexameric enzyme ($\alpha_2\beta_2\gamma_2$) identified in different denitrifying bacteria growing on toluene (Leuthner and Heider, 2000; Krieger et al., 2001). The operon for the genes which encode all three subunits of benzylsuccinate synthase, plus an activating enzyme and a putative chaperone, was identified and expressed in different alkylbenzene utilizing cultures (Leuthner et al., 1998; Coschigano, 2000; Hermuth et al., 2002). The functional proof of the active subunit of benzylsuccinate synthase (BssA), was of a mutant without the BssA encoding gene which can not convert the alkylaromatic substrate, whereas when the *bssA* gene was reintroduced, the initial phenotype was recovered (Achong et al., 2001). The α-subunit of benzylsuccinate synthase was proposed to function by a glycyl-radical attack of the substrate, similar to another glycyl-radical enzyme, pyruvate-formate lyase. The existence of this type of catalytic active site in the α-subunit of benzylsuccinate synthase was demonstrated by: i) the identification of the oxygenolytic cleavage product of the large subunit (α'), which is known to occur in other glycyl-radical enzymes (Knappe and Sawers, 1990) : ii) mutagenesis and genetic complementation studies which showed that the radical-carrying glycine and one conserved cysteine from the catalytic active center were essential for growth on toluene (Coschigano et al., 1998); and iii) GC-MS on the benzylsuccinate produced as intermediate from [deuterated-methyl]toluene which suggested that one hydrogen atom is abstracted from the methyl-group of toluene (Beller and Spormann, 1997).

Similar to other glycyl-radical enzymes, benzylsuccinate synthase is likely activated to the radical form, by an adenosyl-radical produced via one-electron-reduction of S-adenosylmethionine. The adenosyl-radical abstracts a hydrogen from a glycyl-residue within the catalytic subunit of benzylsuccinate synthase. The gylcyl-radical will attack a conserved cysteine forming a thiyl-radical, which finally removes a hydrogen atom from toluene yielding a benzyl-radical. Next, the benzyl-radical adds at the double bond of fumarate forming the benzylsuccinyl-radical. The cycle is completed by the transfer the hydrogen from the hydrogen-bearing enzyme to the benzylsuccinyl radical and the transfer of the radical back to the enzyme (Figure 5.1) (Heider et al., 1999; Boll et al., 2002; Widdel et al., 2006; Heider, 2007).

Presently, either the intermediary metabolites (Beller, 2000; Elshahed et al., 2001; Suflita,

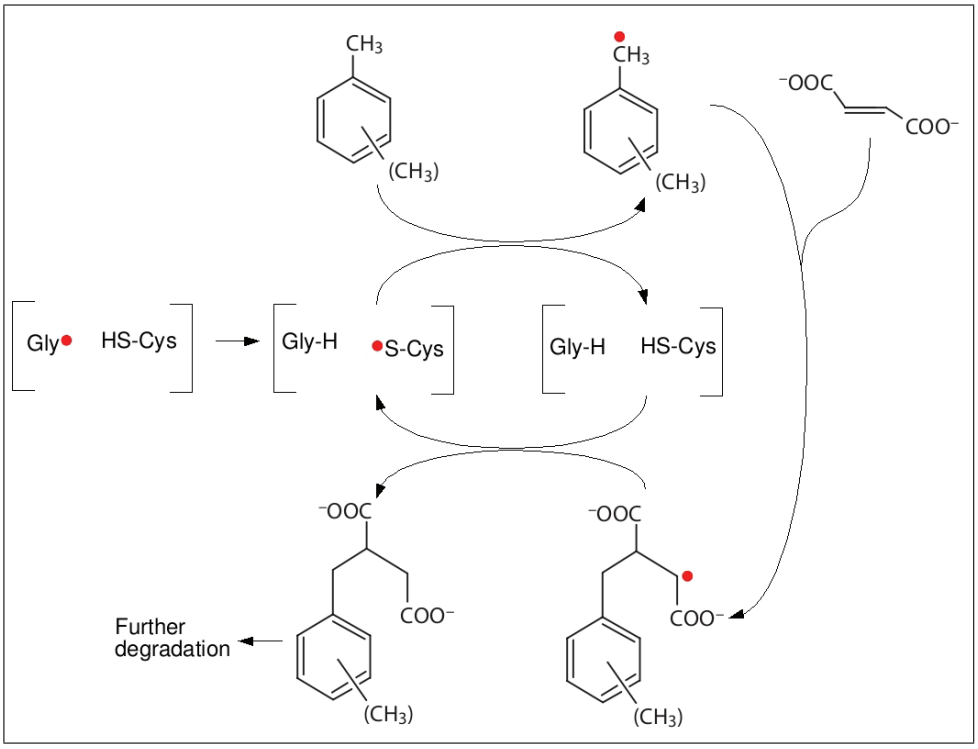

Figure 5.1: Mechanisms of activation by addition to fumarate of toluene and xylenes. The active form of benzylsuccinate synthase has a glycyl-radical which abstracts one hydrogen atom from a cysteine-residue. The resulting thiyl-radical attacks the inactive alkylbenzene at the methyl-group, forming an alkylbenzyl-radical, which attacks the double bound in fumarate, forming an (alkylbenzyl)succinyl-radical. The thiyl-radical in the enzyme is recovered while the (alkylbenzyl)succinyl-radical recombines with a hydrogen atom from the inactive enzyme. The red symbols, show where the radical is positioned. (adapted after Widdel et al., 2006) .

2002), or the presence of the *bssA* gene, that encodes the active subunit of benzylsuccinate synthase (Winderl et al., 2007, 2008), are considered potential environmental biomarkers for monitoring the recovery of contaminated areas.

Ethylbenzene. The same fumarate addition mechanism was observed in a SR culture thriving on ethylbenzene (Figure 5.1). However ethylbenzene, was activated differently in denitrifiers, by hydroxylation at the carbon atom adjacent to the ring, forming 1-phenylethanol. The provenience from water of the hydroxyl group in 1-phenylethanol, was demonstrated by oxygen stable isotope labeling (Ball et al., 1996). This reaction is mediated by ethylbenzene dehydrogenase, a Mo-Fe-S enzyme (Ball et al., 1996; Rabus and Heider, 1998; Johnson and Spormann, 1999; Johnson et al., 2001; Kniemeyer and Heider, 2001). The genome of the denitrifier EbN1 has been sequenced and it provided information on ten anaerobic and four aerobic potential hydrocarbon degrading pathways (Rabus et al., 2005). Some of these pathways were proved by global expression analysis attesting the metabolic versatility and complex regulation of this organism (Kuhner et al., 2005;

Wöhlbrand et al., 2007).

Lower degradation pathway

All BTEX are degraded to a central intermediate, benzylacetate, intermediate which has been identified in cultures thriving on any of these compounds (Altenschmidt and Fuchs, 1991; Beller et al., 1992; Evans et al., 1992; Seyfried et al., 1994; Ball et al., 1996; Coates et al., 2002). However the path to reach benzylacetate differ depending on the activation step, which in the case of benzene is unknown.

(Alkylbenzyl) succinic acids, the products of fumarate addition to toluene, xylenes, and ethylbenzene under SR conditions, enter a modified β-oxidation route after a CoA transferase replaces the succinyl moiety with a succinyl-CoA moiety. This was supported by detection of CoA transferase activity in toluene grown cells of *Thauera aromatica (Leutwein and Heider, 2001).* The next step is oxidation to E-(alkylphenyl) itaconyl-CoA which was supported by the identification of the corresponding acid in cultures grown on toluene and xylenes (Beller and Spormann, 1997; Morasch et al., 2004). It is assumed that the next metabolic step is β-oxidation to (alkyl)benzyl-CoA and succinyl-CoA (Leuthner and Heider, 2000; Leutwein and Heider, 2001).

On the other hand 1-phenylethanol, the activation product of ethylbenzene under denitrifying conditions, is dehydrated further to acetophenone, carboxylated to benzoylacetate, CoA activated, and thiolytically cleaved to benzoyl-CoA and acetyl-CoA (Rabus and Widdel, 1995; Ball et al., 1996; Widdel et al., 2006).

Subsequent degradation of (alkyl)benzyl-CoA involves reductive dearomatization and hydrolytic ring cleavage followed by β-oxidation (Harwood et al., 1999). However in the case of *o*- and *p*-xylene, the second methyl-group interrupts one normal round of β-oxidation, and it is yet unknown how the next degradation steps occur under this circumstances.

6 Polyaromatic hydrocarbon degradation

PAH (poly-nuclear aromatic hydrocarbons) are structurally stable molecules with multiple fused benzene rings that are found in coal tar, petroleum (Tissot and Welte, 1984) or are produced by plants and insects, and some PAH are raw chemicals for industry. They are rather inactive due to a large π-electron cloud of 10 or more electrons, and a lack of alkyl or polar substituents. PAH are soluble and toxic and alike BTEX they are regarded as recalcitrant contaminants in groundwaters. The degradation of these molecules was for a long time considered unlikely under anaerobic conditions, however few stable enrichments were obtained, during the last 15 years (reviewed by Meckenstock et al., 2004b; Foght, 2008). Most reports focused on naphthalene degradation which was the organic energy source for nitrate-, sulfate-, iron(III)- and manganese(IV)-reducing enrichments (Mihelcic and Luthy, 1988a,b; Al-Bashir et al., 1990; Durant et al., 1995; Bregnard et al., 1996; Coates et al., 996a; Langenhoff et al., 1996; Coates et al., 1997; Bedessem et al., 1997; Rockne and Strand, 1998) and of five anaerobic isolates growing under denitrifying (Rockne et al., 2000) or sulfate reducing conditions (Galushko et al., 1999; Musat et al., 2009).

Phylogeny The denitrifying strains, NAP-3-1 and NAP-4-1 (Rockne et al., 2000), were related to *Pseudomonas stutzeri* and *Vibrio pelagicus*, respectively. Whereas the sulfate reducing strains, NaphS2 (Galushko et al., 1999), NaphS3 and NaphS6 (Musat et al., 2009) grouped within *Desulfobacteraceae* together with other aromatic hydrocarbon degraders (Harms et al., 1999; Kniemeyer et al., 2003). Phylogenetic information on more complex PAH was obtained by molecular analysis of a phenantrene enrichment culture, which harbored a phylotype related to the alkene degrading Hxd3, and one related to a microorganisms never shown to be involved in hydrocarbon degradation, *Desulfofrigus oceanense* (Davidova et al., 2007).

Mechanism of activation and proposed degradation The pathway for anaerobic degradation of PAH is incompletely understood. Two mechanisms of activation, similar to benzene activation were proposed for the model PAH, naphthalene: carboxylation to naphtoic acid (Zhang and Young, 1997; Meckenstock et al., 2000; Annweiler et al., 2002) and methylation to 2-methylnaphthalene (Safinowski et al., 2006; Safinowski and Meckenstock, 2006). Alike toluene, 2-methylnaphthalene could react with fumarate producing (2-naphthylmethyl)succinate as observed in 2-methylnaphthalene grown cultures (Annweiler et al., 2000). However, for

the sulfate reducing strains NaphS2, NaphS3 and NaphS6, 2-methylnaphthalene seems unlikely to play the role of an intermediate in naphthalene degradation since: 1) the growth of these strains was delayed on 2-methylnaphthalene; 2) the α subunit of (naphthylmethyl)succinate synthase was absent in naphthalene grown cells, but identified in 2-methylnaphthalene grown cells; and 3) cells grown on a mix of [deuterated]naphthalene and 2-methylnaphthalene produced unlabeled 2-(naphthylmethyl)succinate (Musat et al., 2009). Hence carboxylation seems the most probable route of naphthalene activation in these sulfate reducing strains. A carboxylation reaction was suggested as well for another unsubstituted polyaromatic hydrocarbon, phenantrene (Davidova et al., 2007).

For cultures where methylation is the initial step of activation, the upper degradation pathway for naphthalene degradation seems to be similar to that of toluene degradation (Safinowski and Meckenstock, 2004; Meckenstock et al., 2004b) and finally converge down to naphtoic acid. PAH degradation is a field of active interest since there are unanswered questions regarding i) the occurrence of different activation mechanisms and 2) understanding the lower degradation pathway - the polyaromatic ring opening and ring reduction.

7 Anaerobic *p*-xylene degradation: results and discussions

7.1 Scope of the study

Reason: *p*-Xylene is one of the least degradable substrates from the monoaromatic oil fraction BTEX. Although, pure cultures or stable enrichments were obtained on all the other BTEX under different electron accepting conditions, yet only three stable enrichments have been describes so far on *p*-xylene, one under denitrifying conditions (Haner et al., 1995) and the other two under sulfate reducing conditions (Morasch and Meckenstock, 2005; Nakagawa et al., 2008). The stoichiometric coupling of sulfate reduction and *p*-xylene oxidation was demonstrated, however this is not the case for nitrate-reducing enrichment cultures. The actual knowledge gained on *p*-xylene degradation comes only from sulfate reducing enrichment cultures. Sequence analysis revealed that a DGGE band from one *p*-xylene degrading enrichment was phylogenetically related to the *o*-xylene degrading sulfate-reducer, oXyS1 (Harms et al., 1999), which is reclassified as *Desulfosarcina ovata* (Kuever et al., 2005). However, the mechanism of *p*-xylene activation, was suggested to be similar to that of toluene after the discovery of two intermediary metabolites, 4-(methylbenzyl) succinate and 4-(methylbenzyl) itaconate (Morasch and Meckenstock, 2005).

Under denitrifying conditions there is no information about the microorganisms involved, and the mechanism of *p*-xylene degradation.

Scope: This work on *p*-xylene degrading enrichment cultures growing under denitrifying conditions, addressed the following points :

1. to enrich cultures on *p*-xylene and attempt isolation,
2. to identify and quantify the dominant member,
3. to determine the stoichiometric coupling of *p*-xylene oxidation and nitrate reduction, and
4. to determine the mechanism of *p*-xylene activation under denitrifying conditions.

7.2 Cultivation of *p*-xylene degrading microorganisms under denitrifying conditions

7.2.1 Enrichment

Sediment-containing enrichment cultures were established with nitrate as electron acceptor and *p*-xylene as electron donor. The initial nitrate added (5 mM) was consumed shortly upon addition and 5 mM of nitrate were resupplied several times. Enrichments with *p*-xylene reduced up to 50 mM nitrate and consumed all the *p*-xylene added during circa 650 days (Annex I. Figure 13.1A). In contrast, a *p*-xylene-free control culture consumed 25 mM of nitrate in cca. 450 days, when nitrate reduction ceased. Consecutive sediment-free subcultures consumed 13 to 27 mM nitrate within 270 days (Annex I. Figure 13.1B).

7.2.2 Isolation attempts

Aerobic cultivation Some denitrifiers are capable of growth under oxic conditions therefore we attempted isolation on R2A agar, or on agar supplemented with BHI and AC media. Samples were taken from the second liquid dilution series. Growth occurred after 2 to 5 days. We selected 58 colonies, with different morphologies and colors. These colonies were fed into anaerobic media with 1% *p*-xylene to determine if they are capable of growth on this substrate. However we observed no growth on *p*-xylene under denitrifying conditions after more then six months.

Anaerobic cultivation

Liquid dilution-to-extinction

1. *p-xylene.* Sediment-free subcultures with the highest accumulated nitrate reduction were used as inocula for four successive liquid dilutions-to-extinction (1:10) in the presence of 1% *p*-xylene in HMN (v/v), which resulted in sediment-free enrichment cultures. Growth with *p*-xylene and nitrate in these highly enriched cultures became faster and doubling times of approximately 7 days were observed. The highest dilution with growth was dominated by thin curved rods, 0.5 µm $\times$ 2 µm (Figure 7.2D) which accounted for 96% of the cell morphotypes visualized.

2. *p-xylene and ascorbate*. Liquid dilution-to-extinction (1:10) were established in the presence of *p*-xylene 1% in HMN and 1 mM ascorbate. Growth was observed after nine days, in cultures with 0.01 ml inocula. Upon inspection of the first dilution by phase contrast microscopy, we enumerated less then 18% thin curved rods, whereas the rest of the microorganisms were short oval or large round cells. In higher dilutions, less of the „*p*-xylene morphotype“ was recovered, which suggests that potential contaminants might thrive on ascorbate despite the selective pressure of *p*-xylene.

Solid serial agar dilutions Solid agar dilutions were attempted as described before (Widdel and Bak, 1992) with 1% *p*-xylene in HMN overlaid on top of the solid agar, and with or without 1 mM cysteine as reducing agent. Growth was observed after 3 to 2 months and colonies were selected for further liquid incubations. Five colonies grew in liquid media, however all these isolates were lost at the following transfer step. It is likely that during the handling procedures, these microorganisms get stressed mechanically (pipetting, injecting etc.) and physiologically (presence of oxygen lack of substrate, inhibitors etc.) and do not recover during incubation.

7.2.3 Growth tests on other hydrocarbons

Of all the tested hydrocarbons, besides *p*-xylene, only toluene sustained growth and denitrification. Non-hydrocarbon substrates utilized by our enrichments were *p*-toluic acid, benzoate, fumarate and lactate. Culture did not thrive on cholesterol, and *o*- and *m*-toluic acid. Cultures grown on *p*-toluic acid and toluene were represents by a similar morphotype like *p*-xylene grown cultures whilst more cell morphologies were observed in cultures grown on substrates such as benzoate, fumarate, and lactate.

Cultures did not utilize the following hydrocarbons: benzene, ethylbenzene, *o*-xylene and *m*-xylene, 2-methylnaphthalene, naphthalene, limonene, *n*-hexane, cyclohexane, or *n*-decane.

Strains isolated on other xylene isomers (Harms et al., 1999; Rabus and Widdel, 1995), were isomer specific (*o*- or *m*-xylene), and only strain OX39 was capable to grow with two different xylene isomers, *o*- and *m*-xylene (Morasch et al., 2004). In this study we observed as well no conversion of the other xylene isomers. This could result from differences in the specificity of the activating enzyme. It is likely that the position of the second methyl group is relevant for the enzymatic attack, considering that toluene is a favorable substrate for growth of xylene degrading strains.

7.3 Dominance of novel *Betaproteobacteria* in *p*-xylene degrading enrichment cultures

To identify the phylogeny of microorganisms in two highly enriched *p*-xylene denitrifying cultures, pXyN1 and pXyN3, we constructed clone libraries for the 16S rRNA gene (Figure 7.1). The clone libraries of these enrichments were dominated by one phylotype (sequences with $\geq$98.5% identity). This phylotype was closely related to the *Betaproteobacteria*, *Denitratisoma oestradiolicum* (95% identity). Other close relatives include strain 72Chol (94.4%) and *Sterolibacterium denitrificans* (94.2%). This phylotype was only distantly related to two *Azoarcus* members: the cyclohexane-1,2-diol degrading strain Lin22 (91.4%), the propylbenzene degrading strain PbN1 (90.9%) and to *Dechloromonas aromatica* strain RCB (91.1%). From the *Thauera* group the closest relative is the toluene degrader, *Thauera*

aromatica K172 (Anders et al., 1995). Other five phylotypes were found, three related to *Denitratisoma oestradiolicum* (84.4 to 94%), one to *Chlorobium phaeobacteroides* DSM 266 (80% identity) and one to candidate division OP11 sequences from a river estuary mangrove (89.8% identity) (Figure 12.5).

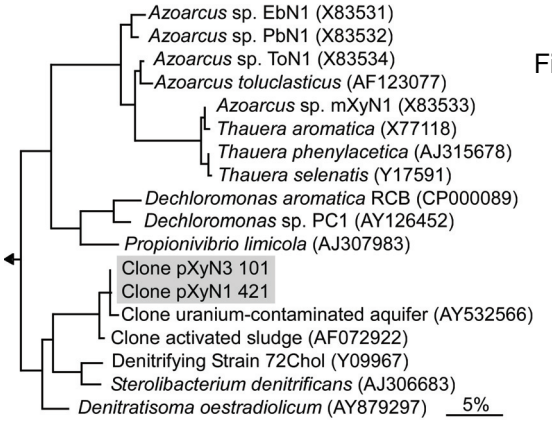

Figure 7.1: Maximum likelihood tree of *Betaproteobacteria* showing the dominant 16S rRNA phylotype (gray boxes) in two enrichment cultures (pXyN3 and pXyN1) grown on *p*-xylene as sole energy source. The arrow points to *Deinococcus* species which were used as outgroup. The scale bar measures distance as 5% similarity per bar length.

To resolve the relative dominance of the organisms in the enrichments, we applied FISH with phylum- to group-specific oligonucleotide probes (Table 12.2) (Amann et al., 1990b). All bacteria were detected with a DNA stain (4'.6-diamidino) 2-phenylindol (DAPI). We determined the relative percentage of probe targeted cells in relation to the number of DAPI stained cells (Figure 12.4). The general *Bacteria* probe (Eub-338 I-III) hybridized 97% of the total cells in both enrichment cultures (Figure 7.2A). The class specific *Betaproteobacteria* probe (Bet-42a), hybridized to more than 93% cells in both enrichments (Figure 7.2B). Newly designed probes (pxyn-440 and pxyn-644) specific for the *Denitratisoma*-related phylotype which dominated both clone libraries, targeted more than 91% of cells in both enrichment cultures (Figure 7.2C).

This is the first time a denitrifying community degrading *p*-xylene has been described. It is surprising that the dominant phylotype did not group with other hydrocarbon-degraders of the *Azoarcus-Thauera* cluster, but with steroid degraders from the *Denitratisoma-Sterolibacterium* group. A similar observation was made on benzene degrading denitrifying enrichments, where *Sterolibacterium*-related organisms dominated as well. It is possible that a new genus related to steroid degraders comprises uncultured hydrocarbon degrading microorganisms.

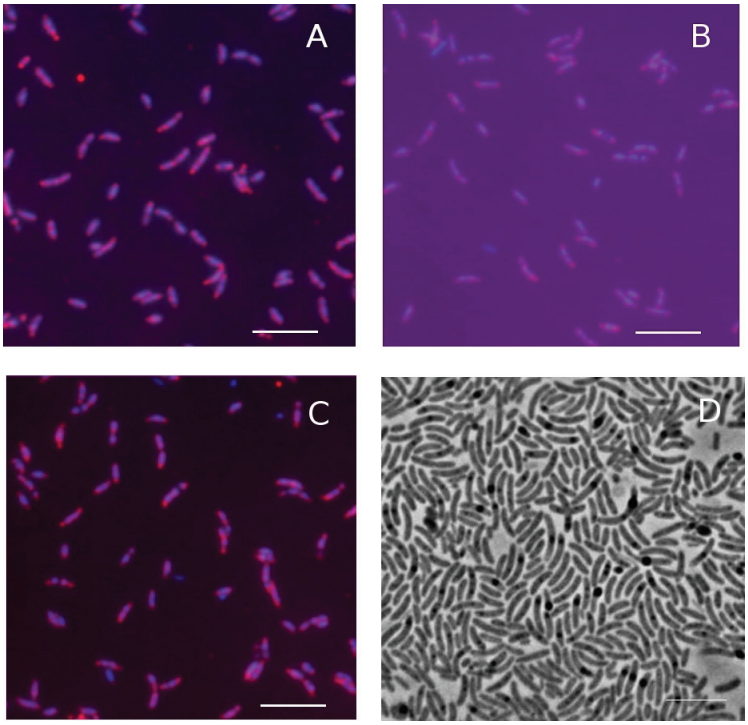

Figure 7.2: Microscopic images of cells in a denitrifying enrichment culture with *p*-xylene. Images A, B and C are superimposed DAPI and probe signals. Cells not targeted by the oligonucleotide probe appear blue (DAPI signal) while probe-targeted cells appear red. The scale bar is 5 µm. A) Cells stained with DAPI and hybridized with a Bacteria-specific probe mix (Eub I-III). B) Cells stained with DAPI and hybridized with probe Bet 42a specific for *Betaproteobacteria*. C) Cells stained with DAPI and hybridized with probe pxyn-440 designed for the dominant phylotype retrieved from two *p*-xylene degrading enrichments. D) Phase contrast micrograph of viable cells.

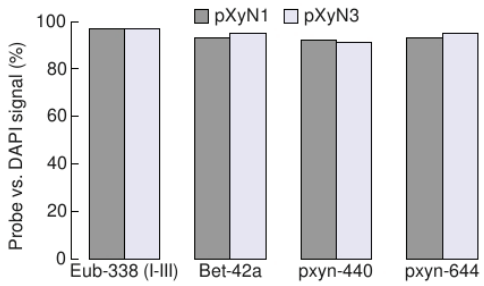

Figure 7.3: Enumeration of cells stained with Cy-3 labeled probes versus DAPI stained cells in two denitrifying cultures highly enriched on *p*-xylene (pXyN1 and pXyN3). The nonsense probe, Non-338, although applied it stained 0% cells, therefore it is not represented here.

7.4 Quantification of *p*-xylene degradation

Catabolism was quantitatively monitored for 80 days in cultures grown with a low amount of hydrocarbon (Table 12.1). Cultures incubated with *p*-xylene consumed all the nitrate added (Figure 7.4). To elucidate whether nitrate is reduced to ammonium, we measured the ammonium concentration in the cell culture medium and observed that at the end of the incubation, it decreased with 1 µM. To determine whether nitrate is reduced to other intermediates instead of dinitrogen gas, nitrite and nitrous oxide were measured. The concentrations of nitrite did not change, whereas nitrous oxide was not detected during cultivation.

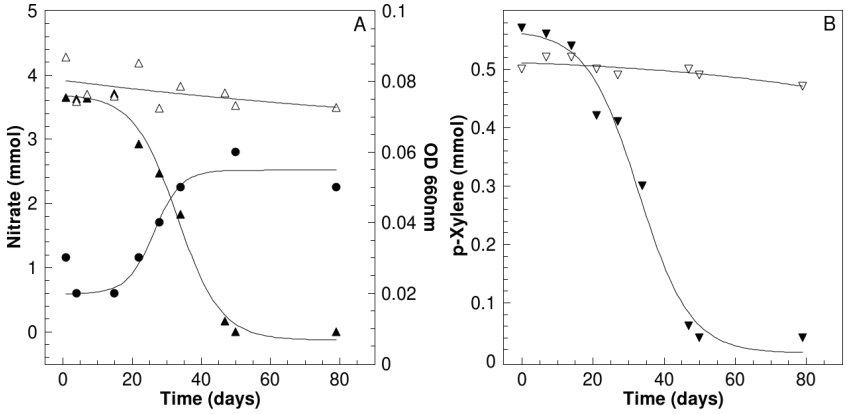

Figure 7.4: Nitrate reduction (filled up-triangles) and optical density (filled circles) increase (panel A) in a sediment free culture, pXyN1 which degrades *p*-xylene (filled down-triangles in panel B). In an inoculated control there is no significant decrease of nitrate (opened up-triangles). In sterile controls no major hydrocarbon loss has been detected (open down-triangles). The values for nitrate and *p*-xylene are average of duplicate measurements.

The nitrate consumed by a *p*-xylene degrading culture was 3.46 $\pm$ 0.07 mmol which could accept 17.3 $\pm$ 0.3 mmol of reducing equivalents (Table 12.1) if completely reduced to dinitrogen gas. The physical loss of *p*-xylene was low, as observed in a sterile control. From dry weight measurements, we estimated that 2.2 µmol *p*-xylene were assimilated into biomass (see Table 12.1). The amount of *p*-xylene incorporated into biomass was calculated using the assimilatory reaction: 17 C_8H_{10} + 32 HCO_3^- + 32 H^+ + 30 $H_2O \rightarrow$ 42 $C_4H_7O_3$. The total amount of *p*-xylene catabolized was of 0.50 $\pm$ 0.08 mmol, which could donate 20.9 $\pm$ 3.3 mmol reducing equivalents if completely oxidized to carbon dioxide. The complete oxidation of *p*-xylene according to the equation C_8H_{10} + 8.4 NO_3^- +

8.4 $H^+ \rightarrow$ 8 CO_2 + 4.2 N_2 + 9.2 H_2O ($\Delta G^{0'}$ is -4202.6 kJ mol^{-1} *p*-xylene), is supported by the electron balance (Table 12.1) and a lack of fatty acids accumulation in the culture media. Here we show for the first time the stoichiometric coupling of *p*-xylene oxidation to nitrate reduction. Another study proposed a complete oxidation of *p*-xylene under denitrifying cultures based on similar rates of electron transferred from the electron donor to the electron acceptor, during exponential growth (Haner et al., 1995). Whereas under sulfate reducing conditions *p*-xylene oxidation was stoichiometrically coupled to reduction of sulfate (Morasch and Meckenstock, 2005; Nakagawa et al., 2008). Although consumption of *p*-xylene was also detected under iron reducing and methanogenic conditions, the process has not been studied quantitatively (Botton and Parsons, 2007).

7.5 Mechanism of *p*-Xylene activation

In acidified and methylated extracts of cultures grown with 5 mM nitrate and 2% *p*-xylene in HMN (v/v), two metabolites were detected as dimethyl esters. Based on relative retention times, mass spectra and comparison to published spectra, we identified the two dimethyl esters in our denitrifying cultures as dimethyl esters of (4-methylbenzyl) succinate, and (4-methylphenyl) itaconate. The mass spectra of (4-methylbenzyl) succinic acid dimethyl ester showed the molecular ion at *m/z* 250, the base peak at *m/z* 105 and further key fragment ions at *m/z* 131, 145, 177 and 190 (Figure 12.6A). The mass spectrum of (4-methylphenyl) itaconic acid dimethyl ester showed the molecular ion at *m/z* 248, the base peak at *m/z* 129 and further key fragments at *m/z* 115, 188 and 216 (Figure 12.6B).

Their mass fingerprints were similar to compounds found in sulfate reducing enrichments which consume *p*-xylene (Elshahed et al., 2001; Morasch and Meckenstock, 2005). The relative retention times of both metabolites were significantly different from those of the corresponding *o*- and *m*- substituted analogues formed from *o*- and *m*-xylene by the sulfate reducing strain OX39, respectively (Morasch et al., 2004). The presence of these metabolites suggest a similar degradation pathway (Figure 7.7) as described for toluene and *m*-xylene in the denitrifiers *Thauera aromatica* and *Azoarcus* sp. strain T, respectively (Biegert et al., 1996; Krieger et al., 1999; Leuthner and Heider, 2000). However, after ring cleavage, the *para*-methyl group would prevent one step of regular β-oxidation. The mechanism by which this „obstacle“ is by-passed is still unknown. Furthermore we amplified and sequenced the *bssA* gene with specific primers (Winderl et al., 2007, 2008) and identified its relationships at the amino acid level to other benzylsuccinate synthases. This amino acid sequence grouped together with other denitrifying microorganisms capable of hydrocarbon degradation (Figure 7.6)having as close relative (84% amino acid similarity) the α-subunit of benzylsuccinate synthase (TutD) from *Thauera aromatica*. The intermediary metabolites and the *bssA* gene are useful molecular biomarkers of alkylbenzene degradation in the environment (Beller et al., 1995; Elshahed et al., 2001; Suflita, 2002; Winderl et al., 2008).

Table 7.1: Quantification of *p*-xylene consumption and nitrate reduction in two highly enriched cultures. The inocula was 25% in a culture volume of 400 ml, overlaid with 40 ml HMN as carrier for *p*-xylene. The theoretical amount of *p*-xylene added was 0.616 mmol corresponding to 0.2% in HMN (v/v). The theoretical amount of nitrate added was 4 mmol.

Culture	NO_3^- initial (mmol)	NO_3^- consumed* (mmol)	*p*-xylene initial (mmol)	*p*-xylene consumed* (mmol)	Cell dry mass (mg)	Electrons accepted*** (mmol)	Electrons donated** (mmol)	Electrons recovered (%)
Cells with *p*-xylene (A)	3.62±0.01	3.62±0.01	0.49 ±0.05	0.46 ±0.08	0.56	17.2 ±0.03	18.0 ±4.00	96 ±22
Cells with *p*-xylene (B)	3.65 ±0.03	3.65 ±0.00	0.57 ±0.02	0.53±0.06	0.58	17.3 ± 0.03	20.9 ± 3.30	83 ± 16
Sterile control with *p*-xylene	-	0.50 ± 0.05	0.03 ±0.05	-	-	-	-	-
Cells without *p*-xylene	3.58 ± 0.06	0.18 ±0.07	-	-	0.11	-	-	-

* The depletion of nitrate and p-xylene was calculated by subtracting the final measured value from the initial one.

**Electrons accepted by nitrate and donated by *p*-xylene were calculated considering the amounts of these energy sources used by cultures. The amount of nitrate used was corrected by subtracting the loss of nitrate observed in an inoculated control culture. Similarly, we corrected the amount of *p*-xylene consumed by enrichment cultures by subtracting the *p*-xylene lost in a sterile control. The amount of electrons donated during dissimilation of *p*-xylene was calculated by excluding the amount assimilated into biomass from the total *p*-xylene that disappeared. We assumed 2.1 mg cell dry mass requires 0.0039 mmol *p*-xylene considering the assimilation reaction:17 C_8H_{10} + 32 HCO_3^- + 32 H^+ + 30 H_2O → 42 $C_4H_7O_3$.

These data support *p*-xylene degradation via fumarate addition.

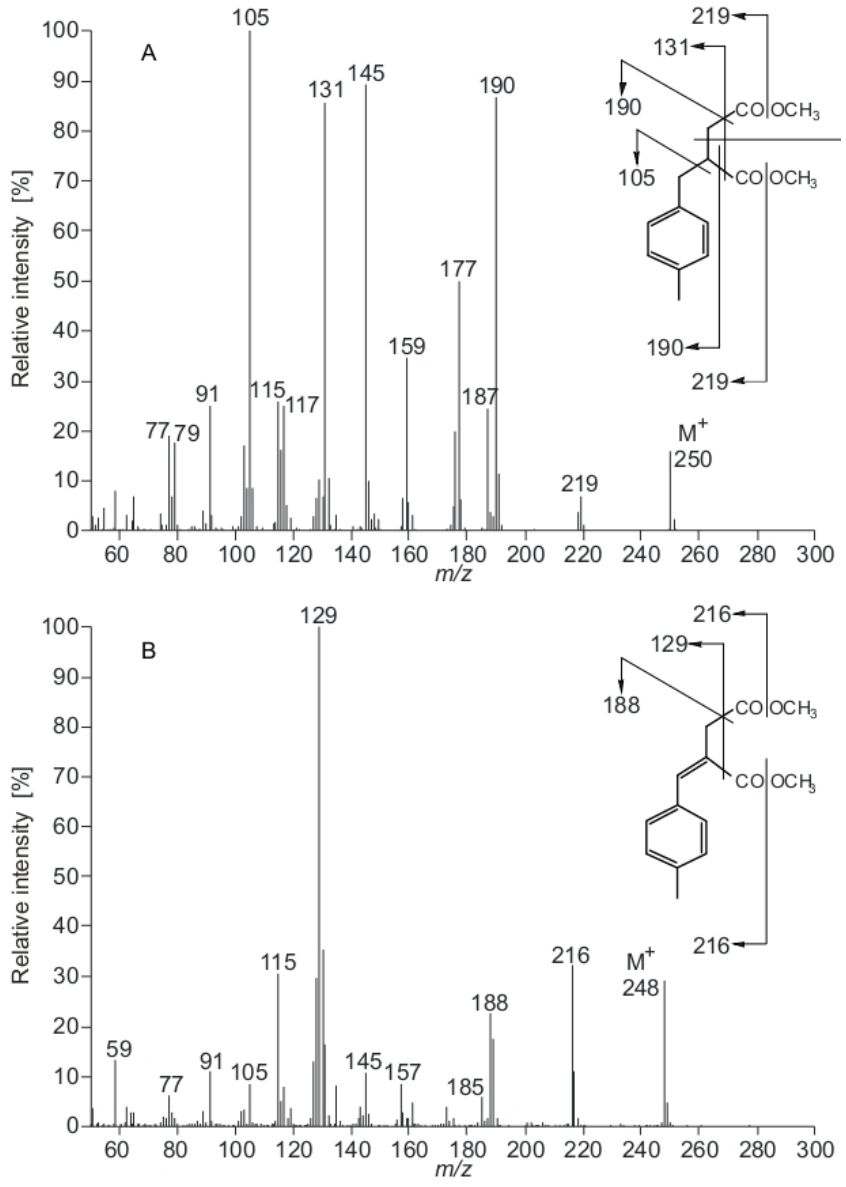

Figure 7.5: Mass spectra of two methylated intermediary metabolites obtained from cultures grown with *p*-xylene as sole organic energy source: A)(4-methylbenzyl) succinic acid dimethyl ester, and B) (4-methylphenyl) itaconic acid dimethyl ester.

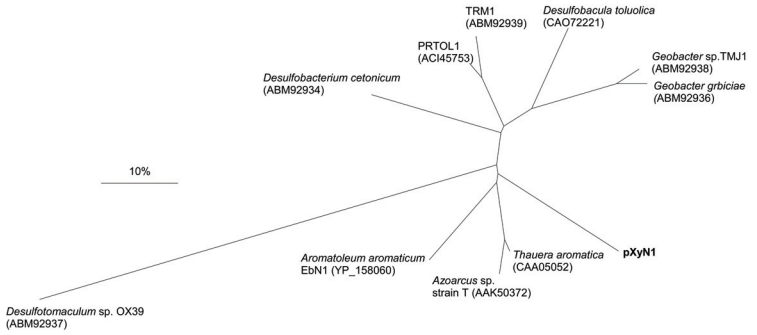

Figure 7.6: Phylip ProML tree of *bssA* gene sequences encoding (alkylbenzyl)succinate synthase. In bold is the BssA sequence retrieved from a highly enriched *p*-xylene degrading culture. In parenthesis are the NCBI accession numbers for the amino acid sequence used for the analysis. The scale bar represents 10% difference in amino acid identity.

7.6 Conclusions and outlook

In this study we obtained a highly enriched culture, with less then 6% contaminants, that is capable to activate *p*-xylene by fumarate addition and oxidize it further, while using nitrate as terminal electron acceptor. The microorganism which degrades *p*-xylene eluded isolation in solid agar. However, liquid dilution-to-extinction series helped to increase their numbers. Inspection of cultures by phase contrast microscopy, showed the dominance of 0.5 µm $\times$ 2 µm thin rods. The same rods were determined with specific FISH probes and represent almost the entire bacterial population. These dominant microorganisms branched separately from other alkylbenzene degraders from *Betaproteobacteria* having as next relative, steroid utilizing denitrifiers, of the genus *Denitratisoma* and *Sterolibacterium*. However, since our cultures showed more then 5% difference to steroid utilizers and were functionally unrelated to these organisms, we suggest they belong to an independent genus within *Betaproteobacteria*. In a denitrifying benzene utilizing enrichment culture slot-blot hybridization revealed as well the dominance of *Sterolibacterium*-related DNA (Ulrich and Edwards, 2003). Hence the capability to utilize aromatic hydrocarbons seems to diverge to unknown genera within *Betaproteobacteria.*

Since no organism was cultured yet on *p*-xylene, of all the BTEX, it is of high relevance to pursue further on with isolation of these microorganisms. There is extensive information on the activation of alkylbenzenes, however two xylene isomers *p*-xylene, and *o*-xylene are likely following a different lower path of degradation bypassing one normal round of β-oxidation. An interesting question that arises is „What enzyme catalyzes this step?“ Substrate induced expression analysis, could clarify more about the enzymes involved in the degradation pathway of toluene versus *p*-xylene. Differences in the latter could be due to different enzymes employed in the lower degradation pathway of *p*-xylene.

Furthermore it has been observed for most alkylbenzene degraders that they are extremely specialized on the type of hydrocarbon substrate used. Hence most xylene degraders, are specialized on the degradation of one xylene isomer. What kind of enzyme stereo-chemistry defines the substrate-enzyme interactions? And how microbes make use of this selective behavior in the environment? These are only some of the questions that remain to be answered.

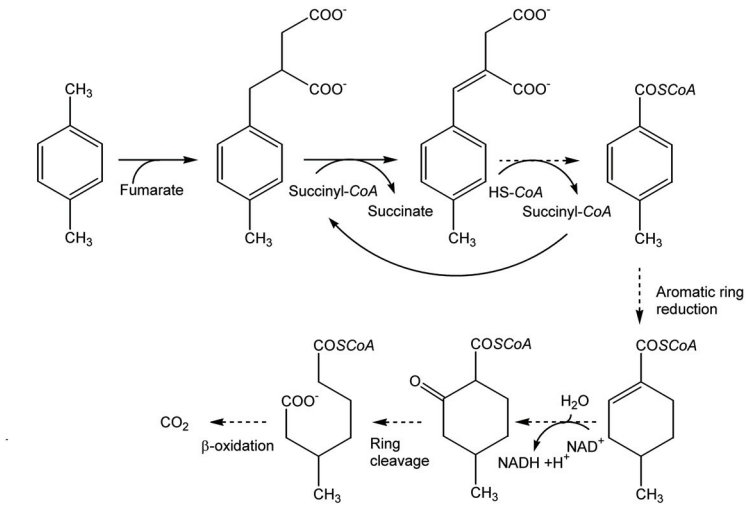

Figure 7.7: Proposed pathway for *p*-xylene degradation by denitrifying cultures. Evidence for the proposed initial reactions are presented in the text. Fumarate addition results in (4- methylbenzyl)succinic acid. The succinyl moiety is likely replaced by succinyl-CoA resulting in (4-methyphenyl) itaconyl-CoA. The hypothesized removal of succinyl-CoA and CoA-thioesterification could generate the central intermediate (4-methylbenzoyl)-CoA. The lower pathway starts with aromatic ring reduction, followed by hydration／dehydrogenation, ring cleavage and β-oxidation. Dotted lines illustrate several intermediary steps.

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9 Explanation of contribution

Manuscript 1. Candidate division OP3 cells in limonene degrading methanogenic cultures

A.-E. Rotaru, R. Schauer, C. Probian, M. Mussmann and J. Harder

Enrichment cultures were maintained by C. Probian. R. Schauer constructed constructed clone libraries, performed the ARDRA analysis and sequencing experiments. M. Mussmann designed the OP3-565 probe. A.-E. Rotaru developed the concept together with J. Harder and performed the growth experiment, the design of probe Eub-338 (VI) testing of the probes and the quantitative cell counts. The manuscript was written by A.-E Rotaru with editorial comments of J. Harder.

Manuscript 2. *Deltaproteobacteria* isolated from limonene degrading enrichments

A.-E.Rotaru, C. Probian and J. Harder

J. Harder initiated the isolation and performed the primary isolation. A.-E. Rotaru performed the second isolation, physiological experiments and molecular analysis. C. Probian was involved in the initial isolation and performed some chemical analysis. A.-E. Rotaru wrote the manuscript with editorial comments of J. Harder.

Manuscript 3. Highly enriched *Betaproteobacteria* growing anaerobically with *p*-xylene and nitrate

A.-E.Rotaru, C. Probian, H. Wilkes and J. Harder

C. Probian and J. Harder established the enrichment cultures and maintained them before this thesis work. A.-E. Rotaru maintained the cultures during the thesis work and performed all the physiological and molecular experiments. H. Wilkes performed GC-MS analysis on metabolite extracts. A.-E. Rotaru wrote the manuscript with editorial assistance of the co-authors.

Part III

Manuscripts

10 Manuscript 1

Candidate Division OP3 cells in limonene degrading methanogenic cultures

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Manuscript in preparation

Abstract

This study describes for the first time a methanogenic community that biodegrades the most widespread monoterpene, limonene. Limonene was degraded to methane gas, with transient formation of acetate. For a better understanding of the community composition clone libraries were established for the *Bacteria* and *Archaea* 16S rRNA gene. The *Bacteria* clone library was represented by 16S rRNA gene sequences related to *Deltaproteobacteria*, *Bacteroidetes* and Candidatus Division OP3. Whereas, the *Archaea* clones were related to members of the genera *Methanosaeta* and *Methanoculleus*. The community composition of limonene degrading enrichment cultures was analyzed by CARD-FISH. During mid exponential growth 40% *Bacteria* and 33% *Archaea* were detected. A specific probe that was newly designed, OP3-565, detected 18% of very small cocci shaped cells that were observed either solitary or aggregated around larger cellular forms. Finally, we propose a model of limonene degradation by syntrophic interactions between partners.

Keywords: limonene, monoterpene, methanogenesis, syntrophy, Candidate Division OP3

Introduction

Limonene, like other monoterpenes, is produced in the plastids of plants by i) fusion of two isoprene units together, catalyzed by isoprene-synthase and ii) cyclization catalyzed by limonene-synthase (Kreuzwieser et al., 1999; Hyatt et al., 2007). The two limonene enantiomers, L- and D- limonene, are produced by different plants. L-limonene has a mint fragrance and is found in trees and herbs, whereas D-limonene is found in the peels of citrus fruits where it gives the specific orange fragrance. In plants, the role of limonene, like other mono-, di- and sesquiterpenoids, is that of a secondary metabolite (Newman and Chappell, 1999). Secondary metabolites are considered non-essential for the basic metabolic functions of plants, however they establish complex ecological interactions, from symbiotic to pathogenic, with microorganisms, fungi, insects or humans (Singer et al., 2003).

The deposition and transport of dead vegetation are the major sources of limonene and other terpenes in freshwater sediments. Presently, limonene is used in industry as scenting agent in the production of cleaning and sterilizing products, or as food flavor. Being one of the new ingredients of detergents it is considered a potential marker for modern sewage recharge in urban groundwaters (Barrett et al., 1999).

In anaerobic environments, such as, underground waters, anoxic soils or anaerobic wastewater treatment plants, limonene is degraded in the absence of oxygen. Facultative anaerobes, like *Thauera terpenica* and *Castellaniella defragrans*, had been isolated on monterpenes under denitrifying conditions and both can thrive on limonene as sole energy source (Foss and Harder, 1998; Foss et al., 1998). However limonene degradation in the

absence of electron acceptors, would be the dominant process in anoxic sediments where the alternative electron acceptors were removed by microbial respiration. Only few studies carried out under methanogenic conditions proved the degradation of unsaturated aliphatic hydrocarbons (Schink, 1985b,a; Harder and Foss, 1999).

The only described organism capable of unsaturated hydrocarbon degradation under methanogenic conditions is the acetylene utilizer, *Pelobacter acetylenicus*. Whereas microorganisms able to degrade alkenes under methanogenic conditions still await isolation and description.

This study aims to understand the methanogenic community established in enrichment cultures thriving on the naturally ubiquitous alkenoic monoterpene, limonene. Limonene was utilized as organic energy source by methanogenic enrichments established previously on α-pinene and 2-carene (Harder and Foss, 1999). After more then 5 successive transfers, we obtained a stable community capable of limonene degradation. The community compositions and structure was analyzed using the full cycle rRNA approach (Amann et al., 1995). A newly designed probe revealed the presence of members of Candidate Division OP3 (Hugenholtz et al., 1998). This is the first time a member of this phylum, had been visualized and quantified.

Materials and methods

Source of organisms and cultivation

Sediment free methanogenic cultures growing on different monoterpenes were established earlier with activated sludge from a waste water treatment plant (Harder and Foss, 1999). These enrichments were transferred in fresh water methanogenic media (Harder and Foss, 1999) overlaid with 30 ml 2,2,4,6,8,8-heptamethylnonane (HMN) as inert carrier phase for 2% or 5% limonene. Cultures were grown in 300 ml methanogenic fresh water media added in sterile 500 ml Duran bottles, sealed with butyl rubber stoppers. The rubber stoppers had no contact to the carrier phase containing the hydrocarbon. The hydrocarbon was replenished by moderate shaking (80 rpm). All chemicals used were of analytical grade. Growth of cells was determined by optical density measurements at 660 nm on Shimadzu UV-VIS 1202 Spectrophotometer.

Chemical analysis

All samples for chemical analysis were taken with N_2 flushed hypodermic needles and syringes.

Limonene concentrations were measured by gas-chromatographic head-space analysis modified after Musat and Widdel (2008). Gas volumes of 0.1 ml were withdrawn at 28°C and injected into a 14B gas chromatograph (Shimadzu) equipped with a Supel-Q PLOT fused silica capillary column (length 30 m, diameter 0.53 mm) and a flame ionization detec-

tor. The carrier gas was N_2. The GC oven temperature was 200℃ isothermal, the injector was at 150℃ and the detector at 280℃.

Methane was measured by gas-chromatography head-space analysis on the same instrument, however at a lower GC oven temperature (110℃ isothermal).

Fatty acids were detected by high performance liquid chromatography on a Sykam HPLC. Filtered samples were diluted 1 to 10 and subsequently injected using a Sykam S5200 auto-sampler. Separation was performed on an Aminex HPX-87H column (300 x 7.8 mm) under isothermal conditions (40℃). The eluent is 5 mM H_2SO_4. Fatty acids were detected on an UV-Detector at 220 nm. Data acquisition and processing was done with DataApex Clarity HPLC Software (Gamma Analysen Technik GmbH). Our standards consisted of the following fatty acids: succinate, lactate, formate, acetate, propionate and butyrate. The detection limit for all of them was 0.1 mM.

Clone library construction and analysis

The genomic DNA of 5 ml cell culture was extracted with the QUIAGEN genomic tip (Quiagen). 25 ng high-molecular weight DNA was used to amplify the 16S rRNA gene with bacterial primers GM3 and GM4 (Hicks et al., 1992; Kane et al., 1993). The reaction mix consisted of 2 µM of each primer, 0.2 mM total dNTP, 0.04 U Red-Taq-Polymerase and $1\times$ PCR buffer in 50 µl. After an initial denaturing step of 4 minutes at 94℃, the polymerase was added at 80℃. The 32 cycles involved a denaturing step for 1 minute at 94℃, primer annealing at 42℃ for 1 minute, and 1 min elongation at 72℃. The A-overhang for cloning was introduced by a final elongation at 60℃ for 60 minutes. The amplicons were purified and cloned into pGEM-T Easy Vector System I (Promega GmbH). The recombinant plasmids were transformed into *E. coli* DH5α (Invitrogen).

Archaea 16S rRNA gene amplicons were obtained with the primer pairs 21F-958R and 21F-1492R (Stahl et al., 1988; DeLong, 1992) as described above with the annealing temperature at 58℃. The amplicons were purified and cloned into pCR4-TOPO vector (Invitrogen, Groningen, Netherlands). The plasmids were transformed into chemically competent *E. coli* TOP10. Inserted genes were amplified with the vector primers M13F and M13R. We analyzed the diversity of clones by amplified rDNA restriction analysis (ARDRA). PCR products were purified, and aliquots of 1 µg of the amplified insert were digested with 7.5 U of the restriction endonucleases *BsuRI* and *RsaI* (Fermentas) for 3 hours at 37℃. The resulting fragments were analyzed on an 3% agarose gels, and restriction patterns within each group were manually compared. Amplicons of 16S rRNA genes were sequenced using an Applied Biosystems kit and analyzed on a 3130 XL Genetic Analyser (Applied Biosystems). Sequences were cleaned of vector data with Sequence Analysis 5.2 (Applied Biosystems) and assembled into contigs with the Sequencer software (Gene Codes Corporation). The nearly complete 16S rRNA gene sequences were aligned with the ARB-Silva software package (Ludwig et al., 2004; Pruesse et al., 2007). A maximum parsimony phylogenetic tree was calculated excluding the influence of highly variable positions. The tree

was reconstructed only using sequences with over 1300 bp.

Probe design and visualization of cells by CARD-FISH

Probe OP3-565 was designed for a group of 16S rRNA sequences related to Candidate division OP3, using the ProbeDesign tool of the ARB software (Ludwig et al., 2004; Pruesse et al., 2007). The accessibility of the probe was verified by inspection of the target sites on the *Rhodopirellula baltica* 16S rRNA accessibility map (Behrens et al., 2003). *In silico* testing was done against the RDP II database vs.9.48 (`http://rdp.cme.msu.edu/probematch/search.jsp`). OP3 565 showed *in silico,* 1.2 weighted mismatches to *Thialkalivibrio halophylus* (DSM 15791) and *Marinitoga piezophila* (DSM 14283). Therefore, an equimolar mix of these two organisms was used as control for hybridization experiments. Probes were synthesized with a horse radish peroxidase (HRP) modification at the 5'-end (`http://www.biomers.net`) and used for hybridization experiments.

Hybridization experiments were performed with CARD-FISH, a method adapted from Pernthaler et al. (2002). Samples were fixed with 1% paraformaldehyde, washed in sterile water and filtered on 0.2 µm GTTP filters. Filters were embedded in 0.2% low melting point agarose (Metaphor) to ensure a minimal cell loss. Dried filters were dehydrated with 96% ethanol prior to permeabilization. We permebilized for 1 hour at 37℃ using 10 mg/ml lysozyme in 0.1 M Tris-HCl and 0.05 M EDTA. The second permeabilization step was in 0.1 M HCl for 30 sec. To inactivate endogenous peroxidases, we performed another incubation step for 10 min in 0.01 M HCl. Hybridizations were carried out for 4 hours at 46℃ with various formamide concentrations to ensure probe specificity (see Table 1). The ratio of probe to hybridization buffer was of 1 to 300. Washing was performed at 48℃ for 10 min. Both hybridization buffer and washing buffer were prepared as described before (Pernthaler et al., 2002). Filters were brought to equilibrium in 1x PBS for 20 min prior to signal amplification. The signal was amplified for 20 min in the dark at 46℃ with Alexa 594 labeled tyramides. All samples were counter-stained with DAPI 1 µg/ml as reference for relative cell counts.

Results

Enrichment of methanogenic cultures with limonene as substrate

In this study, we showed for the first time the ability of methanogenic enrichment cultures to grow on limonene. All cultures were transferred annually. Within this time, 300 ml culture produced more then 1L of gas. Small amounts of acetate (2 mM) and cysteine (1 mM) were supplemented to the freshwater media, to sustain the methanogenic community. However, both compounds could be used as alternative energy sources by a different organisms from a complex microbial community.

We monitored the consumption of limonene and production of methane gas in cultures with or without additional acetate (2 mM) for circa 7 months (Figure 10.1). We observed

a clear increase in cell density after more then 2 months. The maximum cell density was measured after 6 months in enrichment cultures with additional acetate (Figure 10.1A). Limonene decrease occurred after 4 months in enrichment cultures whereas sterile controls did not show a decrease (Figure 10.1B). The methane production was different between cultures incubated with or without additional acetate. The cultures with acetate, showed a steady increase of methane for the entire incubation period, whereas cultures without additional acetate, started methane production with a delay of 3 months (Figure 10.1C). Acetate build up was observed within the first 3 months with a sudden decrease during the 4^{th} month (Figure 10.1A). For cultures with additional acetate, we observed that fatty acids did not accumulate at the end of the incubation whereas the 2 mM acetate supplemented to the media was consumed, as well.

The stochiometry was calculated for two culture duplicates (series 164 and 165) incubated with different amounts of monoterpene in the presence (165) or absence (164) of 2 mM acetate as shown in Table 10.2. The methane produced in a control experiment without limonene, matched (97% methane recovery) the theoretical methane produced by 2 mM acetate and 1 mM cysteine. Moreover, this control culture produced 90% less methane when compared to the least active enrichment. In enrichment cultures the methane recovery was between 45% and 75% from the theoretical methane that could be formed from the total amount of limonene utilized and other energy sources added (acetate, cysteine). Our data support the following stoichiometry: $C_{10}H_{16}$ + 6 $H_2O \rightarrow$ 7 CH_4+ 3 CO_2

Microbial community composition

The microbial community compositions of limonene degrading enrichments was revealed by the full cycle 16S rRNA approach (Amann et al., 1995). We established clone libraries for *Bacteria* and *Archaea* 16S rRNA genes. ARDRA analysis was performed on 327 Bacteria clones and 141 *Archaea* clones. The representative clones for each ARDRA pattern were sequenced. Phylogenetic analysis were performed with 34 *Bacteria* and 28 *Archaea* representative 16S rRNA almost full length sequences. Moreover, we partially sequenced 141 *Archaea* clones and 130 *Bacteria* clones. 16S rRNA gene sequences with more then 98.5% identity were regarded as one OTU. The 21 OTU representatives for *Bacteria* and *Archaea* and their closest cultured and uncultured relatives are shown in table 10.3.

***Bacteria* clone library** *Bacteria* 16S rRNA gene sequences were represented by 11 OTUs (Table 10.3) related to *Bacteroidetes* (i), *Deltaproteobacteria* (ii) Candidate Division OP3 (iii) and as well *Firmicutes* (iv) (Figure 10.2).

(i) Bacteroidetes. All seven *Bacteroidetes* OTUs were far related to *Prolixibacter bellariivorans* (81 to 89% identity) and *Alistipes putredinis* (84.1%). Clones from a long chain fatty acid (LCFA) degrading enrichment showed high similarity with most of our *Bacteroidetes* sequences (92-96% identity). Other *Bacteroidetes* OTUs had very high similarity ($\geq$99%) to sequences retrieved from waste water of paper mil or coking treatment plants. Some

Bacteroidetes sequences showed less then 90% sequence identity to uncultured microorganisms from biofilms of an acid mine drainage or sequences retrieved from the chimney of a hydrothermal vent.

(ii) Deltaproteobacteria. The two identified *Deltaproteobacteria* OTUs were related to *Syntrophobacter* sp. with a 16S rRNA identity of only 92%. Their uncultured relatives whose sequences were retrieved from LCFA methanogenic enrichments and sphagnum peat bog samples showed 4.9% difference in sequence identity (Table 10.3).

(iii) Candidate Division OP3. Interestingly, one OTU represented by 5 sequences, was very far from anything cultured until now, showing as much as 24.2% difference to the closest cultured relative, *Opitus* sp. VeSm13, from phylum *Verucomicrobia*. Moreover, this phylotype showed even a low 16S rRNA gene identity (16.7% different) to members of the uncultured phylum, Candidate Division OP3 (Table 10.3).

(iv) Firmicutes. Our *Bacteria* clone library revealed also the presence of one *Firmicutes* phylotype. This sequence had 91.2% identity to *Syntrophomonas* sp., and only 95% to the closest uncultured relative, a sequence retrieved from a reactor degrading organic waste.

***Archaea* clone library** *Archaea* 16S rRNA gene sequences were represented by 10 *Euryarchaeota* OTUs (Table 10.3), related to microorganisms from the orders *Methanomicrobiales* (v) and *Methanosarcinalles* (vi) (Figure 10.3).

(v) Methanomicrobiales. With one exception, all *Methanomicrobiales* sequences were comprised in one OTU, which showed 91.6% identity to *Methanoculleus* sp. The other *Methanomicrobiales* OTU was far related (88.6%) to a member of the same genus, namely *M. palmolei.*

(vi) Methanosarcinalles. The order *Methanosarcinalles* was represented by 8 OTU, which were very similar or identical to the 16S rRNA gene of *Methanosaeta* sp. AMPB-Zg (more then 99% identity) and *Methanosaeta concilii* (89% to 100% identity) (Table 10.3).

Catalyzed-reporter-deposition fluorescent *in situ* hybridization

By phase contrast microscopy we identified different morphotypes from long and thick filaments to thin filaments, vibrio, and cocci of different sizes (Figure 10.4G and H). To better understand the community composition, we quantified the members of this community with CARD-FISH during mid exponential growth, in cultures thriving only on limonene (without additional acetate).

Kingdom specific probing. The *Eubacteria* general probe, Eub-338 (I), matched *in silico* most sequences from our *Bacteria* clone library, with the exception of Candidate Division OP3 phylotype. This phylotype did not match *in silico* any other published *Eubacteria* probes (Eub-338 II and III). Therefore we designed a new *Eubacteria* probe, Eub-338 (VI), which perfectly paired positions 338-355 on the 16S rRNA gene of Candidate Division OP3 sequences. These two probes when applied in equimolar amount, targeted 40% of the total cells (DAPI stained) of the microbial community. The *Archaea* probe, Arch-915, matched

cells within *Methanosaeta*-like filaments and rods with flat ends, that made up for 33% of the entire microbial community detected by DAPI. Therefore circa 26% of the DAPI stained cells were not detected with HRP-labeled probes (Table 10.4). We observed that the general Arch-915 did not target the entire *Methanosaeta*-like filaments but rather scarce cells through the filaments (Figure 10.4C). The relative percentages of probe targeted cells versus DAPI targeted cells are shown in Table 10.4. As control probe we used Non-338, which gave one signal for each 300 DAPI stained cells.

Group specific probing. To identify the groups within *Bacteria* and *Archaea*, which dominate the enrichments, group specific probes were applied. *(i) Bacteroidetes.* Probe CF-319a matched *in silico* all the *Bacteroidetes* sequences from our clone library. However, it stained only 1% of the total detected cells in the enrichment samples. *(ii) Deltaproteobacteria.* Probe Delta-495a, targeted *in silico* all the *Deltaproteobacteria* sequences from the *Bacteria* clone library and stained 12% of the total cells, which corresponds to less then one third of the *Eubacteria* (Eub-338 I&VI). *(iii)* Candidate Division OP3. The Candidate Division OP3 probe (OP3-565) designed during this study, was used to visualize and quantify for the first time Candidate Division OP3 members. This probe stained very small round cells (Figure 10.4E and F for the corresponding DAPI) which made up for 18% of the total DAPI stained cells, that represents almost half of the total detected *Eubacteria*. When the probe was applied at the same hybridization conditions on 1.2 weighted mismatch control organisms, *Marinitoga piezophila* and *Thialkalivibrio halophylus,* it did not bind. To double check the presence and quantity of Candidate Division OP3, we used probe Pla-46, a *Planctomycete* specific probe*,* which matched *in silico* our Candidate Division OP3 phylotype. When applied, this probe stained the same morphotype like OP3-565, but matched only 13% of the total detected cells. *(vi) Methanosaetaceae.* Since in our enrichments we found numerous filaments with a *Methanosaeta* like morphology, we expected that these phylotype will be representative for *Archaea*. Probe MX-825 is specific for some members of the *Methanosaetaceae* family, and matched *in silico* all the *Methanosaeta* phylotypes from the *Archaea* clone library. However, when tested on our enrichment cultures it only targeted 1% of the DAPI stained cells, that corresponds to about 3% of the Arch-915 detected *Archaea*. MX-825 did not target the entire filaments but rather scarce cells through the filaments as observed earlier with the general *Archaea* probe, Arch-915.

Discussions

Enrichment cultures thriving on limonene under methanogenic conditions

In this study, we demonstrate for the first time, limonene degradation under methanogenic conditions. When the only electron acceptor left is CO_2, microorganisms can gain only very small amounts of energy from limonene compared to other anaerobic respiratory processes with electron acceptors like nitrate, iron or sulfate. To surpass the energetic barriers, microorganisms establish mutualistic co-operations. Such interactions between microbes, are

dependent on transfer of intermediary molecules like hydrogen, formate or acetate between metabolically different types of microbes (Schink and Stams, 2006). Theoretical calculations on limonene degradation in methanogenic conditions were done after Mavrovouniotis, 1991. This is an energy gaining process, with a free energy gain in standard conditions ($\Delta G^{0'}$) of -348 kJoule mol^{-1} limonene consumed. Under methanogenic conditions different metabolic groups could share the free energy released by the mineralization of limonene.

We suggest a model for the degradation of limonene with a first group of microbes, most likely fermenting bacteria, capable of limonene degradation to alcohols and fatty acids. Syntrophic microorganisms would break down short chain fatty acids and alcohols to small molecules like acetate, formate, hydrogen, or carbon dioxide. These could be used as substrates by methanogens, which produce methane and carbon dioxide as end-product. In our enrichments it is more likely that acetate is exchanged between syntrophic bacteria and methanogenic *Archaea*. This was suggested by acetate formation and consumption, in cultures incubated with limonene as organic energy source (without any additional acetate). The addition and accumulation of acetate was reported to inhibit syntrophic degradation of fatty acids and benzoate (Ahring and Westermann, 1987; Fukuzaki et al., 1990; Warikoo et al., 1996). Hence it is possible that larger amounts of acetate could have an „end-metabolite“ inhibitory effect on the syntrophic microbial community, and finally on the limonene degradation process itself.

Acetate and hydrogen releasing reaction:
$C_{10}H_{16}$ + 10 $H_2O \rightarrow$ 5 $C_2H_4O_2$ + 8 H_2
$\Delta G^{0'}$= + 156 kJoule mol^{-1} limonene

Acetate and hydrogen consuming reactions:
$C_2H_4O_2 \rightarrow CH_4 + CO_2$
$\Delta G^{0'}$= - 49 kJoule mol^{-1} acetate
4 H_2+ $CO_2 \rightarrow CH_4$+ 2 H_2O
$\Delta G^{0'}$= - 131 kJoule mol^{-1} methane

Total:
$C_{10}H_{16}$ + 6 $H_2O \rightarrow$ 7 CH_4 + 3 CO_2
$\Delta G^{0'}$= - 348 kJoule mol^{-1} limonene

Microbial community composition

To better understand the microbial community composition in limonene degrading enrichments, we used the 16S rRNA approach. This culture-independent approach revealed the presence of unusual phylotypes spanning through the super-kingdoms of *Bacteria* and *Archaea*. CARD-FISH was preferred to the more usual mono-labeled FISH, to surpass the differences in cell metabolic activities, or the varieties of microbial cell walls that such a complex community might have. Kingdom specific probes accounted for 73% of the total

DAPI stained cells, which could be due to: i) an uneven permeabilization treatment of the different cell types prior to hybridization, ii) dead cells within *Methanosaeta*-like sheaths, supported by phase contrast visualization of *Methanosaeta*-like empty sheath stretches, and iii) the existence of other unknown phylas in our enrichments which are not targeted by the known phylogenetic probes and primers. A recent study showed that *Methanosaeta concilii* gives heterogeneous signals independent of the different permeabilization procedures prior to CARD hybridizations. They observed a doubling in the detection rates of *Archaea* after prolonging the storage of the samples from 2 weeks to 6 months (Kubota et al., 2008). Since our samples were used one or two days after fixation and permeabilization we might have underestimated the abundance of *Archaea*.

All the *Bacteria* phylotypes recovered from methanogenic enrichment cultures were phylogenetically distant from any described genus within the families *Bacteroidetes*, *Deltaproteobacteria*, or *Firmicutes* to which they were ascribed. Whereas one of the encountered phylotypes was related to sequences of microorganisms from the uncultured phylum, Candidate Division OP3.

Bacteroidetes. Phylum *Bacteroidetes* is highly diverse and lately the taxonomy of this phylum went through numerous changes, such as reclassification of members of genus *Bacteroides* as members of a novel genus, *Alistipes* (Rautio et al., 2003). Genus *Alistipes* is represented by strictly anaerobic microorganisms, isolated primarily from human sources, which have as major metabolic product succinate (Rautio et al., 2003; Song et al., 2006). In our *Bacteria* clone library, we found two phylotypes related to *A. putredinis.* Differences in 16S rRNA identity of 16% to 18% suggests that these sequences belong to a new genus, within family *Rikenellaceae*,. The other *Bacteroidetes* phylotypes found in our survey, were related to *Prolixibacter bellariivorans,* with identity differences ranging between 12% and 16%. Genus *Prolixibacter* is represented by facultative anaerobes, which ferment sugars by mixed-acid fermentation (Holmes et al., 2007). The members of this phylum were not in significant numbers (1%), as observed by hybridization experiments with CF-319a. *Bacteroidetes* related organisms could be involved in degradation of long-chain fatty acids, as part of the process of limonene degradation, or a scavengers of residual organic material from dead cells.

Deltaproteobacteria. The two new *Deltaproteobacteria* phylotypes, found during our survey, were related to the non-syntrophic *Syntrophobacter* sp. strain TsuA1 (92%), a sulfate reducer which grows on adipate and is able to utilize C_1- C_{12} straight fatty acids, C_2- C_{10} straight chain primary alcohols, 2-, 3- hexadionate, pyruvate, and lactate (Tanaka et al., 2000). However, these *Syntrophobacter*-related phylotypes, were only far related to this sulfate-reducer, and showed even larger differences to syntrophic microorganisms of the family *Syntrophobacteraceae*. Therefore we suggest they represent a novel genus within the class *Deltaproteobacteria*. *Syntrophobacter*-related organisms together with Candidate Division OP3 - related microorganisms made up for almost the entire *Bacteria* detected in this survey.

Candidate Division OP3. Sequences of Candidate Division OP3 were identified and

grouped within one phylotype. This phylotype was well represented in our enrichment cultures. Small and round shaped cells were targeted by the specific OP3-probe, and were found either alone or attached to a larger cell which was not stained by the OP3 specific probe. 16S rRNA sequences of Candidate Division OP3 have been hitherto recovered solely from anoxic habitats such as the anoxic sediments of Yellowstone Hot Spring (Hugenholtz et al., 1998), the anoxic water body of the Cariaco basin (Madrid et al., 2001), 700 m deep in the Antarctic continental shelf (Bowman and McCuaig, 2003), groundwater of a gold mine (Lin et al., 2006), 15 m deep in a pristine coastal aquifer (Lopez-Archilla et al., 2007) or from an anaerobic waste water digestor (Chouari et al., 2005). Since there is no isolate available, their metabolic capabilities are unknown. We could only suspect that they might be involved in short chain fatty acid degradation, considering that OP3 sequences were found also in two anaerobic and mesophilic chemostats thriving on propionate and butyrate (Shigematsu et al., 2006; Tang et al., 2007).

The methanogenic *Archaea* in limonene degrading enrichments were 33% of the entire community, and were represented by members of the genus *Methanosaeta* and *Methanoculleus*.

Methanosaeta. Most of the *Methanosaeta* phylotypes from our *Archaea* clone library were closely related to members of genus *Methanosaeta* (90% to 100% sequence identity). This genus comprises obligate anaerobes which use as sole energy source acetate, converting it into equimolar amounts of methane and carbon dioxide. The two *Methanosaeta* relatives were *Methanosaeta concilii* which was isolated from a pear waste digestor (Patel and Sprott, 1990) and *Methanosaeta* sp. AMPB-Zg isolated from freshwater sediment (Scholten and Stams, 2000). Both were described as sheathed rods that aggregate into bundles. We identified such filaments with a *Methanosaetaceae* specific probe, MX-825, that targeted *in silico* all the *Methanosaeta* phylotypes from our *Archaea* clone library. However when applied this *Methanosaetaceae* probe stained only 1% of the total cells from our limonene degrading enrichment. Both the *Methanosaetaceae* specific probe, MX-825, and the *Archaea* probe, Arch-915, stained filaments heterogeneously as previously mentioned for *M. concilii* (Kubota et al., 2008).

Methanoculleus. The closest *Methanoculleus* relative for two of our phylotypes was *Methanoculleus palmolei* (Zellner et al., 1998) a highly irregular cocci, isolated from an anaerobic bioreactor treating wastewater of a palm oil mill. *M. palmolei*, like most members of genus *Methanoculleus,* has complex nutritional requirements such as potassium and tungsten ions as growth promoters, or acetate as organic mineral supplement (Zellner et al., 1998; Whitman et al., 2006). In incubation of limonene degrading enrichments without supplementary acetate, we observed a longer period prior to methane build-up because this group of methanogens is likely out-competed.

Conclusions

Limonene degradation under methanogenic conditions revealed a complex syntrophic community spread through the *Bacteria* and *Archaea* kingdoms. Limonene is most likely degraded by *Bacteria* to acetate, that is further on metabolized by methanogenic *Archaea,* to methane and carbon dioxide. We identified unusual *Bacteria* phylotypes, which could be representatives of novel genus within *Rickenelaceae*, and *Syntrophobacteraceae.* We visualized and quantified members of the hitherto uncultured Candidate Division OP3, whose small round cells often attach to the surface of other bacteria.

This enrichments contained unique phylogenetic novelty that could be the objective of more in depth molecular and physiological analysis. We suggest: i) the use of a metagenomic approach for additional information on Candidate Division OP3 members, ii) the use of a more sophisticated ecological approach, Nano-SIMS, to identify the phylogeny of the microorganism degrading C ^{13}- labeled limonene, iii) or isolation of the limonene degrading organisms in well defined co-cultures, using liquid and solid dilution series in the presence of an acetotrophic or hydrogenotrophic methanogen.

Acknowledgments

We thank the Max Plank Society and the International Max Planck Research School for Marine Microbiology for financial support.

Figures and tables

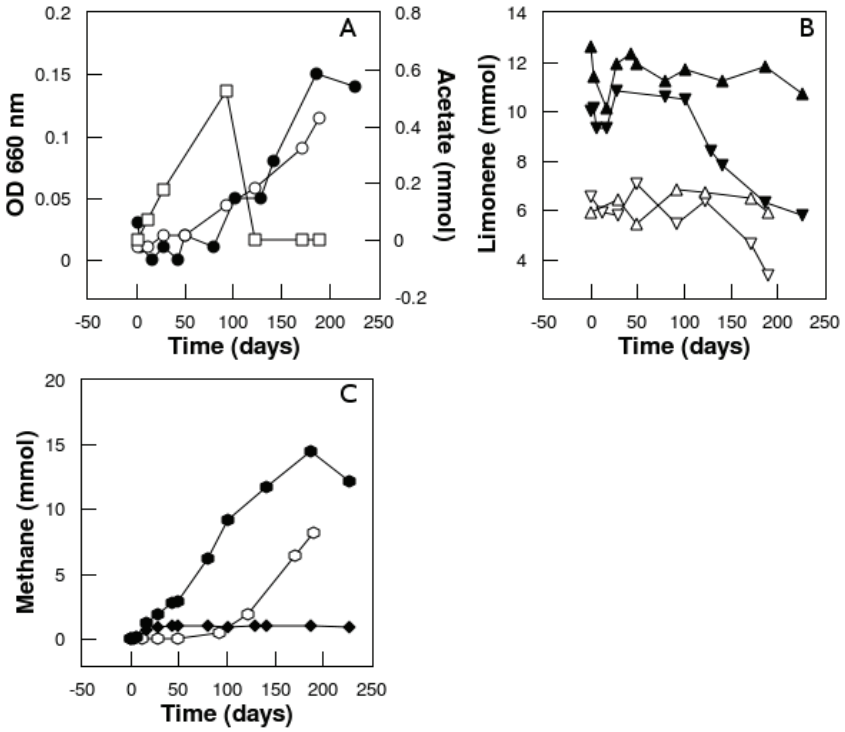

Figure 10.1: Limonene degradation in two enrichment cultures incubated in the presence (filled symbols) or absence (opened symbols) of acetate (2mM) under methanogenic conditions. Panel A shows the optical density increase in the two enrichment cultures, growing with 5% (filled circle) and 2% (opened circles) limonene in HMN. There is a transient accumulation of acetate in enrichment cultures incubated only with limonene (empty squares). Panel B shows the consumption of limonene in the same enrichment cultures, with 5% (filled down-triangles) and 2% (empty down-triangles) limonene in HMN versus incubated controls without inocula (corresponding filled and empty up-triangles). In panel C is shown the methane production in enrichment cultures (corresponding filled and empty hexagons) versus an inoculated control with acetate 2 mM (filled diamonds).

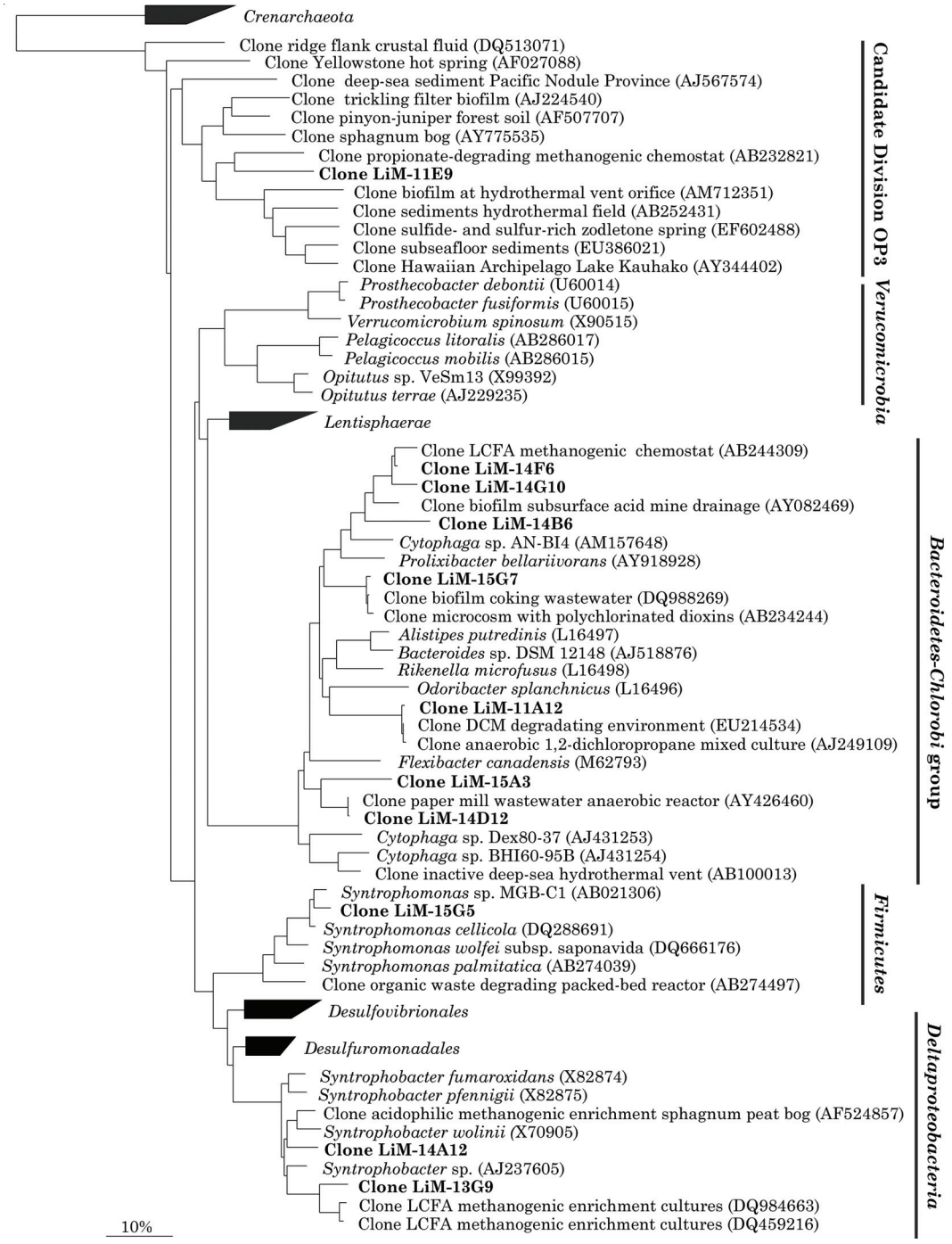

Figure 10.2: *M*aximum parsimony tree of *Bacteria* 16S rRNA gene sequences retrieved from limonene degrading methanogenic enrichment cultures. The representative *Bacteria* sequences obtained in this study are emphasized in bold letters. The accession number of reference sequences is shown in parentheses. *Crenarchaeota* sequences were used as out-group. The scale bar corresponds to 10 substitutions per 100 nucleotides.

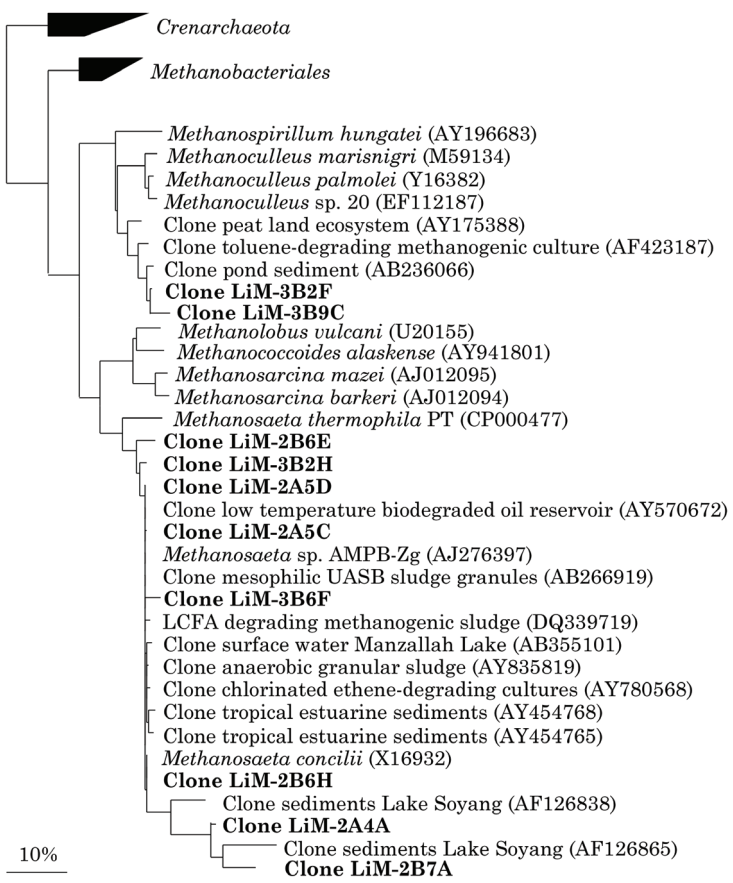

Figure 10.3: *M*aximum parsimony tree of *Archaea* 16S rRNA gene sequences retrieved from limonene degrading methanogenic enrichment cultures. The representative *Archaea* sequences obtained in this study are emphasized in bold letters. The accession number of reference sequences is shown in parentheses. *Crenarchaeota* sequences were used as out-group. The scale bar corresponds to 10 substitutions per 100 nucleotides.

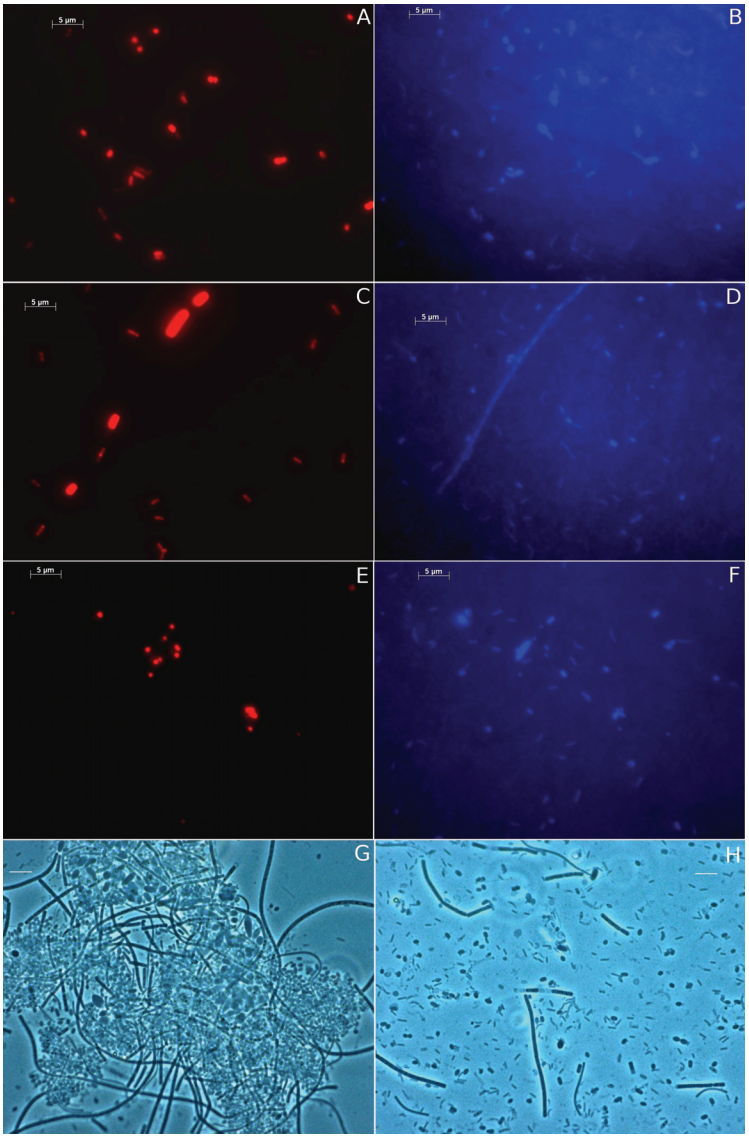

Figure 10.4: Microscopic images of samples from methanogenic enrichment cultures thriving on limonene, as visualized by epifluorescence microscopy (A-F) and phase contrast microscopy (G and H). The left panels represent samples stained by different HRP labeled probes. The red signals are given by the HRP catalyzed deposition of Alexa-594 tyramides. To the right are the same samples visualized by a DNA stain, DAPI. Panel A shows cells stained by the general Eubacteria probes, Eub-338 (I and VI). The same microscopic field is shown to the left, in panel B, with cells only stained by DAPI. In panel C are shown Arch-915 stained cells. The corresponding microscopic field visualized with DAPI is shown to the right, in panel D. Panel E, depicts cells stained with the newly designed probe, OP3-565, specific for the Candidate Division OP3 phylotype from the *Bacteria* clone library. To the right is the same microscopic field visualized by DAPI. The two phase contrast microscopic images, show an aggregate (panel G) and the usual morphotypes (panel H) encountered in limonene degrading enrichment cultures. The scale bar is 5 µm for all images.

Table 10.1: Microbial community composition as determined by HRP labeled probes, applied on samples from enrichment cultures thriving on limonene under methanogenic conditions.

Target group (coverage %)	Probe name	Sequence (5'-3')	16S rRNA target site	Form-amide	References
Domain *Bacteria* (90%)	Eub-338*	GCTGCCTCCCGTAGGAGT	338-355	0-35%	Amann et al., 1990a
Clones LiM related to OP3	Eub-338 (VI)*	GCAGCCTCCCGTAGGAGT	338-355	0-35%	This study
Domain *Archaea* (90%)	Arch-915	GTGCTCCCCCGCCAATTCCT	915-934	35%	Stahl and Amann, 1991
None	Non-338	ACTCCTACGGGAGGCAGC	-	0-35%	Wallner et al., 1993
Class *Deltaproteobacteria* (73%)	Delta-495a**	AGTTAGCCGGTGCTTCCT	495-512	35%	Loy et al., 2002
Phylum *Bacteroidetes* (38%)	CF-319a	TGGTCCGTGTCTCAGTAC	319-336	30%	Manz et al., 1996
Family Planctomycetaceae (75%)	Pla-46	GACTTGCATGCCTAATCC	46-63	30%	Neef et al., 1998
Family *Methanosaetaceae* (some members)	MX-825	TCGCACCGTGGCCGACACCTAGC	825-847	50%	Raskin et al., 1994
Clones LiM related to OP3	OP3-565	TACCTGCCCTTTACACCC	565-583	30%	This study

*An equimolar amount of probes Eub-338 and Eub-338(VI) was applied to enumerate *Bacteria*.

**An equimolar amount of probe Delta-459a and competitor (5' AGTTAGCCGGTGCTTCTT 3') was used to target the *Deltaproteobacteria*.

Table 10.2: Limonene consumption and methane production in enrichment cultures with limonene in the presence or absence of traces of acetate (2mM). Control cultures (Ctrl) showed how much of the methane produced is related to the endogenous carbon sources carried with the inocula, or to the traces of acetate added to the media.

Culture name	Inocula (% v/v)	Initial limonene (mmol)	Final limonene (mmol)	Final methane (mmol)	Methane (mmol) from:			Methane recovery (%)***
					Limonene (T)	Acetate (T)	Cysteine (T)	
165A	10	10	7.8	12.3	15.4	0.6	0.3	75
165B	10	10	6.3	12.0	25.9	0.6	0.3	45
165Ctrl	10	-	-	0.9	-	0.6	0.3	97
164A	**1.3	4.6	2.6	9.7	14.0	-	0.3	65
164B	**1.3	4.6	2.4	8.2	15.4	-	0.3	50
164Ctrl	**1.3	-	-	0.1	-	-	0.3	-

* The theoretical methane formed from limonene was calculated according to equation 1. Whereas the theoretical methane formed from acetate and cysteine was calculated from the equations: i) $C_2H_4O_2 \rightarrow CH_4 + CO_2$ and ii) $4\ C_3H_7NO_2S + H_2O \rightarrow 5\ CH_4 + 7\ CO_2 + 4\ NH_3 + 4\ H_2S$, respectively.

**The inocula was 20 × concentrated cell suspension added to the media in the mentioned amount.

***The methane recovered is the percentage of methane produced by cultures in relation to the theoretical value ($\Sigma\ CH_4$ from limonene acetate and cysteine).

Table 10.3: Phylotypes (OTUs) identified in limonene degrading enrichment cultures growing under methanogenic conditions, and the identity (ID. %) to their closest cultured and uncultured relatives. The accession number of the reference organisms is given in parenthesis.

Phylum	Clone	No.	Cultured relative	Identity (%)	Uncultured relative	Identity (%)
Proteobacteria	LiM-14A12	11	*Syntrophobacter* sp. strain Tsu1 (AJ237605)	92.1	Methanogenic sphagnum peat bog (AF524857)	92.3
Proteobacteria	LiM-13G9	1	*Syntrophobacter* sp. strain Tsu1 (AJ237605)	92.4	LCFA methanogenic enrichment culture (DQ459216)	95.1
Bacteroidetes	LiM-14F6	8	*Prolixibacter bellariivorans* (AY918928)	88.7	LCFA methanogenic enrichment culture (AB244309)	96.5
Bacteroidetes	LiM-14D12	3	*Prolixibacter bellariivorans* (AY918928)	85.3	Anaerobic bioreactor paper mill waste water (AY426460)	99.7
Bacteroidetes	LiM-11A12	2	*Alistipes putredinis* (L16497)	84.1	DCM degrading environment (EU214534)	99.3
Bacteroidetes	LiM-14G10	1	*Prolixibacter bellariivorans* (AY918928)	86.9	LCFA methanogenic enrichment culture (AB244309)	92.5
Bacteroidetes	LiM-14B6	1	*Prolixibacter bellariivorans* (AY918928)	83.9	Subsurface acid mine drainage biofilm (AY082469)	88.1
Bacteroidetes	LiM-15G7	1	*Prolixibacter bellariivorans* (AY918928)	86.5	Biofilm coking waste water (DQ988269)	99.5
Bacteroidetes	LiM-15A3	1	*Alistipes putredinis* (L16497)	81.8	Inactive deep-sea hydrothermal vent chimneys (AB100013)	83.8
Firmicutes	LiM-15G5	1	*Syntrophomonas cellicola* (DQ288691)	96.3	Undescribed *Syntrophomona*s strain MGB-C1 (AB021306)	96.5
Candidate Division OP3	LiM-11E9	5	*Opitus* sp. VeSm13 (X99392)	75.8	Pinion-juniper forest soil (AF507707)	83.3
Euryarchaeota	LiM-3B2F	10	*Methanoculleus palmolei* (Y16382)	91.6	Pond sediment (AB236066)	99.0
Euryarchaeota	LiM-3B9C	1	*Methanoculleus palmolei* (Y16382)	88.7	Pond sediment (AB236066)	96.4
Euryarchaeota	LiM-2A5C	5	*Methanosaeta* sp. AMPB-Zg (AJ276397)	99.7	Low-temperature biodegradable oil reservoir (AY570672)	99.7
Euryarchaeota	LiM-2A5D	4	*Methanosaeta* sp. AMPB-Zg (AJ276397)	99.8	UASB sludge granules (AB266919)	99.8
Euryarchaeota	LiM-2B6E	2	*Methanosaeta concilii* (X16932)	95.0	Tropical estuarine sediments (AY454768)	98.8
Euryarchaeota	LiM-2A4A	2	*Methanosaeta concilii* (X16932)	95.2	Sediments of Lake Soyang (AF126838)	92.5
Euryarchaeota	LiM-3B6F	1	*Methanosaeta concilii* (X16932)	97.6	Surface water Manzallah Lake (AB355101)	98.0
Euryarchaeota	LiM-3B2H	1	*Methanosaeta concilii* (X16932)	98.1	Tropical estuarine sediments (AY454768)	98.8
Euryarchaeota	LiM-2B7A	1	*Methanosaeta concilii* (X16932)	89.8	Sediments of Lake Soyang (AF126865)	91.9
Euryarchaeota	LiM-2B6H	1	*Methanosaeta concilii* (X16932)	100.0	Anaerobic granular sludge (AY835819)	100.0

Table 10.4: Relative abundance of different groups of *Bacteria* and *Archaea* in methanogenic enrichment cultures thriving on limonene as quantified by CARD-FISH.

Probe	Target	% population *hybridized with: Kingdom specific probes	Phylum or group specific probes
Eub-338 (I & VI)	*Eubacteria*	40	-
Non-338	Nonsense	0	-
Arch-915	*Archaea*	33	-
Delta-495a	*Deltaproteobacteria*	-	12
CF-319a	*Bacteroidetes*	-	1
Pla-46	*Planctomycetes*	-	**13
OP3-565	Candidate Division OP3	-	18
MX-825	*Methanosaeta*	-	1
Total recovery		73	32

*Numbers show the percentages of cells that hybridized to a probe versus DAPI-stained cells in the same visual field. **This probe was used to target Candidate Division OP3 , since *in silico* it fully matched our OP3 sequences. Therefore we excluded the 13% Pla-46 targeted cells from the total recovery.

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11 Manuscript 2

Deltaproteobacteria isolated from limonene degrading enrichments

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Manuscript in preparation

Abstract

The microorganisms involved in hydrocarbon degradation under methanogenic conditions are predominantly unknown. Here, we pursued to isolate and identify microorganisms potentially involved in limonene degradation under methanogenic conditions. We isolated, from limonene degrading enrichments, microorganisms growing on lactate or fumarate under fermenting conditions. These isolates grouped within *Deltaproteobacteria*, with only 89% identity to their next cultured relative, *Desulfoarculus baarsii*. Strains isolated on lactate fermented their substrate to acetate, propionate and butyrate, whereas strains isolated on fumarate accumulated acetate and succinate. We propose that these new strains represent a new genus enclosed in *Deltaproteobacteria* which could play the role of secondary fermenters or syntrophs, in limonene degradation.

Introduction

Methanogenesis prevails in anoxic environments where electron acceptors other then carbon dioxide are limiting. Examples of such environments are anoxic sediments, flooded soils, sewage digestors and the intestinal tract of animals and insects (Whitman et al., 2006; Hattori, 2008; Dolfing et al., 2008). Organic matter is degraded to methane and carbon dioxide, a low energy gaining process in comparison to other respiratory processes. Some microorganisms have the ability to catabolize complex organic matter only if their end metabolites are removed by a metabolically different group of microbes (Schink, 1997; Schink and Stams, 2006; McInerney et al., 2008). The mutualistic relationship established between metabolically different organisms is defined as syntrophy (Schink and Stams, 2006; McInerney et al., 2008).

Numerous studies on syntrophic co-cultures revealed the capacity of such cooperative interactions to degrade fatty acids (Boone and Bryant, 1980; McInerney et al., 1981; Stieb and Schink, 1985; Roy et al., 1986; Wallrabenstein et al., 1994; Plugge et al., 2002; Wu et al., 2006; Dolfing et al., 2008), aminoacids (Zindel et al., 1988), alcohols (Bryant et al., 1967; Ben-Bassat and Zeikus, 1981; Schink and Stieb, 1983; Schink, 1985b) and aromatics (Szewzyk and Schink, 1989; Jackson et al., 1999; Qiu et al., 2003, 2006, 2008). Hydrocarbon degradation under methanogenic conditions was demonstrated by earlier studies in enrichment cultures (Schink, 1985a; Edwards and Grbic-Galic, 1994; Harder and Foss, 1999; Meckenstock, 1999; Zengler et al., 1999; Anderson and Lovley, 2000) or by field measurements (Reinhard et al., 2005). However, important questions are left unanswered such as: “i) Who degrades hydrocarbons under methanogenic conditions?” and “ii) What are the potential intermediates shuttled between microorganisms to establish a co-operative interaction?”

This study tackles the identification of microorganisms involved in the degradation of the most widespread monoterpene, limonene under methanogenic conditions. Limonene is

produced by plants and is the most widespread monoterpene in nature (Trudgill, 1990). The degradation of this monoterpene, and the microbial community compositions was previously studied (Rotaru et al., Prep). Here we pursue isolation of syntrophic microorganisms from limonene degrading enrichment cultures. The strains were isolated on two fermentable substrates, fumarate and lactate. We determined the phylogeny of these strains, and the ability to grow in the presence of limonene, in co-culture with a methanogen, *Methanosarcina mazei*.

Materials and methods

Cultivation of microorganisms

Enrichment. Sediment free methanogenic cultures growing on different monoterpenes were established earlier from waste water treatment plant on other monoterpenes (Harder and Foss, 1999) and later grown with 5% limonene in 2,2,4,6,8,8-hepta-methylnonane (HMN) (Rotaru et al., Prep). Methanogenic fresh water media was prepared after Harder and Foss (1999).

Isolation of microorganisms from limonene degrading enrichments. Potential syntrophic microorganisms were isolated anaerobically with the roll tube technique (Hungate, 1969) using fermentable substrates, like fumarate (50 mM) or lactate (50 mM), as energy source. A total of 40 colonies were selected. Each colony was resuspended and inoculated in freshwater liquid media. These resuspended colonies were inoculated in 3 tubes to ensure the chance of survival during transfer. The cultures were transferred and maintained under fermenting conditions in 15 ml Hungate tubes with 10 ml freshwater media, lactate (50 mM) or fumarate (50 mM). Purity of cultures was routinely verified by phase contrast microscopy.

Co-cultivation of isolated strains with *Methanosarcina mazei.* The newly isolated strains were incubated in co-culture with *M. mazei.* The methanogen was kindly supplied by Jana Milucka. For this experiment the energy source was 5% limonene in HMN (vol/vol) overlaid on top of freshwater mineral media. The freshwater media was prepared as mentioned above. The inocula was 10% isolate (vol/vol) and 10% (vol/vol) *M. mazei*. Each of the isolated cultures were incubated with 5% limonene (in HMN) in the absence of *M. mazei*. Another control was *M. mazei* incubated with acetate (10 mM) in the presence of the monoterpene. The optical density increase was monitored by direct measuring of culturing tubes on a Shimadzu UV-VIS Spectrophotometer.

Chemical analysis

All samples for chemical analysis were retrieved with N_2 flushed hypodermic needles and syringes.

Fatty acids were detected by high performance liquid chromatography on a Sykam HPLC (Sykam). Filtered samples were injected using a Sykam S5200 autosampler. Separation was performed on an Aminex HPX-87H column (300 x 7.8 mm) under isothermal conditions (40℃). The eluent was 5 mM H_2SO_4. Fatty acids were detected with a LINEAR UVIS Detector. Data acquisition and processing was done with DataApex Clarity HPLC Software (Gamma Analysen Technik GmbH). Our standards consisted of the following fatty acids: succinate, lactate, formate, acetate, propionate and butyrate. The detection limit was 0.1 mM. Fumarate and formate exhibit the same retention time on the column.

Molecular analysis of isolated strains

Denaturing gradient gel electrophoresis (DGGE). As template for DGGE PCR-amplification we used lyzed cells of isolated strains and total DNA extracted from a limonene degrading enrichment. The DNA extraction was done as published elsewhere (Zhou et al., 1996). The amplification reaction mix for DGGE consisted of: 2 µM each primer (GM5-GC and 907-RM), 0.2 mM total dNTPs, 0.04 U Taq-Polymerase, 30 µg bovine serum albumin, 1× PCR Enhancer and 1× PCR buffer in a final volume of 100 µl. The DGGE amplification was carried on an Eppendorf Mastercycler (Eppendorf) with an initial denaturing step of 5 min at 95℃, followed by polymerase addition at 80℃, primer annealing at 65℃ for 1 min, and 3 min elongation at 72℃. The next 23 cycles comprised one denaturing step of 1 min at 94℃, primer annealing - 65℃ for 1 min, and 3 min elongation at 75℃. For the following 19 cycles there was a decrease of the annealing temperature as well as elongation temperature, down to 65℃ and 72℃, respectively. The final elongation step was 10 min at 72℃. The denaturing gradient gel was prepared as published elsewhere (Muyzer et al., 1996) and run for 3.5 hours at 200 mV. Bands with high intensity were chosen for sequencing. The sequencing reactions were done as mentioned below. Sequences of circa 500 bp were added by maximum parsimony to the phylogenetic tree calculated as explained below.

Sequencing analysis. Freeze-thaw lyzed cells were used as template for the amplification of the 16S rRNA gene with bacterial primers, GM3 and GM4 (Hicks et al., 1992; Kane et al., 1993). The reaction mix consisted of 1 µM of each primer, 1 mM total dNTPs, 0.04 U Taq-Polymerase, 15 µg bovine serum albumin and 1× PCR buffer in 50 µl. PCR amplification was carried on an Eppendorf Mastercycler (Eppendorf) with an initial denaturing step of 5 min at 96℃, followed by 30 cycles that involved denaturing for 1 min at 96℃, primer annealing at 48℃ for 1 min, and 3 min elongation at 72℃. These cycles were followed by a final elongation step of 10 min at 72℃. The amplicons were purified by gravity centrifuging at 910 RCF on Sephadex Superfine G50 columns (Amersham Bio-

sciences). Purified amplicons were used as template for sequencing reactions generated according to the manufacturer's manual and analyzed on a 3130 XL Genetic Analyser (Applied Biosystems). Sequences were cleaned of vector data and assembled into contigs using DNA-Baser (www.dnabaser.com). The nearly complete 16S rRNA gene sequence data were aligned with the ARB-Silva software package (Pruesse et al., 2007; Ludwig et al., 2004). A maximum parsimony phylogenetic tree was calculated excluding the influence of highly variable positions. The tree was reconstructed only using sequences with more then 1200 bp.

Results and discussions

Isolation of novel *Deltaproteobacteria* from limonene degrading enrichments

From limonene degrading methanogenic enrichments, we isolated seven strains in fermenting conditions, with fumarate or lactate as energy sources. The use of such substrates that are more oxidized then the initial one is a well described procedure for isolation of syntrophic microorganisms (Schink, 1985a; Beaty and McInerney, 1987; Wallrabenstein et al., 1994, 1995a).

Numerous yellow-white colonies were obtained using the roll tube technique (Hungate, 1969). We selected 40 of them, for transfer in freshwater methanogenic liquid media. Transfers took more then 6 months for growth and only 8 colonies developed into stable liquid cultures. From these, three fermented lactate (SynL-5-9-c1, SynL-5-9-c2 and SynL-65) and the other five fermented fumarate (SynF-5-17-c1, SynF-5-17-c2, SynF-5-17-c3, SynF-5-18-c1). Lactate isolated strains were represented by large vibrio shaped cells with an average size of 0.9 μm $\times$ 2.5 μm. The fumarate isolated strains were less curved and slightly smaller with an average size of 0.8 μm $\times$ 2.4 μm (Figure 11.1).

Fermentation profile

We analyzed the fatty acid profile for lactate and fumarate utilizing strains.

The isolates which grew on lactate produced acetate, propionate and butyrate. In average, for each 1 mM lactate consumed, the products were 1 mM acetate and less then 0.05 mM propionate and butyrate (Table 11.2). Lactate fermentation to acetate in a ratio of 1 to 1 was also observed in the thermophilic syntrophic, propionate oxidizer *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge et al., 2002). *D. thermobenzoicum* can grow by fermentation besides lactate, also with benzoate, fumarate, hydrogen, and pyruvate.

The isolates which grew on fumarate produced acetate and succinate. For each 1 mM fumarate consumed we identified the evolution of 1.5 to 2 mM acetate, and less then 0.4 mM succinate (Table 11.2).

Syntrophobacter strains are capable of growth under sulfate reducing conditions, with

different substrates. A full metabolic profile of these strains could reveal more on different aspects of their physiology. Questions that remain to be addressed are: „If they use other electron acceptors?“ „Considering the full identity between lactate and fumarate - utilizing strains, are they as similar at the physiological level?“„What other substrates can they ferment?“ „What substrates could they specifically ferment in co-culture with a methanogen?“

Co-cultures with *Methanosarcina mazei*

The growth of lactate and fumarate isolated syntrophs in co-culture with *M. mazei* in the presence of 5% limonene (vol/vol in HMN) was monitored for 71 days. Strains grew at higher optical density then when cultivated alone in the presence of limonene (Figure 11.4). Regardless of the higher cell densities that were found in co-cultures thriving on limonene, the fatty acid profiles suggest that limonene degradation was not on the account of the new isolates (Table 11.2). The difference in the fatty acid profiles for lactate- and fumarate-isolated strains alone or in co-cultures was most likely due to the utilization of endogenous carbon sources (lactate, fumarate or acetate), carried with the inocula and not due to limonene utilization.

Phylogenetic analysis

The phylogenetic position was determined by 16S rRNA sequence analysis (Figure 11.3). We obtained sequences of each isolate and compared them using the ARB software. The resulting isolates were representatives of a single phylotype with less then 1% difference between each other. Their closest uncultured relative (91.2%) was a sequence retrieved from 4-methyl-benzoate degrading methanogenic enrichment (Wu et al., 2001). Whereas the closest cultured relative (11% difference), was a sulfate reducer that utilizes formate, and cannot utilize lactate, *Desulfoarculus baarsii* (Widdel, 1980, 1981). Moreover the isolates were far related to genus *Syntrophobacter*. Even the nearest neighbor within this genus, *Syntrophobacter phennigii* (Wallrabenstein et al., 1995b), showed only 85% 16S rRNA gene identity to these strains. We suggest that these novel isolates establish a novel branch within *Deltaproteobacteria,* since they are far from their next in culture relative, *D. baarsii,* both at the phylogenetic and physiologic level.

Another tool for determining the relationship between isolates was DGGE pattern analysis. We observed a similar position for their amplified 16S rDNA gene in denaturing gel (Figure 11.2). However two of the fumarate isolates showed a second band at a higher position on the gel. This could be due to: i) impurity of cultures, so different DGGE patterns emerged; ii) each strains has two different 16S rRNA operons. This could result from an insertion or a higher G+C content in one of the 16S operons. The dominant DGGE band that was common for all the isolated strains, was sequenced and was 100% identical to the 16S rRNA gene sequences of the isolates (Figure 11.2). The phylotype was present in limonene degrading enrichment cultures, since a similar DGGE band was observed.

We suggest that these newly isolated *Deltaproteobacteria* do not play the role of primary fermenters in limonene degradation, but of secondary fermenters (syntrophs), likely being involved in short chain fatty acid or alcohol degradation. A better understanding of the physiology of these strains, could offer more insights on the mechanism of limonene methanogenic degradation. Isolation of primary fermenters capable of limonene degradation could be pursued by incubation with larger fatty acids and alcohols as substrates for fermentation. Another question which could be addressed is „If three is the minimum number of members necessary for limonene degradation under methanogenic conditions, or is there a need for more?“ Successful isolation of primary fermenters could be the start point for tri-culture experiments in the presence of *Deltaproteobacteria*-related isolates and different types of methanogens thriving solely on limonene.

Here we isolated lactate and fumarate fermenting *Deltaproteobacteria* and we observed that they can grow in the presence of limonene in co-culture with an acetotrophic and/or hydrogenotrophic methanogen. Conclusive data on their involvement in limonene degradation remain to be brought in a further study.

Acknowledgments

We would like to thank Jana Milucka for supplying growing cultures of *Methanosarcina mazei*, and Regina Schauer for introduction to DGGE. We thank for financial support the Max Planck Society and for partial funding the International Research School for Marine Microbiology.

Figures and tables

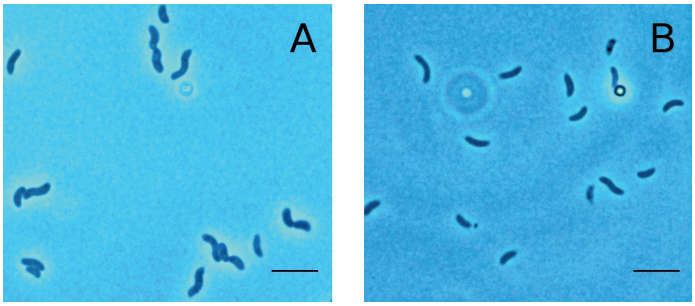

Figure 11.1: Phase contrast micrographs of cultures isolated from limonene degrading methanogenic enrichments. A culture isolated on lactate is shown in panel A and one isolated on fumarate is shown in panel B. The scale bar is 5 µm.

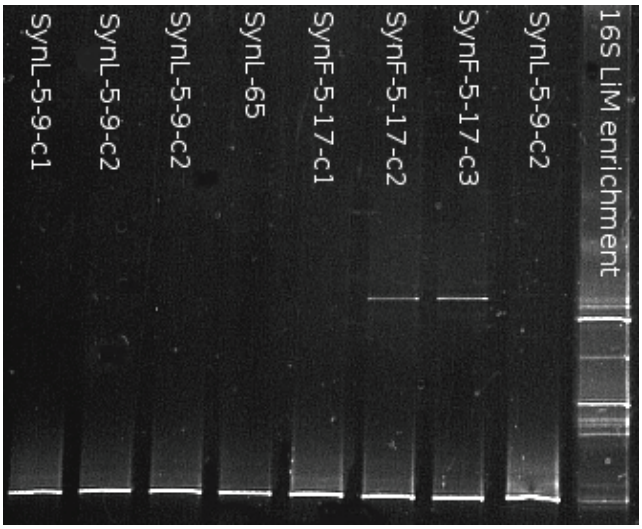

Figure 11.2: DGGE patterns of newly isolated strains and extracted 16S rDNA of an enrichment thriving on limonene.

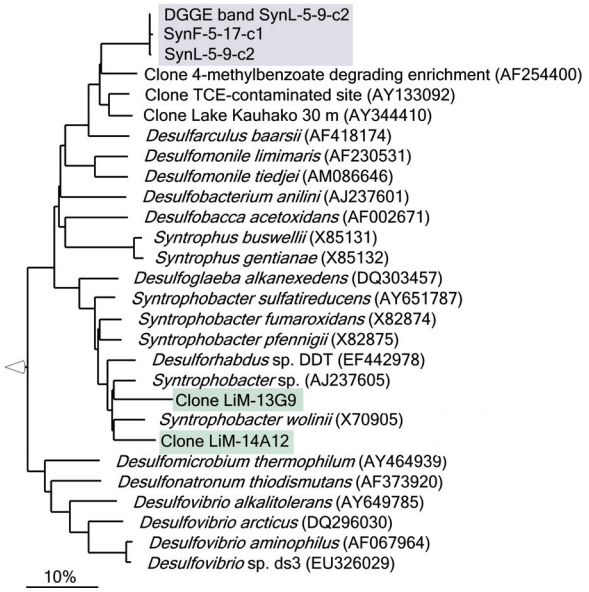

Figure 11.3: Maximum parsimony phylogenetic tree of 16S rRNA gene sequences of microorganisms isolated from limonene degrading methanogenic enrichment cultures (blue). The closest related clones from limonene degrading enrichments are emphasized in green. The tree was rooted using *Chlorobi* sequences as an out-group. The scale bar represents 10 substitutions per 100 bp .

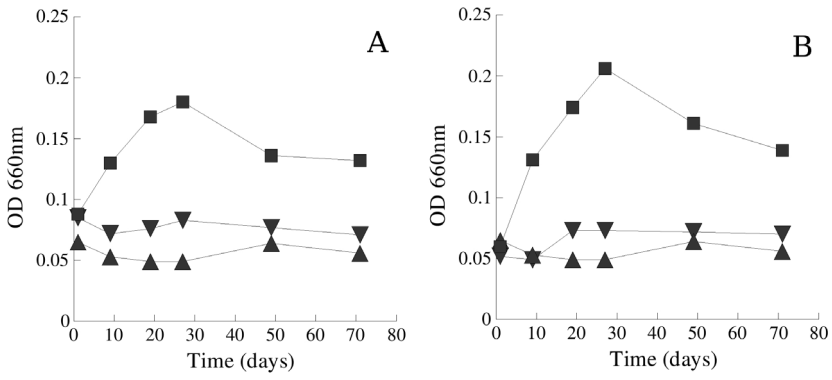

Figure 11.4: Growth of co-cultures with 5% limonene in HMN (vol/vol) as energy source (squares) versus isolates alone (down triangles) or *M. mazei* alone (up triangles) with limonene as energy source. Panel A shown the corresponding data for a lactate isolated organism SynL-5-9-c2, while panel B shows the data for a fumarate isolated organism SynF-5-17-c1.

Table 11.1: 16S rRNA primers used for denaturing gradient gel electrophoresis

PRIMER	SEQUENCE (5'-3')	POSITION	REFERENCE
GM5F-GC[α]	CCTACGGGAGGCAGCAG	341-357	(Muyzer et al., 1993)
907R	CCGTCAATTCCTTTGAGTTT	907-927	(Muyzer et al., 1995)

F (forward) and R (reverse) indicate the orientation of the primers in relation to the rRNA sequence

[α] GC clamp: 5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´

Table 11.2: Fatty acid profile of newly isolated *Deltaproteobacteria,* grown on lactate and fumarate, in the absence or presence of limonene, and in co-culture with *Methanosarcina mazei.*

	Substrate added:			Substrate final (mM)		Fatty acids produced (mM)			
Strain	Limonene (mM)*	Lactate (mM)*	Fumarate (mM)*	Lactate	Fumarate	Acetate**	Succinate	Propionate	Butyrate
SynL-5-9-c1	-	50	-	31.6	-	24.6	-	0.8	0.8
SynL-5-9-c2	-	50	-	36.2	-	18.2	-	0.8	0.6
SynL-65	-	50	-	31.6	-	18.6	-	1.3	0.6
SynF-5-17-c1	-	-	50	-	23.9	31.1	2.1	-	-
SynF-5-17-c2	-	-	50	-	36.0	30.4	2.4	0.4	-
SynF-5-17-c3	-	-	50	-	29.7	38.8	8.8	-	-
SynF-5-18-c1	-	-	50	-	28.6	39.8	0.2	-	-
SynL-5-9-c1	27	5	-	1.2	-	6.7	-	0.1	-
SynL-5-9-c2	27	5	-	0.4	-	7.2	-	0.1	-
SynL-65	27	5	-	0.6	-	6.8	-	0.1	-
SynF-5-17-c1	27	-	5	-	13.8	0.2	-	-	-
SynF-5-17-c2	27	-	5	-	18.8	-	0.7	-	-
SynF-5-17-c3	27	-	5	-	11.8	0.2	-	-	-
SynF-5-18-c1	27	-	5	-	9.0	0.5	0.2	-	-
SynL-5-9-c1 + M. mazei	27	5	-	0.8	-	-	-	-	-
SynL-5-9-c2 + M. mazei	27	5	-	2.4	-	2.3	-	3.7	-
SynL-65 + M. mazei	27	5	-	1.5	-	-	-	-	-
SynF-5-17-c1 + M. mazei	27	-	5	-	3.0	0	0.3	-	-
SynF-5-17-c2 + M. mazei	27	-	5	-	3.1	0	0.5	-	-
SynF-5-17-c3 + M. mazei	27	-	5	-	2.7	0.3	0.6	-	-
SynF-5-18-c1 + M. mazei	27	-	5	-	4.0	0	0.2	-	-

* These substrates were added in these amounts to the freshwater media, however these are not measured data.

** The amount of acetate is calculated by subtracting the amount of acetate known to be added to the media (detected as 2 mM in control cultures without inocula) from the measured acetate in cultures media at the end of the incubation.

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12 Manuscript 3

Highly enriched *Betaproteobacteria* growing anaerobically with *p*-xylene and nitrate

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Intended for FEMS Microbial Ecology

Abstract

The identity of the microorganisms responsible for anaerobic *p*-xylene degradation and the mechanism of activation under denitrifying conditions are hitherto unknown. Here we report highly enriched cultures of freshwater denitrifying microorganisms that grow anaerobically with *p*-xylene as sole electron donor. A 16S rRNA gene-based approach was used to identify the dominant microorganisms in these cultures, which are long curved rods with 95% 16S rRNA gene sequence identity to *Denitratisoma oestradiolicum*. This phylotype belongs to the *Rhodocyclaceae* family, rather distant to other denitrifying hydrocarbon degraders which cluster within *Azoarcus-Thauera*. According to quantitative growth experiments and chemical analysis, *p*-xylene was completely oxidized to CO_2, via (4-methylbenzyl) succinate and (4-methylphenyl) itaconate as intermediary metabolites.

Introduction

The aromatic hydrocarbon *p*-xylene (1,4-dimethylbenzene), is a component of petroleum (Tissot and Welte, 1984) and raw material for the chemical industry. *p*-Xylene production reaches 2.2 $\times$ 10^3 tones per year (Association of petrochemicals producers in Europe, A.P.P.E.), since it is used to manufacture solvents and precursors, such as terephthalic acid, the precursor of polyester.

The discharge of *p*-xylene into freshwater from petroleum seeps is thought to be surpassed by anthropogenic release, such as accidental spills of fuel and leakage from tankers and hauling pipes.

p-Xylene is a non-polar compound with a relatively high water solubility (0.18 g / L at 25℃) (Shiu and Ma, 2000). Such properties place *p*-xylene among the most mobile and toxic petroleum-derived groundwater contaminants, along with benzene, toluene, ethylbenzene, and *o*- and *m*-xylene (Anderson and Lovley, 1997). The fate of *p*-xylene in the environment is determined by chemical and microbial processes (Tuazon et al., 1984; Atkinson et al., 1991; Head et al., 2006). Aerobic microorganisms activate *p*-xylene to 3,6-dimethylcatechol, a process catalyzed by dioxygenases (Wackett, 2006). Whereas under anaerobic conditions, which often prevail in underground waters and aquifers, *p*-xylene is removed by microorganisms thriving under nitrate-, sulfate-, iron (III) - reducing or methanogenic conditions.

p-Xylene is of the least degradable consitutents of the entire BTEX fraction, along with benzene and *o*-xylene (Heider et al., 1999; Widdel et al., 2006; Foght, 2008). In contrast to *p*-xylene, benzene and *o*-xylene supported anaerobic growth of pure cultures (Harms et al., 1999; Coates et al., 2001; Morasch et al., 2004; Kasai et al., 2006), whereas only three studies obtained stable enrichment cultures and confirmed anaerobic degradation of *p*-xylene under denitrifying and sulfate reducing conditions (Haner et al., 1995; Morasch and Meckenstock, 2005; Nakagawa et al., 2008). 16S rRNA gene fragments from denatur-

ing gels showed the presence of a unique sequence type (Nakagawa et al., 2008) related to *Desulfosarcina ovata* strain oXyS1 an *o*-xylene degrading sulfate-reducing bacteria (Harms et al., 1999). Moreover *p*-xylene loss was also detected under iron-reducing conditions (Botton and Parsons, 2007) and the 16S rRNA gene fragments retrieved from these enrichments were related to *Geobacter* and *Deltaproteobacteria* (Botton et al., 2007). The identity of denitrifying microorganisms involved in *p*-xylene degradation was never addressed. Therefore in this study we identified and quantified the members of a highly enriched community that degrades *p*-xylene under nitrate reducing conditions. The dominant phylotype of the microbial community was detected using a 16S rRNA gene-based approach (Amann et al., 1995).

Benzylsuccinic acid and its methylated analogues have been proposed as indicators of anaerobic toluene and xylene metabolism in contaminated environments (Beller, 2000; Elshahed et al., 2001; Suflita, 2002). To confirm that *p*-xylene is activated in denitrifiers similarly to sulfate reducers, by addition to fumarate (Morasch and Meckenstock, 2005), we performed gas chromatography-mass spectrometric analysis of metabolites.

Materials and Methods

Source of organisms and cultivation

A mixed inocula of 150 ml freshwater pond sediment and 50 ml sludge from a wastewater treatment plant (Osterholz-Schambeck, Germany) was added to 800 ml defined anoxic freshwater medium containing 5 mmol nitrate (after Widdel and Bak, 1992). As electron donor, we added 1% (v/v) *p*-xylene in 50 ml 2,2,4,4,6,8,8-heptamethylnonane (HMN). A control culture was prepared without *p*-xylene. Cultures were sealed with butyl rubber stoppers that had no contact with the organic phase, and incubated at 28℃ under a N_2/CO_2 atmosphere (90/10, v/v). A subsequent transfer with 10% (v/v) inocula from the initial enrichment cultures was incubated with 5 mM nitrate as electron acceptor and 1% p-xylene (v/v) as electron donor. Mud-free cultures were continuously incubated under moderate shaking (80 rpm).

Furthermore, from mud-free cultures we attempted isolation by four consecutive dilution-to-extinction series established in 156 ml serum bottles. The second dilution-to-extinction was done with a 1 to 2 dilution, and the remaining series with a 1 to 10 dilution. Each subsequent series was inoculated from the highest dilution that provided a grown culture. Isolation was attempted in solid agar, overlaid with 10% HMN as carrier for 1% *p*-xylene, colonies were picked and cultured in denitrifying liquid media with 1% *p*-xylene in HMN (v/v). Five colonies grew in liquid media, but were lost at subsequent transfers.

Growth tests were performed with the following hydrocarbons in HMN (conc. are v/v in %): benzene (0.5), toluene (1), ethylbenzene (1), *o*- and *m*-xylene (1), 2-methylnaphthalene (2), naphthalene (2), limonene (1), *n*-hexane (0.5), cyclohexane (0.5), *n*-decane (0.5). In addition we tested cholesterol (granules), *o-, m-* and *p*-methylbenzoate (each 5 mM), ben-

zoate (5 mM), fumarate and lactate (each 10 mM).

The consumption of *p*-xylene and nitrate, formation of nitrite, nitrous oxide, and cell dry mass, were measured in cultures grown in 400 ml medium, 12% (v/v) inocula, and 40 ml HMN as carrier for 0.2% *p*-xylene (v/v). Inoculated media without *p*-xylene and media with *p*-xylene and without inocula served as controls. Cell dry mass was quantified as described elsewhere (Rabus and Widdel, 1995).

Chemical analysis

All samples for chemical analysis were taken with N_2 flushed hypodermic needles and syringes.

Nitrate and nitrite samples were diluted 1:10 prior to ion chromatography on a Sykam HPLC IBJ A3 anion exchange column (4 $\times$ 60 mm) (Sykam, Munich, Germany). Separation under isothermal conditions (50 ℃) was achieved with 20 mM NaCl in 45% ethanol at a flow rate of 1 ml min^{-1}. Nitrate (retention time 3 min) and nitrite (2.1 min) were detected at 220 nm on a LINEAR Spectrophotometer. The signals were processed with DataApex Clarity HPLC Software (Gamma Analysen Technik GmbH, Bremerhaven, Germany). For fast monitoring, Merckoquant Test Strips (Merck, Darmstadt, Germany) were used to detect consumption of nitrate and nitrite. Ammonium was determined colorimetrically as described elsewhere (Weatherburn, 1967).

N_2O gas was detected on a Shimadzu GC-8A gas chromatograph equipped with a Poraplot Q column (length 2 m, inner diameter 3 mm). The carrier gas was N_2 and the flow rate was 32 ml/min. Separation occurred isothermally at 40 ℃, with the injector port and the thermal conductivity detector at 110 ℃.

p-Xylene concentrations were measured by head-space gas chromatography when all three phases (gaseous, HMN and aqueous) were in equilibrium (adapted from Musat and Widdel, 2007). Gas volumes of 0.1 ml were withdrawn at 28 ℃ and injected into a Shimadzu GC-14B (Duisburg, Germany) equipped with a Supel-Q PLOT fused silica capillary column (length 30 m, diameter 0.53 mm) and a flame ionization detector. Isothermal separation was performed at 200 ℃ with N_2 as carrier gas, the injector at 150 ℃ and the FID at 280 ℃.

Samples for volatile fatty acid analysis were filtered and diluted 1:10 before injection onto a Sykam HPLC (Fürstenfeldbruck, Germany) equipped with an Aminex HPX-87H HPLC column (300 x 7.8 mm). The eluent was 5 mM H_2SO_4. The separation was isothermal at 40 ℃ with the UV detector at 210 nm.

Metabolite were extracted from 100 ml of cells disrupted by heating at 85 ℃ for 40 min. Samples were acidified to pH 1 with H_3PO_4 prior to extraction with dichloromethane (DCM). The DCM extract was dried with anhydrous Na_2SO_4 and derivatized prior to gas chromatographic-mass spectrometric (GC-MS) analysis using a solution of diazomethane in diethyl ether. GC-MS measurements were performed using a Trace GC-MS (Thermoelectron, Dreieich, Germany) equipped with a temperature-programmable injection system and a BPX5 fused silica capillary column (length 50 m, inner diameter 0.22 mm, film

thickness 0.25 µm). Helium was used as carrier gas. The GC oven temperature was programmed from 50℃ (1 min isothermal) to 310℃ (30 min isothermal) at a rate of 3° per minute. The mass spectrometer was operated in electron impact mode and at an ion source temperature of 230℃. Full scan mass spectra were recorded over the mass range of 50 to 600 Dalton at a rate of 2,5 scans per second.

Molecular analysis

Extracted and purified genomic DNA from highly enriched cultures (Zhou et al., 1996) was used to amplify the almost full 16S rRNA gene sequence with specific bacterial primers, 8F (Hicks et al., 1992) and 1492R (Kane et al., 1993). The PCR products were cleaned with a QIAquick Purification Kit (Quiagen, Hilden, Germany) cloned into pCR4-TOPO vector (Invitrogen) and transformed into TOP10 chemically competent *E. coli* cells (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator vs. 3.0 cycle sequencing kit and an ABI Prism 3130XL Genetic Analyser (Applied Biosystems). Sequences were cleaned of vector data with Sequence Analysis 5.2 (Applied Biosystems) and assembled into full length 16S rRNA sequences using the Sequencher software (Gene Codes Corporation). 16S rRNA gene sequences were aligned with the sequences in the Silva database vs. 94 (http://www.arb-silva.de) using the ARB software package (Ludwig et al., 2004; Pruesse et al., 2007). Different phylogenetic trees were calculated with nearly complete sequences (more than 1300 nucleotides) by methods such as maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ).

Two probes for fluorescence in situ hybridization (FISH), pxyn-440 and pxyn-644, were specifically designed for the most dominant phylotype in our clone libraries (ARB PROBE Design Tool). Probes were tested *in silico* against RDP II database version 9.48 (http://rdp.cme.msu.edu/probematch/search.jsp). As two mismatch control for the specificity of pxyn-440 strain 72Chol was used (Harder and Probian, 1997). Probe pxyn-644 did not match any cultivated microorganism in the database. Specificity of the probes was ensured at 40% formamide during hybridization experiments. Nested FISH was performed with phylum to group-specific probes (Table 12.2) as described elsewhere (Amann et al., 1995). All probes used were synthesized with a 5'- Cy3 modification.

Nucleotide sequence accession numbers FM207901 to FM207960 are the EMBL-EBI accession numbers, of 16S rRNA gene sequences retrieved from two different enrichment cultures thriving on *p*-xylene.

Results and discussions

Enrichment and cultivation

Sediment-containing enrichment cultures with 1% *p*-xylene in HMN as organic energy source consumed the initial nitrate added (5 mM) after 80 days. Nitrate (5 mM) was re-supplied several times. The *p*-xylene-free control culture consumed 25 mM of nitrate in cca. 450 days, then nitrate reduction ceased. In contrast, enrichments with *p*-xylene continued to reduce up to 50 mM nitrate. After 650 days, *p*-xylene was no longer detected in these cultures. Consecutive subcultures consumed 13 to 27 mM nitrate within 270 days. The subcultures with the highest cumulative nitrate reduction were used as inocula for 4 successive liquid dilutions-to-extinction series, which resulted in sediment-free highly enriched cultures. Growth with *p*-xylene and nitrate in highly enriched cultures became faster and doubling times of approximately 7 days were observed. These enrichments were dominated by thin curved rods, 0.5 µm $\times$ 2 µm.

Growth tests on other hydrocarbons

Of all the tested hydrocarbons, besides *p*-xylene, only toluene sustained growth and denitrification. Cultures did not utilize the following hydrocarbons: benzene, ethylbenzene, *o*-xylene and *m*-xylene, 2-methylnaphthalene, naphthalene, limonene, *n*-hexane, cyclohexane, or *n*-decane. Other substrates which were utilized by our enrichments were *p*-toluic acid, benzoate, fumarate and lactate. Culture did not thrive on cholesterol, and *o*- and *m*-toluic acid. Cultures grown on *p*-toluic acid and toluene were represents by a similar morphotype like *p*-xylene grown cultures whilst more cell morphologies were observed in cultures grown on substrates such as benzoate, fumarate, and lactate.

Anaerobic pure cultures with *p*-xylene as electron donor have not been obtained yet, whereas pure cultures of denitrifiers and sulfate-reducers which utilize the other two xylene isomers are readily described (Harms et al., 1999; Rabus and Widdel, 1995). *m*-Xylene is the most easily degraded of the xylene isomers and some microorganisms isolated on this hydrocarbon are the sulfate-reducing strain mXyS1 (Harms et al., 1999), and the denitrifying strain mXyN1 (Rabus and Widdel, 1995). *o*-Xylene is less prone to degradation, however some sulfate-reducing strains, such as OX39 (Morasch et al., 2004) and oXyS1 (Harms et al., 1999), were isolated on this monoaromatic compounds. Although these strains were tested for growth on all xylene isomers, only strain OX39 was capable to grow with two different xylene isomers, o- and *m*-xylene (Morasch et al., 2004).

Quantification of *p*-xylene degradation

Catabolism was quantitatively monitored for 80 days in cultures grown with a low amount of hydrocarbon (Table 12.1). Cultures incubated with *p*-xylene consumed all nitrate added (Figure 12.1). To elucidate whether nitrate is reduced to ammonium, we measured the

ammonium concentration in the cell culture medium. At the end of the incubation, ammonium decreased by 1 µM. To determine whether nitrate is reduced to dinitrogen gas, we looked after intermediates, like nitrite and nitrous oxide. The concentrations of nitrite did not change, and dinitrogen gas was not detected during cultivation.

The nitrate consumed by a *p*-xylene degrading culture was 3.46 $\pm$ 0.07 mmol which could accept 17.3 $\pm$ 0.3 mmol of reducing equivalents (Table 12.1) if completely reduced to dinitrogen gas. The physical loss of *p*-xylene was low, as observed in a sterile control. From dry weight measurements, we estimated that 2.2 µmol *p*-xylene were assimilated into biomass (see Table 12.1). The amount of *p*-xylene incorporated into biomass was calculated using the assimilatory reaction: 17 C_8H_{10} + 32 HCO_3^- + 32 H^+ + 30 $H_2O \rightarrow$ 42 $C_4H_7O_3$. The total amount of *p*-xylene catabolized was of 0.50 $\pm$ 0.08 mmol, which could donate 20.9 $\pm$ 3.3 mmol reducing equivalents if completely oxidized to carbon dioxide. The complete oxidation of *p*-xylene according to the equation C_8H_{10} + 8.4 NO_3^- + 8.4 $H^+ \rightarrow$ 8 CO_2 + 4.2 N_2 + 9.2 H_2O ($\Delta G^{0'}$ is -4202.6 kJ mol^{-1} *p*-xylene), is supported by the electron balance (Table 12.1) and a lack of fatty acids accumulation in the culture media. Here we show for the first time the stoichiometric coupling of *p*-xylene oxidation to nitrate reduction. Another study proposed a complete oxidation of *p*-xylene under denitrifying cultures based on similar rates of electron transferred from the electron donor to the electron acceptor, during exponential growth (Haner et al., 1995). Whereas under sulfate reducing conditions *p*-xylene oxidation was stoichiometrically coupled to reduction of sulfate (Morasch and Meckenstock, 2005; Nakagawa et al., 2008). Although consumption of *p*-xylene was also detected under iron reducing and methanogenic conditions, the process has not been studied quantitatively (Botton and Parsons, 2007).

Mechanism of *p*-xylene activation

In acidified and methylated extracts of cultures grown with 5 mM nitrate and 2% *p*-xylene in HMN (v/v), two metabolites were detected as dimethyl esters. Based on relative retention times, mass spectra and comparison to published spectra, we identified the two dimethyl esters in our denitrifying cultures as dimethyl esters of (4-methylbenzyl) succinate, and (4-methylphenyl) itaconate. The mass spectra of (4-methylbenzyl) succinic acid dimethyl ester showed the molecular ion at *m/z* 250, the base peak at *m/z* 105 and further key fragment ions at *m/z* 131, 145, 177 and 190 (Fig. 5A). The mass spectrum of (4-methylphenyl) itaconic acid dimethyl ester showed the molecular ion at *m/z* 248, the base peak at *m/z* 129 and further key fragments at *m/z* 115, 188 and 216 (Figure SM 12.6).

Their mass fingerprints were similar to the compounds found in sulfate reducing enrichments degrading *p*-xylene (Elshahed et al., 2001; Morasch et al., 2004; Morasch and Meckenstock, 2005). The presence of these metabolites suggest a similar degradation pathway as described for toluene and *m*-xylene in the denitrifiers *Thauera aromatica* and *Azoarcus* sp. strain T, respectively (Biegert et al., 1996; Krieger et al., 1999; Leuthner and Heider, 2000). However, after ring cleavage, the *para*-methyl group would prevent one step

of regular β-oxidation. The mechanism by which this „obstacle“ is by-passed is unknown.

Phylogeny and cell hybridization

To identify the phylogeny of microorganisms in two highly enriched *p*-xylene denitrifying cultures, pXyN1 and pXyN3, we constructed 16S rRNA gene libraries. The clone libraries of these enrichments were dominated by one phylotype ($\geq$98.5% sequence identity) (Figure 12.2). This phylotype was closely related to the *Betaproteobacteria*, *Denitratisoma oestradiolicum* (95% identity). Other close relatives include strain 72Chol (94.4%) and *Sterolibacterium denitrificans* (94.2%). This phylotype was only distantly related to two *Azoarcus* members: the cyclohexane-1,2-diol degrading strain Lin22 (91.4%), the propylbenzene degrading strain PbN1 (90.9%) and to *Dechloromonas aromatica* strain RCB (91.1%). From the *Thauera* group the closest relative is the toluene degrader, *Thauera aromatica* K172. Other five phylotypes were found, three related to *Denitratisoma oestradiolicum* (84.4 to 94%), one to *Chlorobium phaeobacteroides* DSM 266 (80% identity) and one to candidate division OP11 sequences from a river estuary mangrove (89.8% identity) (Figure SM 12.5). To resolve the relative dominance of the organisms in the enrichments, we applied FISH with phylum- to group-specific oligonucleotide probes (Figure 12.4). All bacteria were detected with a DNA stain (4'.6-diamidino) 2-phenylindol (DAPI). We determined the relative percentage of probe targeted cells in relation to the number of DAPI stained cells. The general *Bacteria* probe (Eub-338 I-III) hybridized 97% of the total cells in both enrichment cultures (Figure 12.3A). The class specific *Betaproteobacteria* probe (Bet-42a), hybridized to more than 93% cells in both enrichments (Figure 12.3B). Newly designed probes (pxyn-440 and pxyn-644) specific for the *Denitratisoma*-related phylotype which dominated both clone libraries, targeted more than 91% of cells in both enrichment cultures (Figure 12.3C).

Within the *Betaproteobacteria* class, *Azoarcus* and *Thauera* clade, comprises all described denitrifying alkylbenzene degraders (Widdel and Rabus, 2001). In contrast, the dominant microorganism in our denitrifying cultures was only distantly related to *Azoarcus* and *Thauera* species. The next relatives at the 16S rRNA level are denitrifying microorganisms capable of steroid degradation, the 17-estradiol degrader *D. oestradiolicum* (Fahrbach et al., 2006), and two cholesterol degrading isolates, *S. denitrificans* and strain 72Chol (Harder and Probian, 1997; Tarlera and Denner, 2003). In another study the dominant phylotype of denitrifying benzene degrading enrichment cultures, was also related to a steroid degrader, *Sterolibacterium denitrificans* (Ulrich and Edwards, 2003). Until now, the ability of steroid degraders to use monocyclic aromatics is unknown. Our enrichments were capable to grow on two alkylbenzenes (toluene, *p*-xylene) and two polar monoaromatics (benzoate, *p*-toluic acid), and did not utilize cholesterol. These physiological and phylogenetic differences suggest the positioning of the dominant phylotype in a new genus within the family *Rhodocyclaceae*.

Acknowledgments

The authors wish to thank Prof. Friedrich Widdel, Dr. Florin Musat, and Dr. Niculina Musat for helpful discussions and critical review of the manuscript. We thank Dr. Niculina Musat for introduction and help with the ARB software, Melissa Duhaime for proof reading the manuscript, Ulrike Jaekel for providing phase contrast microscopy photos, Olav Grundmann, Anke Sobotta and Cornelia Karger for assistance with GC-MS analysis. This work was supported by the Max-Planck-Gesellschaft and, partly through the International Max Planck Research School for Marine Microbiology.

Figures and tables

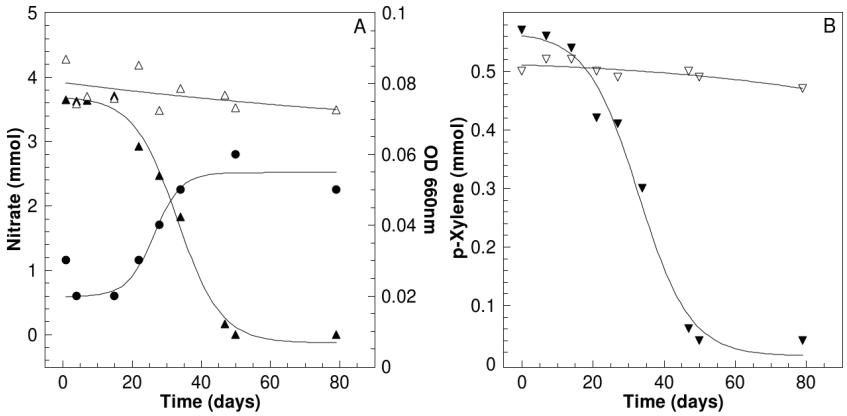

Figure 12.1: Nitrate reduction (filled up-triangles) and optical density (filled circles) increase (panel A) in a sediment free culture, pXyN1 which degrades *p*-xylene (filled down-triangles in panel B). In an inoculated control there is no significant decrease of nitrate (opened up-triangles). In sterile controls no major hydrocarbon loss has been detected (open down-triangles). The values for nitrate and *p*-xylene are average of duplicate measurements.

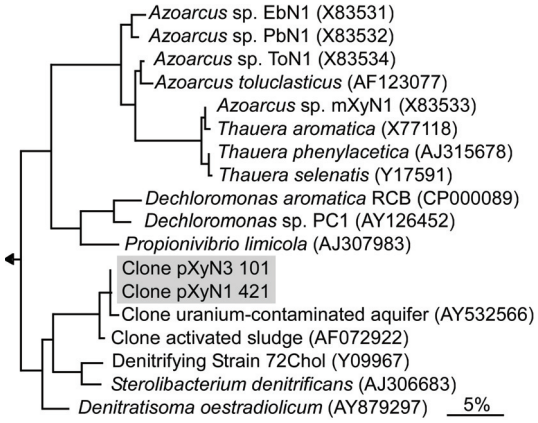

Figure 12.2: Maximum likelihood tree of *Betaproteobacteria* showing the dominant 16S rRNA phylotype (gray boxes) in two enrichment cultures (pXyN3 and pXyN1) grown on *p*-xylene as sole energy source. The arrow points to *Deinococcus* species which were used as out-group. The scale bar measures distance as 5% similarity per bar length.

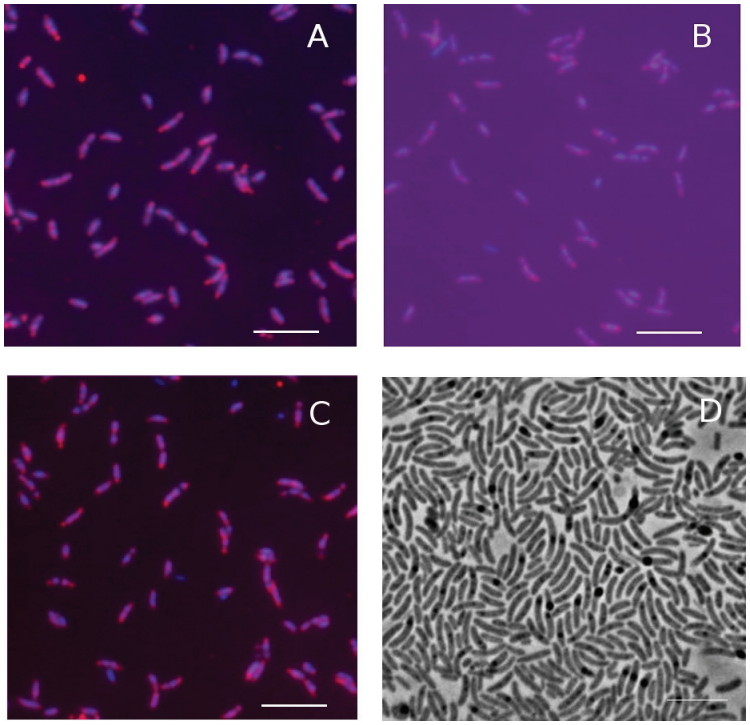

Figure 12.3: Microscopic images of cells in a denitrifying enrichment culture with *p*-xylene. Images A, B and C are superimposed DAPI and probe signals. Cells not targeted by the oligonucleotide probe appear blue (DAPI signal) while probe-targeted cells appear red. The scale bar is 5 µm. A) Cells stained with DAPI and hybridized with a Bacteria-specific probe mix (Eub I-III). B) Cells stained with DAPI and hybridized with probe Bet 42a specific for *Betaproteobacteria*. C) Cells stained with DAPI and hybridized with probe pxyn-440 designed for the dominant phylotype retrieved from two *p*-xylene degrading enrichments. D) Phase contrast micrograph of viable cells.

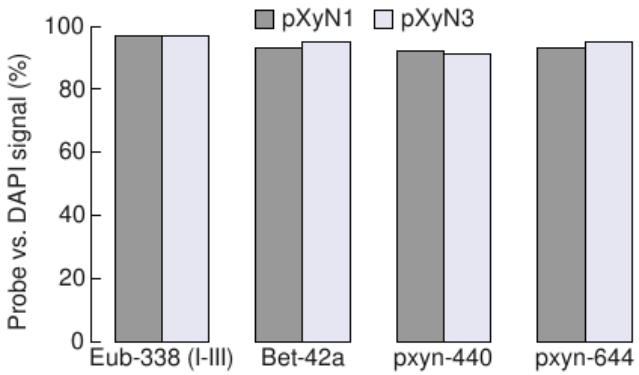

Figure 12.4: Enumeration of cells stained with Cy-3 labeled probes versus DAPI stained cells in two denitrifying cultures highly enriched on *p*-xylene (pXyN1 and pXyN3). The nonsense probe, Non-338, although applied it stained 0% cells, therefore it is not represented here.

Table 12.1: Quantification of *p*-xylene consumption and nitrate reduction in two highly enriched cultures. The inocula was 25% in a culture volume of 400 ml, overlaid with 40 ml HMN as carrier for *p*-xylene. The theoretical amount of *p*-xylene added was 0.616 mmol corresponding to 0.2% in HMN (v/v). The theoretical amount of nitrate added was 4 mmol.

Culture	NO_3^- initial (mmol)	NO_3^- consumed* (mmol)	*p*-xylene initial (mmol)	*p*-xylene consumed* (mmol)	Cell dry mass (mg)	Electrons accepted*** (mmol)	Electrons donated** (mmol)	Electrons recovered (%)
Cells with *p*-xylene (A)	3.62±0.01	3.62±0.01	0.49 ±0.05	0.46 ±0.08	0.56	17.2 ±0.03	18.0 ±4.00	96 ±22
Cells with *p*-xylene (B)	3.65 ±0.03	3.65 ±0.00	0.57 ±0.02	0.53±0.06	0.58	17.3 ± 0.03	20.9 ± 3.30	83 ± 16
Sterile control with *p*-xylene	-	0.50 ± 0.05	0.03 ±0.05	-	-	-	-	-
Cells without *p*-xylene	3.58 ± 0.06	0.18 ±0.07	-	-	0.11	-	-	-

* The depletion of nitrate and p-xylene was calculated by subtracting the final measured value from the initial one.

**Electrons accepted by nitrate and donated by *p*-xylene were calculated considering the amounts of these energy sources used by cultures. The amount of nitrate used was corrected by subtracting the loss of nitrate observed in an inoculated control culture. Similarly, we corrected the amount of *p*-xylene consumed by enrichment cultures by subtracting the *p*-xylene lost in a sterile control. The amount of electrons donated during dissimilation of *p*-xylene was calculated by excluding the amount assimilated into biomass from the total *p*-xylene that disappeared. We assumed 2.1 mg cell dry mass requires 0.0039 mmol *p*-xylene considering the assimilation reaction: $17\ C_8H_{10} + 32\ HCO_3^- + 32\ H^+ + 30\ H_2O \rightarrow 42\ C_4H_7O_3$.

Table 12.2: Oligonucleotide probes used for fluorescence in situ hybridization of enrichment cultures pXyN1 and pXyN3.

Probe name	Sequence (5'-3')	rRNA target site (*E. coli* numbering)	Target group coverage	Formamide	References
Eub-338 (I)*	GCTGCCTCCCGTAGGAGT	16S (338-355)	90% domain *Bacteria*	0-50%	(Amann et al., 1990a)
Eub-338 (II)*	GCAGCCACCCGTAGGTGT	16S (338-355)	69% order *Planctomycetales*	0-50%	(Daims et al., 1999)
Eub-338 (III)*	GCTGCCACCCGTAGGTGT	16S (338-355)	93% order *Verrucomicrobiales*	0-50%	(Daims et al., 1999)
Bet-42a**	GCCTTCCCACTTCGTTT	23S (1027-1043)	86% class *Betaproteobacteria*	35%	(Manz et al., 1992)
pXyn-440	ACCACCGTTTCGTTCCTGCT	16S (440-460)	*p*-xylene phylotype specific	40%	This study
pXyn-644	CGTATTAGGGACCACCGTTT	16S (644-664)	*p*-xylene phylotype specific	40%	This study

* An equimolar amount of Eub-338 (I, II and III) was used to enumerate most *Bacteria*.

** This probe was used in equimolar amount with an unlabeled Gam-42a competitor (5'-GCCTTCCCACTTCGTTT-3'), to enhance specificity.

Supplementary material: Highly enriched *Betaproteobacteria* growing anaerobically with *p*-xylene and nitrate

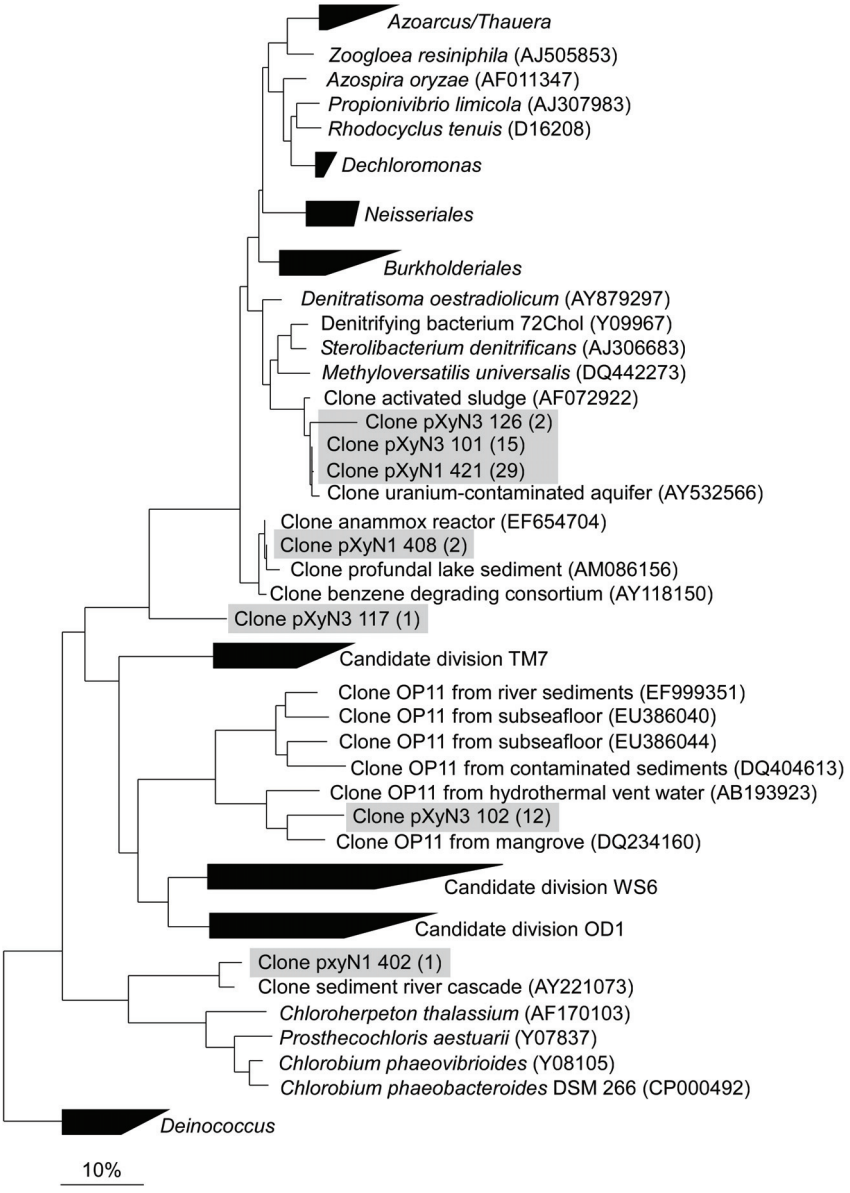

Figure 12.5: Maximum likelihood tree of 16S rRNA sequences from two enrichment cultures (pXyN3 and pXyN1) grown on *p*-xylene as sole carbon and energy source. Gray boxes indicate the different sequence types encountered in the two clone libraries. Each clone library contained 30 clones. The number of clones of each phylotype is mentioned in parenthesis. *Deinococcus* species were used as outgroup. The scale bar measures distance as 10% similarity per bar length.

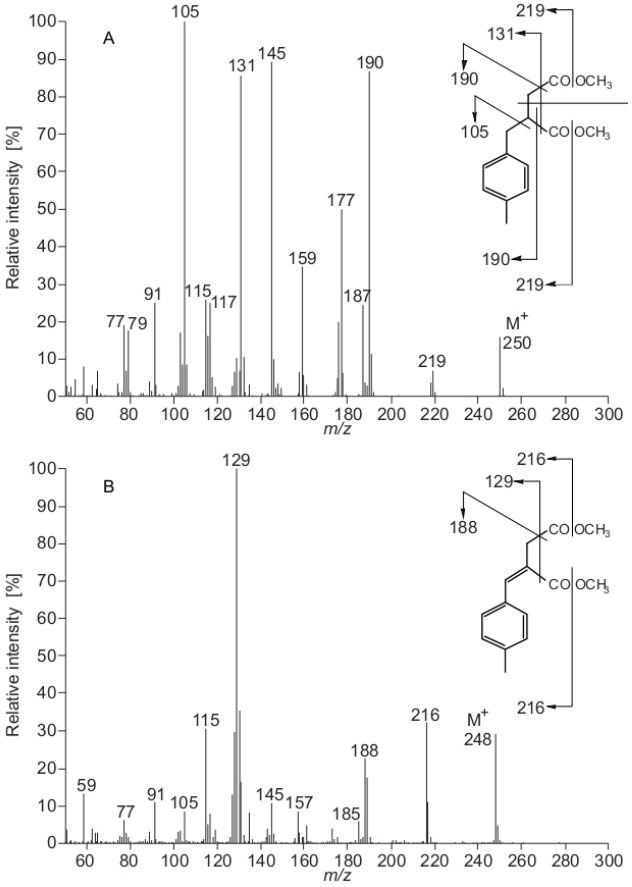

Figure 12.6: Mass spectra of two methylated intermediary metabolites obtained from cultures grown with *p*-xylene as sole organic energy source: A)(4-methylbenzyl) succinic acid dimethyl ester, and B) (4-methylphenyl) itaconic acid dimethyl ester.

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13 Annexes

Annex I. Initial sediment-containing enrichments

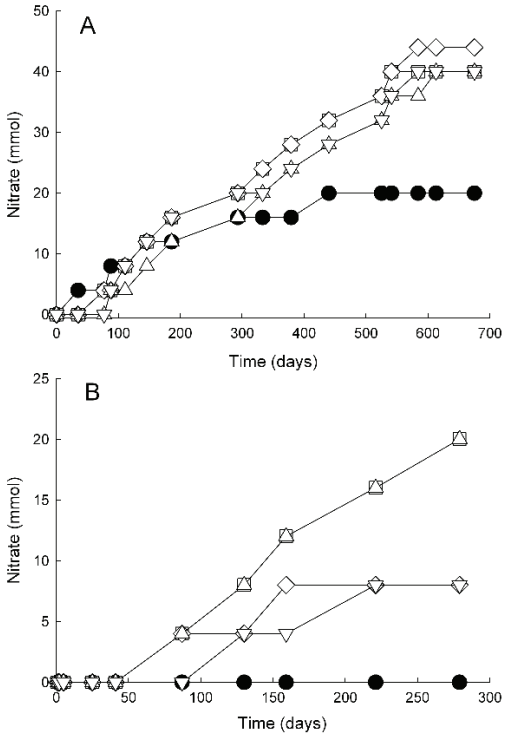

Figure 13.1: Denitrifying activity measured in sediment-containing enrichments (panel A) and subsequent cultures (panel B) grown with 1% *p*-xylene in HMN (v/v). A) Nitrate reduction started after 80 days and persisted for circa 670 days in sediment-containing enrichments with *p*-xylene (empty symbols). The control culture without *p*-xylene ceased the denitrifying activity after approximately 450 days (filled circles). B) Two of the subsequent enrichments (empty up-triangles and squares) grown on 1% *p*-xylene were active for circa 300 days, whereas the other two (empty down-triangles and diamonds) ceased nitrate reducing activity after 150 days. The control (filled circles) showed no denitrifying activity during the entire period of incubation.

Annex II. *bssA*-like gene amplification and protein fingerprints of *p*-xylene degrading cultures

The *bssA* gene was amplified directly from cultures with primers from literature (Winderl et al., 2007) bssA-7772F (5'-GACATGACCGACGCSATY-3') and bssA-8846R (5'-TCGTCGTC-RTTGCCCCAYTT-3'). The sequence quality of the *bssA* sequences was manually inspected and a contig was assembled using an appropriate software (DNA-baser). The ARB software (Ludwig et al., 2004) was used for the alignment and tree construction. The sequence closest relatives were identified by tBLASTx analysis (translated nucleotide query versus translated protein database) (www.ncbi.nih.gov/tBLASTx) an imported into ARB. The alignment was done using ClustalW with Blosum62 as matrix. The relationships were inferred by Phylip ProML maximum likelihood analysis using the Dayhoff filter.Preparation of protein extracts, SDS-PAGE and Commasie blue staining were performed as described (Rabus and Heider, 1998).

GAAGCGGCCAAGCGCATCCGCACGCCAGAGCCCTCGATCGTATTCCGCTACTCGAAGAAGAACAGGGAAAAGACCCTGCGCTGGGTGTTCGATTGCGTCCGCGACGGCCTCGG
CTATCCGTCGATCAAGCACGACGACATCGGCACGCAACAGATGAAGGATCTTGGCAAGTACAGCCTCACTGGCAACGGCGCCACCGATGCGGAAGCGCATGACTGGGCGTGTGT
CTTGTGCATGTCGCCTGGACTGGCTGGCCGCCGCAAGACGCAGAAAACCCGTTCCGAAGGCGGGGGGTCGATCTTCCCGGCGAAAATCCTGGAAATCACTTTGACCAATGGATA
CGACTGGTCCTACGCCGATATGCAGCTTGGCCCGAAGACCGGAGATATCTCGACATTCAAGACCTTCGAAGACGTCTGGGAAGCTTTCCGCAAGCAATACCAATACGCCATCAAT
CTGTGCATCAGCACCAAGGATGTTTCACGCTACTTCGAAGGCCGCTATCTGCAGATGCCCTTCGTCTCCGCGATCGACGACGGCTGCATGGAGTTGGGTGCCGAGGCCTGCGC
GCTGTCCGAGCAACCGAACGCTTGGCACAACCCGATCACCACCATTGTGGCGGCCAATTCGCTGGTCGCCATCAAGAAACTGGTGTTCGACGACAAAAAGTACACGCTTGAGCA
ACTCACCGAGGCGCTGTTGGCCAACTGGGAAGGGTTCGAAGAGATGCGTGTAGCCTTCAAAAAAGCGCCGAAGTGGGACAAAAACAACAAACGGTTTCCTTGATGGCAACCCCA
GCGAATTGGCCGCCACAATGGTGGTGATCGGGTT

Figure 13.2: BssA sequence retrieved from *p*-xylene degrading highly enriched cultures.

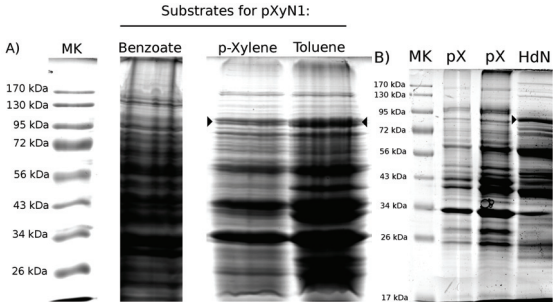

Figure 13.3: Protein profiles of enrichment culture pXyN1 grown with different substrates (Panel A). Arrows point at the position where the α-subunit of (alkylbenzyl)succinate synthase could migrate according to its size in SDS denaturing gels. A dual band α' of this protein is expected to occur due to oxygenolytic cleavage. This dual band was visible only in toluene and *p*-xylene grown enrichments, however it was absent from benzoate and fumarate (not shown) grown cultures. To make sure we look at the right migration behavior we used strain HxN1 that has a (methyl) succinate synthase which migrates around 95 kDa. The arrow in panel B points at the α subunit of MssA. However, the double bands from our enrichments were distant from MssA.

14 Acknowledgments

Thank you!

My PhD supervisor, PD Dr. Jens Harder - for believing in me, and for giving me the opportunity to work on this project.

Prof. Friedrich Widdel - for helpful discussions during my PhD, corrections of my first paper, and for reviewing my thesis.

My thesis committee members - Dr. Jens Harder, Dr. Bernhard Fuchs, Prof. Dr. Ulrich Fisher, and Dr. Florin Musat.

My lab-rotation students Paola and Ulrike for their work, and especially Ulrike, for making wonderful phase contrast photos and helping to sequence the *bssA*-like sequence.

My office mates Alex, Maya, Jacob, and later Mathias and Marcel, for making the office a space dedicated to peace and thinking, but also for laughs and ardent discussions.

Cristina Moraru for „FISH emergency“-help and cookie chats.

My „Romanian family in Bremen “, Niculina and Florin who were my inspiration and gave me comfort and strength whenever I needed it.

My best friend Alberto who shared coffees, „super-sandwiches“, ideas and dreams about science and life.

My friend Adriana, who was there at the other end of the line, sometime for hours, listening, understanding and passing through a similar struggle.

My friends that made the life in Bremen a joy: Melissa, Steffi, Gulcin, Luciana, Pablo, Gabi, Foxxy.

My wonderful boyfriend Stefan whose strength, love and care kept me going over the most difficult times.

Familiei mele care m-au ajutat sa ajung aici. Mamei mele, Crina si tatalui meu, Nicolae, pentru incredere si putere, dar si pentru dragostea pentru natura cladita treptat de cand eram de-o schioapa; bunicilor mei, Lucretia, si Titus care mi-au pus creionul in mana. Sorei mele dragi, Diana, pentru veselia si dragostea pe care le emana - care de multe ori m-au eliberat din „inchisoarea propriei tristeti“ . De fapt ii multumesc intergii mele familii care mi-au fost aprope in suflet la bine si la greu.

Anlage zur Dissertation

Name: Amelia-Elena Rotaru Ort, Datum: Bremen, 30 April 2009
Anschrift:

Erklärung

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Ich erkläre, dass ich
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2. keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzen Werken wörtllich oder inhaltlich entnommenen Stellen als solche kentich gemacht habe.

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