RESEARCH ARTICLE

Anaerobic degradation of the aromatic hydrocarbon biphenyl by a sulfate-reducing enrichment culture

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Received 1 October 2008; revised 17 December 2008; accepted 21 December 2008.
First published online 29 January 2009.

DOI:10.1111/j.1574-6941.2009.00652.x

Editor: Max Häggblom

Keywords
biodegradation; *Desulfotomaculum*; PAH; BTEX; contamination.

Abstract

The aromatic hydrocarbon biphenyl is a widely distributed environmental pollutant. Whereas the aerobic degradation of biphenyl has been extensively studied, knowledge of the anaerobic biphenyl-oxidizing bacteria and their biochemical degradation pathway is scarce. Here, we report on an enrichment culture that oxidized biphenyl completely to carbon dioxide under sulfate-reducing conditions. The biphenyl-degrading culture was dominated by two distinct bacterial species distantly affiliated with the Gram-positive genus *Desulfotomaculum*. Moreover, the enrichment culture has the ability to grow with benzene and a mixture of anthracene and phenanthrene as the sole source of carbon, but here the microbial community composition differed substantially from the biphenyl-grown culture. Biphenyl-4-carboxylic acid was identified as an intermediate in the biphenyl-degrading culture. Moreover, 4-fluorobiphenyl was converted cometabolically with biphenyl because in addition to the biphenyl-4-carboxylic acid, a compound identified as its fluorinated analog was observed. These findings are consistent with the general pattern in the anaerobic catabolism of many aromatic hydrocarbons where carboxylic acids are found to be central metabolites.

Introduction

Because of its toxic effect on living organisms, the occurrence of biphenyl in the environment has raised public concern (MacDonald *et al.*, 2000). The aromatic hydrocarbon biphenyl is used for a vast variety of industrial purposes (e.g. dye carrier in textile industry, heat transfer agent, and preservative agent of citrus fruits). In addition, it is a component of creosote that is widely used as a wood preservative. Much of the biphenyl present in creosote and in industrial wastewater leaches into the environment or volatilizes into the atmosphere. Despite biphenyl's release into the environment, quantitative data of its presence therein are scarce. For example, 25–43 $\mu g\,L^{-1}$ biphenyl was detected in groundwater at a former coal gasification site (EPA, 1984). As a hydrophobic substance, it is suggested that biphenyl has a moderate tendency to accumulate in terrestrial environments. Because it adsorbs to the solid matrix to a high extent (K_{oc} 1400 $mL\,g^{-1}$) (CHEMFATE, 1994), biphenyl is not well transported with the groundwater flow.

Indigenous microorganisms have developed diverse strategies to metabolize a wide range of aromatic substances. Numerous studies have recorded the isolation of bacterial strains able to degrade biphenyl with oxygen as an electron acceptor (Bevinakatti & Ninnekar, 1992; Kiyohara *et al.*, 1992; Nielsen & Christensen, 1994; Hernandez *et al.*, 1995). However, it is of particular interest to detect anaerobic biodegradation of aromatic hydrocarbons, because the contamination of ecosystems leads to the formation of anoxic conditions (Christensen *et al.*, 1994). For a long time, it was assumed that nonsubstituted aromatic hydrocarbons were resistant to microbial degradation under anoxic conditions. In the last few years, it could be shown that aromatic compounds such as phenanthrene (Zhang & Young, 1997; Davidova *et al.*, 2007), naphthalene (Zhang & Young, 1997; Galushko *et al.*, 1999; Meckenstock *et al.*, 2000; Musat *et al.*, 2008), and benzene (Phelps *et al.*, 1998; Rooney-Varga *et al.*, 1999; Ulrich & Edwards, 2003; Kunapuli *et al.*, 2007; Musat & Widdel, 2008) are well biodegradable with anaerobic electron acceptors. However, anaerobic biphenyl degradation has been rarely observed. From creosote-contaminated

sediments, several bacterial cultures have been enriched that were able to oxidize biphenyl under methanogenic (Sharak Genthner *et al.*, 1997; Natarajan *et al.*, 1999), nitrate-reducing, and sulfate-reducing conditions (Rockne & Strand, 1998). Additionally, Ambrosoli *et al.* (2005) and Yang *et al.* (2008) demonstrated that the potential for natural attenuation of biphenyl is even present in uncontaminated terrestrial environments.

So far, the biochemical pathway of anaerobic biphenyl degradation is unknown. Analysis of metabolites provided indications that the monoaromatic compound *p*-cresol is an intermediate product in methanogenic biphenyl degradation (Natarajan *et al.*, 1999). Moreover, neither a phylogenetic characterization nor the isolation of an anaerobic biphenyl-degrading pure culture has been reported.

In the present study, we report on a sediment-free enrichment culture able to oxidize biphenyl as the sole carbon source with sulfate as an electron acceptor. Here, the finding of biphenyl carboxylic acid as a metabolite provides indications of the biochemical mechanism of anaerobic biphenyl degradation.

Materials and methods

Cultivation and growth characteristics of the bacterial enrichment culture BiphS1

The culture BiphS1 was enriched from contaminated soil at a former coking plant in Gliwice, Poland. The cells were cultivated in 120-mL serum bottles, half-filled with bicarbonate-buffered fresh water medium, pH 7.1, containing 10 mM Na_2SO_4, and reduced with 1 mM Na_2S (Widdel *et al.*, 1983; Widdel & Bak, 1992). Three millimolar $FeCl_2$ was added in order to scavenge the sulfide produced. Substrates were added as solid crystals, 5–10 mg per bottle, combined with 0.3 g absorber resin XAD7 (Morasch *et al.*, 2001), before autoclaving to the bottles. Cometabolism experiments were performed with 1.5% biphenyl in heptamethylnonane containing 0.1% of 4-fluorobiphenyl, 2-PCB, 3-PCB, 4-PCB, 3,4-PCB, or 3,5-PCB. The bottles were flushed with N_2/CO_2 (80 : 20), closed with butyl rubber stoppers, and incubated at 30 °C. New cultures were inoculated with 10% inoculum (v/v) and were usually grown out after 3 months.

Bacterial growth was monitored by microbial cell counts. For counting, bacterial cells were fixed in 4% paraformaldehyde for 10 min and stained with 4,6-diamidino-2-phenylindole (DAPI) (Merck, Darmstadt, Germany) at a final concentration of 10 μg mL^{-1} for 10 min. The samples were filtered onto black 0.2 μm Nucleopore polycarbonate filters (Whatman, Brentford, UK) under vacuum pressure. Counting was performed with a fluorescence microscope (Zeiss Axioplan 2, Oberkochen, Germany) at 1000-fold magnification. From each sample, one filter was counted with 10 randomly selected fields.

In order to monitor CO_2 production from biphenyl, the enrichment culture was cultivated with ^{13}C-labeled biphenyl (Sigma Aldrich, Steinheim, Germany, 99% purity) as a substrate in the presence of XAD7. For control experiments, the cultures were autoclaved three times to ensure no survival of bacteria. One-milliliter gas samples were taken from the head space and stored 1 : 10 diluted in helium-filled vials. Stable carbon isotope ratios were determined by GC-combustion/isotope ratio MS (GC-C/IRMS) consisting of a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific Corporation, Milan, Italy), which was coupled to a Finnigan^{TM} MAT 253 IRMS (Thermo Fisher Scientific Corporation, Bremen, Germany) via a Finnigan^{TM} GC combustion III interface (Thermo Fisher Scientific Corporation). The GC was equipped with an Optic 3^{TM} temperature-programmable high-performance injector system of ATAS GL International B.V. (Veldhoven, the Netherlands) and a DB-5 column (0.25 μm film thickness, 0.25 mm i.d., and 30 m length, Agilent Technologies, Böblingen, Germany). The split flow of the Optic 3^{TM} injector was set to 14 mL min^{-1}, i.e. a split ratio of 1 : 10, and the temperature was held isothermally at 280 °C. The GC oven temperature was programmed as follows: 10 °C (hold 2 min), at 40 °C min^{-1}, to 220 °C (hold 1 min). Helium of grade 5.0 was used as the carrier gas with a constant flow rate of 1.4 mL min^{-1}. Data acquisition was carried out using the ISODAT NT software (Thermo Fisher Scientific Corporation). The carbon isotopic compositions of all compounds were reported in the δ-notation relative to Vienna PeeDee Belemnite (V-PDB):

$$\delta^{13}C = [(^{13}C/^{12}C_{Sample} - ^{13}C/^{12}C_{V-PDB\,Standard})/^{13}C/^{12}C_{V-PDB\,Standard}] \times 1000$$

$$^{13}CO_2/^{12}CO_2\ \text{sample} = [1 + (\delta^{13}CO_2/1000)] \times [^{13}CO_2/^{12}CO_{2\,V-PDB\,standard}]$$

$$^{13}CO_2[\text{mM}] = ^{13}CO_2/^{12}CO_{2\,sample} \times \text{initial}\ ^{12}CO_2[\text{mM}].$$

Analytes were measured against a laboratory CO_2 reference gas that was calibrated to V-PDB by referenced CO_2 isotope standards (RM 8562, RM 8563, and RM 8564) provided by the International Atomic Energy Agency (IAEA, Vienna, Austria).

Molecular analysis

Bacterial cells were centrifuged for 10 min at 3345 ***g*** and washed with 1 × PBS. Extraction of genomic DNA from the

cells was performed with a FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's protocol. Amplification of the 16S rRNA coding genes was performed using the primer set Ba27f/Ba1492r (Weisburg *et al.*, 1991), resulting in a full-length PCR product. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into *Escherichia coli* JM109 using the pGEM-T cloning kit (Promega, Mannheim, Germany). Selected clones were screened for correct insert size using the vector-specific PCR primers M13 reverse and T7 promoter and agarose gel electrophoresis. Sequencing was performed on a 3730 DNA Analyser (Applied Biosystems, Weiterstadt, Germany) using the BigDye terminator v3.1 chemistry according to the manufacturer's protocol.

16S rRNA gene-based terminal restriction fragment polymorphism (T-RFLP) analysis was performed using the primer set Ba27f-FAM/907r (Lane *et al.*, 1985). Amplicons were digested with MspI (Promega). One microliter of the desalted digest was mixed with 13 μL Hi-Di formamide (Applied Biosystems) containing a 1 : 300 dilution of ROX-labeled MapMarker 1000 ladder (Bioventures Incorporation, Murfreesboro), denatured for 5 min at 95 °C, cooled on ice, and size separated on a 3730 DNA Analyser (Applied Biosystems).

Phylogenetic analysis

The 16S rRNA gene sequencing reads were manually assembled and checked for quality using the SEQMAN II software module (Lasergene 6 suite, DNAstar, Madison). The sequences were tested for chimeric structures using the Chimera Check analysis function of the Ribosomal Database Project II (http://rdp.cme.msu.edu/). The 16S rRNA gene sequences were added to an existing database of about 25 000 small-subunit rRNA gene sequences using the fast alignment tool of the ARB software package (http://www.arb-home.de) (Ludwig *et al.*, 2004). Alignments were checked visually. Phylogenetic analyses based on nucleotide sequences were performed and verified by applying maximum likelihood, maximum parsimony, and neighbor-joining methods using the respective tools in the ARB software package. All 16S rRNA gene sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov/) under accession no. EU651861 to EU651886.

Analysis of metabolites

Culture supernatants were acidified with HCl to pH 1 and extracted three times with diethyl ether. The organic phase was collected, dried over water-free sodium sulfate, and evaporated under a continuous nitrogen stream. The residue was redissolved in 1.8 mL methanol. Trimethylchlorosilane (200 μL) was added and the samples were derivatized for 1 h at 75 °C in order to transform the carboxylic acids into trimethylsilyl esters. After cooling, the samples were dried under a continuous nitrogen stream and dissolved with 1 mL ethyl acetate. The detection of the metabolites was performed with GC-MS (Trace GC ultra, Thermo) equipped with a DB-5 column (0.25 μm film thickness, 0.25 mm i.d., 30 mL length, Agilent Technologies). Sample injection was splitless (1 μL), and the flow rate of the carrier gas helium was 0.9 mL min^{-1}. The oven temperature was 43 °C for 5 min, then ramped at a rate of 4 °C min^{-1} to 280 °C, and was held for 5 min. The MS was operated at 315 °C in the scan mode.

Results

Growth characteristics of the biphenyl-degrading enrichment culture

Enrichment of biphenyl-degrading sulfate-reducing bacteria was performed with contaminated soil as the inoculum and biphenyl with XAD7 as the carrier phase. After 15 transfers into a new sterile, soil-free medium, a culture with a stable microbial community was obtained. To prevent a potential toxic effect of sulfide on the microorganisms, ferrous chloride was added to the culture medium to scavenge free sulfide, resulting in the formation of greigite (Fe_3S_4).

Degradation of biphenyl under anoxic conditions was confirmed by production of $^{13}CO_2$ from uniformly labeled ^{13}C-biphenyl. Because of the low solubility of biphenyl in water (28 μM), solid biphenyl was always present in the bottles rendering a proper mass balance impossible. Nevertheless, 1 mM $^{13}CO_2$ was recovered from the conversion of ^{13}C-biphenyl after a time period of 202 days, proving a total oxidation to CO_2 (Fig. 1). The biphenyl degradation process was biotic as no $^{13}CO_2$ production was observed in control experiments with heat-killed cells. Simultaneously, biphenyl oxidation was coupled to an increase in cell numbers as evaluated microscopically (Fig. 1).

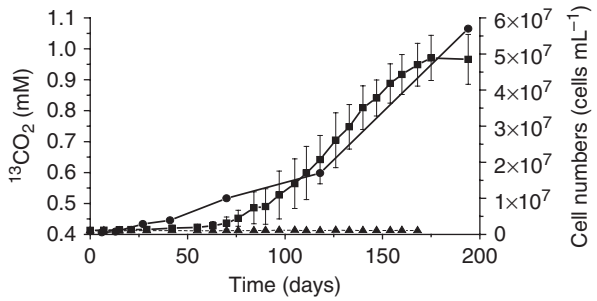

Fig. 1. Growth of the enrichment culture BiphS1: increase in cell numbers (closed circles) and $^{13}CO_2$ production with $^{13}C_{12}$-biphenyl as a carbon source in inoculated cultures (closed squares) and in sterile controls (closed triangles). Error bars represent the SDs of three parallel incubations.

In addition to biphenyl, the degradation potential of the enrichment culture BiphS1 was tested with alternative aromatic compounds as sole sources of carbon. The culture was also able to grow on benzene, a mixture of anthracene and phenanthrene, and benzoate whereas no growth with 2-naphthoic acid or naphthalene could be observed. We also tested whether halogenated biphenyls could be degraded by the enrichment culture BiphS1. Whereas neither of the tested chlorinated biphenyl analogs (2-PCB, 3-PCB, 4-PCB, 3,4-PCB, and 3,5-PCB) has been used as sole sources of carbon or as cosubstrates, only 4-fluorobiphenyl could be transformed cometabolically with biphenyl. No microbial activity was observed when 4-fluorobiphenyl was provided as the sole carbon source.

Molecular analysis based on 16S rRNA gene sequences

The diversity of the bacterial community structure in the enrichment culture BiphS1 was assessed directly by T-RFLP analyses of 16S rRNA gene sequences. The fingerprinting profile of the biphenyl-grown culture was dominated by two terminal restriction fragments (T-RFs) with a length of 178 and 209 bp (Fig. 2a). These T-RFs were also identified as the prevalent peaks in the benzoate-grown culture (Fig. 2d). When grown on a mixture of anthracene and phenanthrene, the culture became dominated by two T-RFs of 149 and 209 bp that appeared in addition to the T-RFs of 178 and 237 bp (Fig. 2b). The fingerprinting results of the benzene-grown culture differed substantially from the others, because here only one T-RF of 139 bp length was obtained (Fig. 2c).

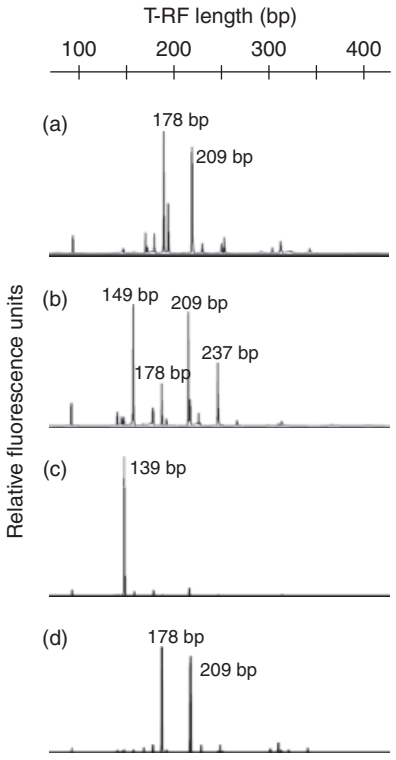

Fig. 2. T-RFLP analyses of 16S rRNA gene sequences amplified from the enrichment culture BiphS1 grown on (a) biphenyl, (b) a mixture of phenanthrene and anthracene, (c) benzene, and (d) benzoate. The length of major T-RFs is indicated in base pairs.

To correlate the results of the T-RFLP analysis with the microbial phylogenetic affiliation, we performed cloning and sequencing of full-length 16S rRNA gene sequences. The majority of the 16S rRNA gene sequences, which accounted for 16 out of 26 analyzed sequences, have been affiliated to the *Desulfotomaculum* cluster I (Fig. 3). There, the sequences form two related phylogenetic groups that correspond to the T-RFs of 178 and 209 bp. Both genotypes were distantly related to a known cultivated representative of the *Desulfotomaculum* genus with 92% sequence similarity to *Desulfotomaculum* sp. TPOSR (A.J.H. Stams, unpublished data) and *Desulfotomaculum thermosubterraneum* (Kaksonen *et al.*, 2006), respectively. An additional 16S rRNA gene sequence was detected that exhibited 96% sequence similarity to *Desulfotomaculum arcticum* (DQ148942) and that could have been correlated to the 237 bp T-RF in the anthracene-phenanthrene-grown culture. However, the 16S rRNA gene sequence of the additional dominant 149 bp T-RF was not detected in the anthracene–phenanthrene–grown culture. Moreover, the T-RF of 139 bp identified in the benzene-grown culture could be assigned with 95% sequence similarity to the 16S rRNA gene sequence JI of an uncultured bacterium related to the genus *Pelotomaculum* (AB091325) (Qiu *et al.*, 2004) and with 94% similarity to the next cultivated species *Pelotomaculum isophthalicum* (Qiu *et al.*, 2006). Most of the 16S rRNA genes detected in the enrichment culture BiphS1 were affiliated to Gram-positive bacteria (22 out of 26), whereas the remaining four sequences were affiliated to diverse bacterial phylogenetic groups (uncultured bacterium Btol, AF282178, 98% sequence similarity; *Acidovorax* sp. JS42, CP000539, 99%; uncultured *Cellulomonadaceae* bacterium HT06Ba02, EU016429, 99%; and uncultured bacterial clone SK27B-06, AB300072, 94%). In order to verify the formation of T-RFs from specific 16S rRNA gene sequences, T-RFLP analysis of selected 16S rRNA gene clone sequences was performed and was successfully assigned to three different sequence types (139, 178, and 209 bp). For the remaining sequence types, the potential T-RFs were deduced from *in silico* analysis of their 16S rRNA gene sequences.

Metabolite analyses

To obtain indications on the anaerobic biphenyl degradation pathway, we analyzed culture supernatants for putative metabolites. Biphenyl-4-carboxylic acid was the only

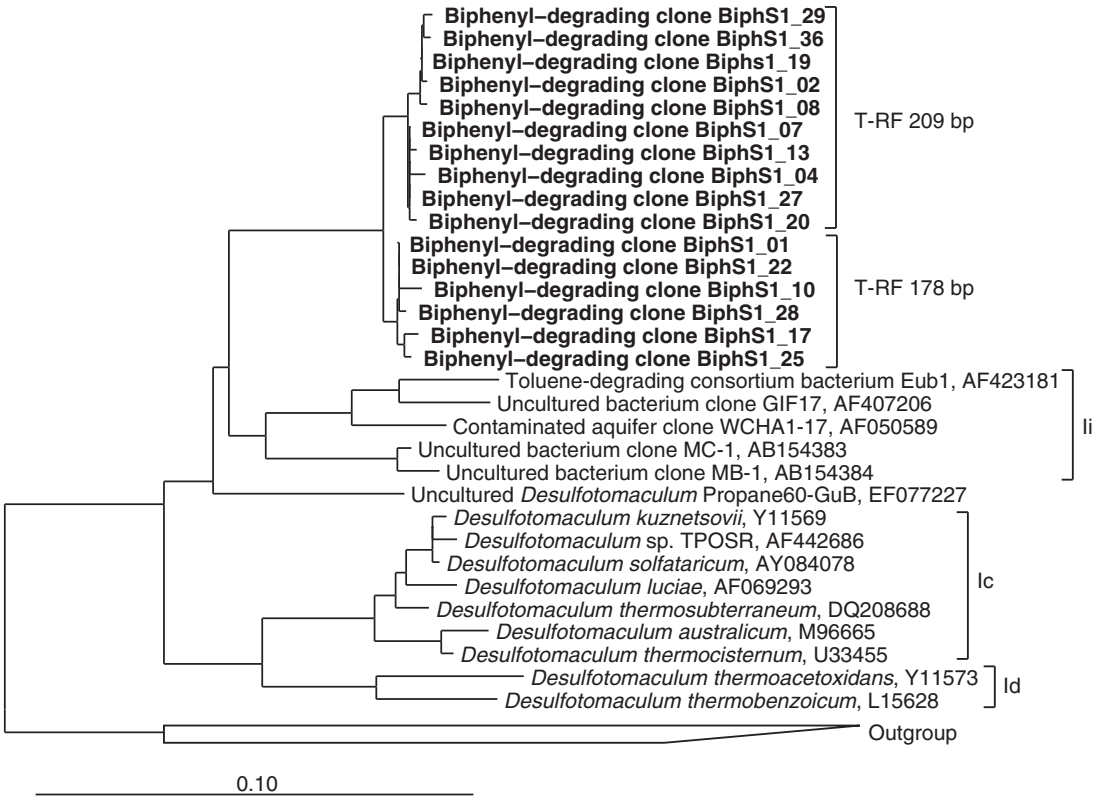

Fig. 3. Phylogenetic consensus tree reflecting the relationship of selected 16S rRNA gene sequences obtained from the biphenyl-degrading enrichment culture BiphS1 with selected sequences of the *Desulfotomaculum* cluster I. Sequences of the enrichment culture BiphS1 are shown in bold. Subdivisions Ic, Id, and Ii of the *Desulfotomaculum* cluster I are listed. An encompassing collection of organisms representing all major lineages of *Archaea* and *Bacteria* was used as an outgroup for tree calculations. The bar indicates 10% estimated sequence divergence.

intermediate we could identify by coelution with a commercially available standard and comparison of the mass spectra by GC-MS analysis (Fig. 4a). No other isomer of biphenyl carboxylic acid could be found. However, when 4-fluorobiphenyl was added as a cosubstrate, a metabolite appeared, in addition to the biphenyl-4-carboxylic acid, that was 18 mass units heavier, which corresponds to the mass difference between a fluorine and a hydrogen atom (Fig. 4b).

Discussion

In the present study, a bacterial culture was enriched that has the ability to degrade biphenyl under sulfate-reducing conditions. Indigenous microbial populations have been shown to have the potential for natural attenuation of biphenyl from uncontaminated sites (Ambrosoli *et al.*, 2005; Yang *et al.*, 2008). Interestingly, here, the addition of molybdate, an inhibitor of sulfate reduction (Hayes *et al.*, 1999), had an inhibitory effect on biphenyl degradation, suggesting that sulfate-reducing bacteria played an important role in anaerobic biphenyl metabolism. All known studies dealing with anaerobic biphenyl degradation so far were restricted to environmental samples or enrichment cultures (Sharak Genthner *et al.*, 1997; Rockne & Strand, 2001; Ambrosoli *et al.*, 2005; Yang *et al.*, 2008). To our knowledge, this is the first report on the determination of 16S rRNA gene sequences associated with an anaerobic biphenyl-degrading culture. Here, we postulate that Gram-positive bacteria of the genus *Desulfotomaculum* are involved in this metabolism because the T-RFLP analysis and sequencing of 16S rRNA genes revealed the dominance (62% of the total microbial population) of two closely related *Desulfotomaculum* species (> 98% sequence identity). Based on 16S rRNA gene sequence similarities, both species comprise several dominant strains that are, apart from the sequence pairs BiphS1_07 and BiphS1_27 (T-RF 209 bp) as well as BiphS1_22 and BiphS1_01 (T-RF 178 bp), all unique. Thus, it is likely that more than two strains, although perhaps belonging to two species, are involved in anaerobic biphenyl degradation. Furthermore, despite extensive enrichment efforts, various microorganisms are still present in the culture. This leads to the hypothesis that these bacteria could play an essential, so far unknown, role in biphenyl degradation.

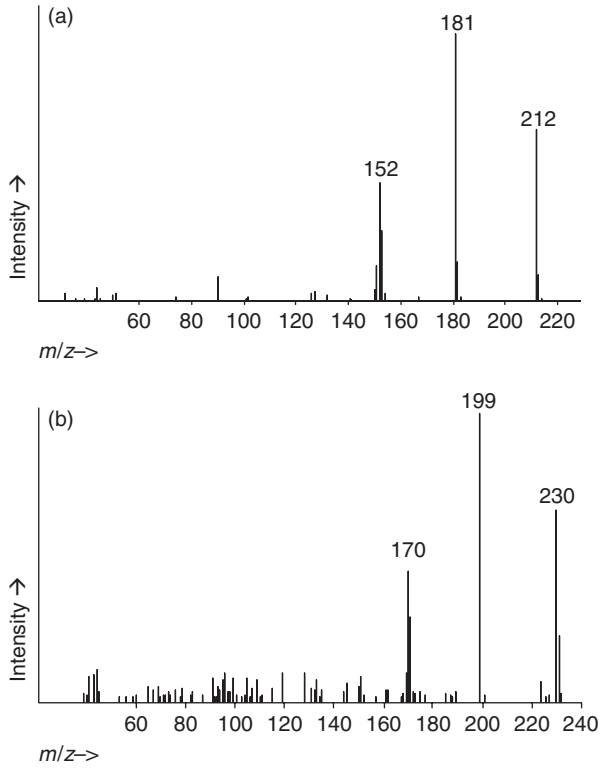

Fig. 4. Mass spectrum of biphenyl-4-carboxylic acid as a trimethylsilyl ester that was found in the supernatants of the enrichment culture BiphS1 (a). When 4-flourobiphenyl was added as a cosubstrate, 4-fluorobiphenyl carboxylic acid could be detected (b).

The functional role and interaction of the two *Desulfotomaculum* species in the biphenyl metabolism is still unidentified. Possibly, one of the *Desulfotomaculum* strains oxidizes biphenyl to some not yet determined and more biodegradable intermediate compound that was used as a growth substrate by the remaining bacterial strain. Alternatively, one strain oxidizes biphenyl to carbon dioxide, whereas the second strain generates the appropriate environment for the first strain. Such a microbial relation has been observed in a phenol-degrading anaerobic coculture that consists of two low-G+C Gram-positive bacteria (Letowski *et al.*, 2001). There, the authors clearly demonstrated that one strain degrades phenol, whereas the other strain provides an unknown factor that has a positive effect on the metabolism of the phenol-degrading strain. Finally, in our culture it is also possible that both strains metabolize biphenyl and thus simply coexist.

As our biphenyl-degrading phylotypes are distantly related to cultivated representatives of the *Desulfotomaculum* cluster I, the corresponding microorganisms may be regarded as a new line of descent within this bacterial group. Therefore, it is difficult to draw a hypothesis on the physiological properties of the respective organisms. Nevertheless, bacteria of the *Desulfotomaculum* cluster I are characterized as sulfate-reducing spore-forming bacteria with a great nutritional versatility (Stackebrandt *et al.*, 1997). In the present study, we obtained the enrichment culture BiphS1 from the mud of a surface pit, a habitat where the conditions are selective for the enrichment of Gram-positive sulfate reducers (Widdel, 2006). The formation of spores enables these species to survive periods of dryness and ensures an efficient survival under varying redox conditions. In fact, the recently growing number of Gram-positive bacteria involved in hydrocarbon degradation indicates that this group has been largely overlooked in the past concerning contaminant degradation (Cord-Ruwisch & Garcia, 1985; Tasaki *et al.*, 1991; Kuever *et al.*, 1999; Morasch *et al.*, 2004; Kunapuli *et al.*, 2007).

In the present study, biphenyl-4-carboxylic acid was identified as a metabolite during anaerobic transformation of biphenyl. This finding is consistent with the general pattern in the anaerobic degradation of diverse aromatic hydrocarbons. There, the degradation is characterized by various peripheral pathways that route structurally diverse aromatic compounds to a common carboxylic acid intermediate that is further degraded via a central pathway (Heider *et al.*, 1999; Widdel & Rabus, 2001; Meckenstock *et al.*, 2004). Thus, benzoate is the common intermediate compound of the anaerobic degradation of monoaromatic compounds, such as toluene (Leutwein & Heider, 1999; Spormann & Widdel, 2000) or benzene (Caldwell & Suflita, 2000; Phelps *et al.*, 2001; Ulrich *et al.*, 2005; Kunapuli *et al.*, 2007). Concerning the degradation of PAHs, it could be shown that 2-naphthoic acid is a metabolite of 2-methylnaphthalene (Annweiler *et al.*, 2000; Sullivan *et al.*, 2001) and naphthalene (Zhang & Young, 1997; Meckenstock *et al.*, 2000) degradation. Moreover, carboxylic acids play a role in the anaerobic metabolism of larger molecular weight PAHs. In a sulfate-reducing enrichment culture capable of phenanthrene oxidation, the incorporation of labeled ^{13}C-bicarbonate into phenanthrene suggested carboxylation as the initial reaction mechanism (Zhang & Young, 1997). Recently, another sulfate-reducing phenanthrene-metabolizing culture was obtained, where it could be shown that the carboxylation leads to the formation of phenanthrene-2-carboxylic acid (Davidova *et al.*, 2007).

In recent years, increasing information on the biochemical mechanism of anaerobic naphthalene and benzene degradation has become available, which might provide an indication of the putative pathway for biphenyl degradation. Direct carboxylation of naphthalene to 2-naphthoic acid was proposed as the initial activation step due to the incorporation of ^{13}C-bicarbonate into 2-naphthoic acid (Zhang & Young, 1997). Carboxylation as the initial activation mechanism was also proposed by Musat *et al.* (2008). Recently, the initial activation reaction of naphthalene was found to be methylation to 2-methylnaphthalene, to which

fumarate is subsequently added (Safinowski & Meckenstock, 2006). With this, the authors clearly demonstrated that the intermediate 2-naphthoic acid is not a direct product of an initial carboxylation. Therefore, despite the finding of biphenyl carboxylic acid as an intermediate, we cannot discern whether biphenyl is directly carboxylated or whether additional reactions lead to the formation of biphenyl-4-carboxylic acid.

Although the persistence of biphenyl in anoxic terrestrial environments is evident from many studies, the present work shows unambiguously that biphenyl can be oxidized by sulfate-reducing microorganisms.

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