RESEARCH ARTICLE

Anaerobic benzene degradation by Gram-positive sulfate-reducing bacteria

Nidal Abu Laban[1], Draženka Selesi[1], Carsten Jobelius[2] & Rainer U. Meckenstock[1]

[1]Helmholtz Zentrum München-German Research Center for Environmental Health, Institute of Groundwater Ecology, Neuherberg, Germany; and [2]Department of Water Chemistry, Engler-Bunte-Institute, University of Karlsruhe, Karlsruhe, Germany

Correspondence: Draženka Selesi, Helmholtz Zentrum München-German Research Center for Environmental Health, Institute of Groundwater Ecology, Ingolstädter Landstraße 1, D-85764, Neuherberg, Germany. Tel.: +49 89 3187 3122; fax: +49 89 3187 3361; e-mail: drazenka.selesi@helmholtz-muenchen.de

Received 7 November 2008; revised 19 February 2009; accepted 20 February 2009. First published online 27 March 2009.

DOI:10.1111/j.1574-6941.2009.00672.x

Editor: Max Häggblom

Keywords
benzene; sulfate reduction; Gram-positive bacteria.

Abstract

Despite its high chemical stability, benzene is known to be biodegradable with various electron acceptors under anaerobic conditions. However, our understanding of the initial activation reaction and the responsible prokaryotes is limited. In the present study, we enriched a bacterial culture that oxidizes benzene to carbon dioxide under sulfate-reducing conditions. Community analysis using terminal restriction fragment length polymorphism, 16S rRNA gene sequencing and FISH revealed 95% dominance of one phylotype that is affiliated to the Gram-positive bacterial genus *Pelotomaculum* showing that sulfate-reducing Gram-positive bacteria are involved in anaerobic benzene degradation. In order to get indications of the initial activation mechanism, we tested the substrate utilization, performed cometabolism tests and screened for putative metabolites. Phenol, toluene, and benzoate could not be utilized as alternative carbon sources by the benzene-degrading culture. Cometabolic degradation experiments resulted in retarded rates of benzene degradation in the presence of phenol whereas toluene had no effect on benzene metabolism. Phenol, 2-hydroxybenzoate, 4-hydroxybenzoate, and benzoate were identified as putative metabolites in the enrichment culture. However, hydroxylated aromatics were shown to be formed abiotically. Thus, the finding of benzoate as an intermediate compound supports a direct carboxylation of benzene as the initial activation mechanism but additional reactions leading to its formation cannot be excluded definitely.

Introduction

Monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are frequent pollutants in contaminated groundwater systems. Compared with other aromatic hydrocarbons (Anderson & Lovley, 1997), BTEX compounds are highly water soluble and carcinogenic (Dean, 1978) and consequently are described as hazardous organic substances that endanger the quality of drinking water resources (USEPA, 1998). Benzene is rapidly biodegraded under aerobic conditions in soils and aquifers (Gibson & Parales, 2000). However, the input of heavy organic loads into saturated sediments results in the rapid depletion of available oxygen, turning aquifers anoxic (Christensen *et al.*, 1994). Based on field and batch studies, it could be shown that benzene can be effectively degraded under iron-reducing (Anderson *et al.*, 1998; Rooney-Varga *et al.*, 1999; Kunapuli *et al.*, 2007), sulfate-reducing (Lovley *et al.*, 1995; Kazumi *et al.*, 1997; Phelps *et al.*, 1998; Caldwell & Suflita, 2000), denitrifying (Burland & Edwards, 1999; Coates *et al.*, 2001; Ulrich & Edwards, 2003), and methanogenic conditions (Grbic-Galic & Vogel, 1987; Ulrich & Edwards, 2003; Chang *et al.*, 2005).

Previous studies dealing with 16S rRNA gene based fingerprinting analysis of benzene-degrading enrichment cultures revealed the presence of phylogenetically diverse microorganisms (Ulrich & Edwards, 2003; Chang *et al.*, 2005). Under iron-reducing conditions, bacterial members of the family *Geobacteraceae* were determined as dominant organisms (Rooney-Varga *et al.*, 1999). Recently, Gram-positive bacteria related to *Clostridia* were discovered to play an essential role in benzene degradation with iron or sulfate as electron acceptor (Kunapuli *et al.*, 2007; Kleinsteuber *et al.*, 2008). In addition, members of the

family *Desulfobacteraceae* have been identified as dominant organisms in several benzene-degrading cultures under sulfate-reducing and methanogenic conditions (Phelps *et al.*, 1998; Ulrich & Edwards, 2003; Chang *et al.*, 2005; Da Silva & Alvarez, 2007; Musat & Widdel, 2008; Oka *et al.*, 2008). In a highly enriched benzene-degrading culture, *Desulfobacterium* was identified as the prominent phylotype (Musat & Widdel, 2008). So far, only two denitrifying benzene-degrading strains of the genera *Dechloromonas* and *Azoarcus* have been described as pure cultures (Coates *et al.*, 2001; Kasai *et al.*, 2006). However, despite the availability of several enrichment cultures, repeated attempts to isolate obligate anaerobic pure cultures failed and consequently the physiological properties of these microorganisms are still largely unknown.

Our knowledge of the biochemical mechanism of anaerobic benzene degradation is still scarce. The analysis of metabolites in diverse benzene-degrading cultures led to the identification of phenol (Caldwell & Suflita, 2000; Chakraborty & Coates, 2005; Kunapuli *et al.*, 2008), toluene (Ulrich *et al.*, 2005), and benzoate (Caldwell & Suflita, 2000; Phelps *et al.*, 2001; Kunapuli *et al.*, 2008) as putative intermediates. Consequently, three different biochemical mechanisms were hypothesized for the initial activation reaction of benzene, including carboxylation to benzoate (Caldwell & Suflita, 2000; Phelps *et al.*, 2001), hydroxylation to phenol (Vogel & Grbic-Galic, 1986; Grbic-Galic & Vogel, 1987; Caldwell & Suflita, 2000; Chakraborty & Coates, 2005), and methylation to toluene (Ulrich *et al.*, 2005).

The present study delivers insights into physiological, phylogenetic, and biochemical characteristics of an enrichment culture able to metabolize benzene under sulfate-reducing conditions. Here, the participation of Gram-positive bacteria in anaerobic benzene metabolism and carboxylation as the putative benzene activation mechanism are highlighted.

Materials and methods

Enrichment of the benzene-degrading culture and growth conditions

The anaerobic benzene-degrading culture BPL was enriched from soil at a former coal gasification site in Gliwice, Poland, with benzene as growth substrate in the presence of the adsorber resin Amberlite XAD-7 (Morasch *et al.*, 2001) and 5 mM Na_2SO_4 as electron acceptor. The enrichment culture was transferred 15 times with 10% inoculum (v/v) into a new 50 mL sediment-free medium. The enrichment culture was cultivated in bicarbonate-buffered (30 mM) freshwater medium (pH 7.2) under an anaerobic atmosphere of N_2/CO_2 (80 : 20 v/v) (Widdel & Bak, 1992). The freshwater medium was reduced with 1 mM Na_2S, and 3 mM $FeCl_2$ was added to scavenge produced sulfide. After repeated transfers (10% inoculum, v/v) into fresh medium, a stable anaerobic benzene-degrading enrichment culture was obtained. All culture bottles were incubated at 30 °C in the dark.

Alternative substrate and electron acceptor utilization

The benzene-degrading enrichment culture BPL was tested for growth on different organic substrates. A 1 mM total concentration of the aromatic substrates biphenyl, toluene, phenol, 4-hydroxybenzoate, and benzoate was added separately as liquids with a glass syringe or as solid crystals to 120-mL serum bottles containing 50 mL of bicarbonate-buffered mineral medium and 0.3 g of Amberlite XAD-7 (Morasch *et al.*, 2001). Bacterial growth on different substrates was checked visually by turbidity formation of the medium in triplicate incubations over a period of 3 months. For cometabolism experiments, 500 μM of the respective aromatic substrates without amberlite XAD-7 were added to the culture bottles. Formate, pyruvate, and lactate were added to the medium from autoclaved or filter-sterilized aqueous stock solutions to a final concentration of 10 mM. H_2/CO_2 gas (30 : 70 v/v) was directly injected to the culture bottles. The tested electron acceptors included Na_2SO_3 (10 mM), $Na_2S_2O_3$ (10 mM), S^0 (1 g L^{-1}), $NaNO_3$ (5 mM), and $Fe(OH)_3$ (50 mM), respectively. Electron acceptors were autoclaved and added to the culture bottles from anoxic stock solutions. $Fe(OH)_3$ was prepared as described elsewhere (Lovley & Phillips, 1986).

Analytical procedures

For quantification of benzene and CO_2 in the electron and carbon balance experiments, the culture was cultivated with 350 μM $^{13}C_6$-benzene (Sigma Aldrich, Steinheim, Germany; 99% purity) without Amberlite XAD-7 in 250-mL serum bottles and sealed with Viton rubber stoppers (Mag Technik, Dübendorf, Switzerland).

$^{13}C_6$-benzene, $^{12}C_6$-benzene, and toluene concentrations were determined by headspace analysis with GC/MS (GC, Trace-DSQ; MS, Thermo Finnigan, San Jose, CA) in selective ion monitoring mode with a fused-silica capillary column DB-5 [30 m length (L), 0.25 mm inside diameter (ID), 0.25 μm film thickness (T); Agilent, Palo Alto]. The injector temperature was 220 °C, the carrier gas was helium (grade 5.0) at a flow rate of 1 mL min^{-1} and the split ratio was split : splitless 1 : 10. The temperature was held at 40 °C for 1 min, raised to 200 °C at 15 °C min^{-1}, to 300 °C at 25 °C min^{-1}, and held for 1 min. The total molar mass of benzene in the culture was calculated from benzene in the liquid phase and Henry's constant of benzene in the headspace as given by Peng & Wan (1997).

Culture samples for phenol quantification were taken inside an anoxic chamber and 25 μL of sample was analyzed directly using the auto injector (SIL-10A*i*, Shimadzu) of a Shimadzu HPLC equipped with Shimadzu RF-10A XL fluorescence and Shimadzu SPD–10AVP UV diode array detectors at 30°C. The column was a C_{18} Prontosil Eurobond (250 mm L, 4 mm ID, 5 μm particle size; Bischoff, Leonberg, Germany). The mobile phase was (1) 1% acetic acid in water pH 2.7 and (2) 1% acetic acid in methanol at a flow rate of 0.19 and 0.20 mL min^{-1}, respectively. UV absorption was measured at 270 nm; fluorescence excitation was at 265 nm and the emission wavelength 315 nm.

The total amount of $^{13}CO_2$ produced from $^{13}C_6$ benzene degradation was determined by measuring the $^{13}CO_2/^{12}CO_2$ isotope ratio in the headspace. The headspace sample (1 mL) was diluted in a 10-mL serum vial filled with helium and stored until analysis. The diluted gas samples (100 mL) were injected to a GC/C/IRMS system consisting of a TRACE GC Ultra gas chromatograph with split/splitless injector (GC) (Thermo Fisher Scientific Corporation, Milan, Italy) coupled to a Finnigan MAT 253 isotope ratio mass spectrometer (IRMS) via Finnigan GC combustion III interface (Thermo Fisher Scientific Corporation, Bremen, Germany). The GC was equipped with a DB-5 column (30 mL, 0.25 mm ID, 0.25 μm T). The temperature of the injector was held at 180 °C isothermally with a split ratio 1 : 10 and column flow of 1.4 mL. Helium (grade 5.0) was used as carrier gas with a constant flow rate of 1.4 mL min^{-1}. The initial oven temperature was 50 °C, ramped at a rate of 45 °C min^{-1} to 100 °C, and held for 1.4 min. The $^{13}CO_2$-concentration was calculated from carbon isotope compositions of CO_2 expressed in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB):

$$\delta^{13}C\,(‰) = \frac{\left(^{13}C/^{12}C_{\text{sample}} - ^{13}C/^{12}C_{\text{V-PDB standard}}\right)}{\left(^{13}C/^{12}C_{\text{V-PDB standard}}\right)} \times 1000 \quad (1)$$

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

$$^{13}CO_2(\text{mM}) = ^{13}CO_2/^{12}CO_{2\,\text{sample}} \times \text{initial}\ ^{12}CO_2\ (\text{mM}) \quad (3)$$

The initial $^{12}CO_2$-concentration was calculated as the sum of $^{12}CO_2$ from the $NaHCO_3$ (30 mM) buffer in the liquid phase and 20% of $^{12}CO_2$ gas in the headspace of the culture bottles.

Sulfate concentrations were analyzed by ion chromatography using a Dionex 300 ion chromatograph (Dionex Corporation, Sunnyvale) equipped with an IonPac AS14 analytical column (4 × 250 mm) (Dionex Corporation). The eluent was Na_2CO_3 (3.5 mM) and $NaHCO_3$ (1 mM) and the flow rate was 1.2 mL min^{-1}.

Cell counts by FISH and 4,6-diamidino-2-phenylindole (DAPI) staining

Oligonucleotide probes used for FISH were (1) EUB338 I-III mix specifically labeling all *Bacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999), (2) DEM1164r targeting all *Desulfotomaculum* cluster I bacteria (Stubner & Meuser, 2000), (3) BET42a in combination with the unlabeled GAM42a oligonucleotide specifically labeling *Betaproteobacteria* (Manz *et al.*, 1992), and (4) GAM42a in combination with the unlabeled BET42a oligonucleotide used to detect *Gammaproteobacteria* (Manz *et al.*, 1992). For the determination of cell numbers via FISH and DAPI staining, 1 mL of the benzene-degrading enrichment culture BPL and the control bacterium *Thiobacillus thiophilus* D24TNT (Kellermann & Griebler, 2009) were fixed for 1–3 h with 1 mL 100% ethanol. The cells were harvested by centrifugation at 6000 ***g*** for 5 min and the cells were stored in phosphate-buffered saline (PBS)-ethanol (1 : 1) at −20 °C. The cells were filtered onto black 0.2 μm Nucleopore polycarbonate filters (Whatman, Brentford, UK) under vacuum pressure and dehydrated in 50%, 80%, and 100% ethanol for 3 min each. Hybridization of the filters was performed at 46 °C for 1.5 h with 40 μL hybridization buffer [0.9 M NaCl, 20 mM Tris/HCl, pH 8, 10% formamide, 0.1% sodium dodecyl sulfate (SDS)], 5 μL of Cy3-labelled probe (30 ng μL^{-1}) and 5 μL of fluorescein-labelled probe (50 ng μL^{-1}). The filters were treated for 20 min with washing buffer (0.45 M NaCl, 20 mM Tris/HCl, pH 8, 0.01% SDS) at 48 °C, washed with distilled water and air dried. For staining with the DNA-binding dye DAPI, the hybridized polycarbonate filters were covered with 5 μL DAPI solution (1 mg mL^{-1}). Counting was performed with an Epifluorescence microscope (Zeiss Axioplan 2, Oberkochen, Germany) equipped with the filters Zeiss49 for DAPI, Zeiss9 for fluorescein and Zeiss43 for Cy3. For cell counting, two separate filters were prepared from duplicate incubations and 20 randomly selected fields were counted for each filter.

Metabolite analysis

Metabolite analysis was performed using a LC-ESI-MS-MS system with a method developed to measure 2-hydroxybenzoate, 4-hydroxybenzoate, benzoate, and benzylsuccinate. Samples of 15 mL from the cultures were spiked with 1 mL of a 100 μg L^{-1} fluorinated benzoate solution as internal standard, filtered (0.45 μm pore size; Millipore, Eschborn, Germany), acidified to pH 1 and stored at 4 °C. Metabolites were extracted from the samples and preconcentrated by solid phase extraction with ISOLUTE ENV$^+$ (200 mg adsorbent; Biotage, Uppsala, Sweden). The analysis was performed using an HPLC Agilent 1100 (Agilent Technologies, Santa Clara) equipped with a binary pump, a degasser, a column oven, and an auto sampler, which was

coupled with an electrospray ionization source (TurboIon Spray, Applied Biosystems MDS/SCIEX, Foster City) to the triple quadrupole mass spectrometer ABI 3000 (Applied Biosystems MDS/SCIEX) (Ohlenbusch *et al.*, 2002). The separation column was a Purospher RP-18e (125×2 mm L, 5 μm particle size; Merck, Darmstadt, Germany). The column oven was set to 35 ± 2 °C. The eluent consisted of (A) water with 0.1% acetic acid (v/v) and (B) 100% acetonitrile. The eluent B was raised from 20% to 90% within 10 min. The flow rate was 0.3 mL min^{-1} and the sample injection volume was 50 μL. At the ESI interface the nebulizer gas flow was set to 1.5 L min^{-1}, the curtain gas flow to 1.6 L min^{-1}, and the dry gas flow to 6 mL min^{-1}. The dry gas temperature was 450 °C and the ion spray voltage of the ESI system was set to -5500 V. MS/MS measurements were performed in the negative multiple reaction mode and the most abundant fragment ion was recorded after collision induced dissociation. For benzylsuccinate, a second, but much less sensitive, fragment (*m/z* 91) was used to confirm the detection of this analyte. The ratio of *m/z* 91 to *m/z* 163 is 0.07 ($\pm$ 20%).

Molecular and phylogenetic analysis

Cells of 50 mL enrichment culture were harvested by centrifugation at 3345 ***g*** for 15 min at 4 °C. The pellet was washed twice with 1 mL of $1 \times$ PBS. Genomic DNA was extracted and purified using the FastDNA Spin Kit for Soil according to the manufacturer's protocol (MP Biomedicals, Illkirch, France).

Terminal restriction fragment length polymorphism (T-RFLP) was performed using the 16S rRNA gene primer set Ba27f-FAM/907r (Lane *et al.*, 1985). T-RFLP analysis of benzene-grown cells was performed from two separate incubations. T-RFLP analyses of cells grown on other substrates were performed once. The T-RFLP analysis was carried out with 20 ng of the amplicon as described previously (Lueders *et al.*, 2006).

16S rRNA gene sequences for construction of the clone library were amplified using the universal primers Ba27f/Ba1492r (Weisburg *et al.*, 1991) resulting in almost full-length products. The PCR reaction, cloning, and sequencing were conducted as described previously (Winderl *et al.*, 2007). The CHECK_CHIMERA program of the ribosomal database project II (Biomedical and Physical Sciences Building, Michigan State University) was used to check for chimeric sequences. The aligned sequences were compared with the closely related sequences available in the public database using BLASTN program (http://ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene sequences were added into a database existing of about 25 000 small-subunit rRNA gene sequences (http://arb-home.de) (Ludwig *et al.*, 2004). Phylogenetic analysis was performed by parsimony, maximum-likelihood, and verified by the neighbour-joining as method implemented in the ARB software package. The sequences determined in this study are available at GenBank under accession nos. EU523065–EU523097.

Results

Physiological properties of the benzene-degrading culture

Enrichment of benzene-degrading, sulfate-reducing bacteria was performed with contaminated soil of a former coal gasification site as inoculum and was since then transferred 15 times into new sterile, soil-free medium. The enrichment culture was able to tolerate benzene up to 1 mM without Amberlite XAD-7. Carbon and electron balances of the culture showed that the complete degradation of 350 μM $^{13}C_6$-benzene was coupled to the production of 1.8 mM $^{13}CO_2$ (Eqn. 1–3; Fig. 1) and to the reduction of 1.2 mM sulfate (Fig. 1). These values correspond to an electron recovery of 88.8 ± 10.7% in the reduced sulfate for total reduction to HS^- and to the production of $83.6\% \pm 2.6\%$ $^{13}CO_2$ from $^{13}C_6$-benzene mineralization. These experimental results were close to the theoretically stoichiometric values for the complete degradation of benzene to carbon dioxide (Eqn. 4):

$$C_6H_6 + 3.75SO_4^{2-} + 3H_2O \rightarrow 6HCO_3^- + 3.75HS^- + 2.25H^+ \quad (4)$$

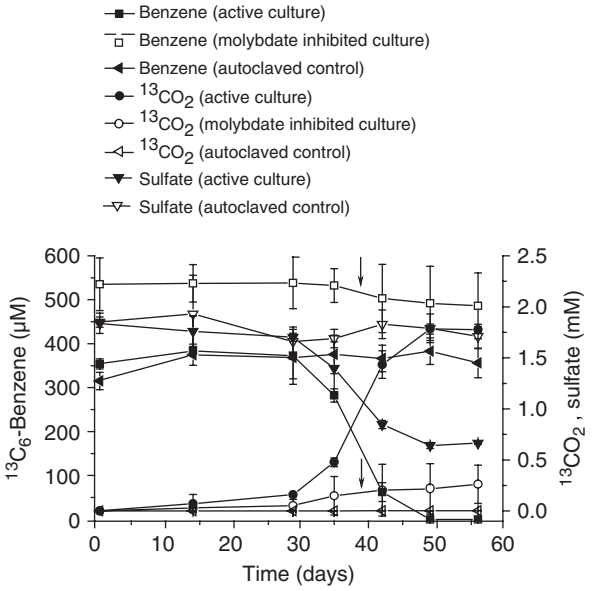

Fig. 1. Degradation of $^{13}C_6$-benzene by the sulfate-reducing enrichment culture BPL. Carbon and electron balance between $^{13}C_6$-benzene and $^{13}CO_2$-evolution and sulfate reduction. Na_2MoO_4 5 mM was added after 38 days (arrows). Data are the means of triplicate incubations and error bars represent SDs.

There was no decrease of $^{13}C_6$-benzene or sulfate in autoclaved control cultures. The addition of 5 mM molybdate (Na_2MoO_4), a specific inhibitor of sulfate reduction, at day 38 after inoculation, caused a complete inhibition of $^{13}C_6$-benzene degradation and $^{13}CO_2$-production (Fig. 1). To prevent a potential toxic effect of sulfide on the microorganisms in the enrichment culture BPL, ferrous chloride was added to the culture medium and consequently a solid iron sulfide mineral (greigite) precipitated.

The enrichment culture BPL was tested for utilization of several monoaromatic, polycyclic, and nonaromatic compounds as carbon source with sulfate as electron acceptor. Besides benzene, the culture was able to grow with biphenyl, whereas benzoate, 4-hydroxybenzoate, phenol, and toluene could not be utilized. The enrichment culture was also tested for its capability to use phenol and toluene as cosubstrates (Fig. 2). Here, neither phenol nor toluene could be degraded cometabolically. Interestingly, the rate of benzene degradation was retarded in the presence of phenol (Fig. 2a) whereas toluene had no effect on the rate of benzene degradation (Fig. 2b). Additionally, growth of microorganisms in the culture BPL could be observed with the organic acids formate, lactate, and pyruvate as well as H_2/CO_2 (30 : 70 v/v) as evaluated by turbidity formation of the growth medium. None of the tested electron acceptors sulfite, thiosulfate, elemental sulfur, nitrate, or ferrihydrite was utilized with benzene as carbon source.

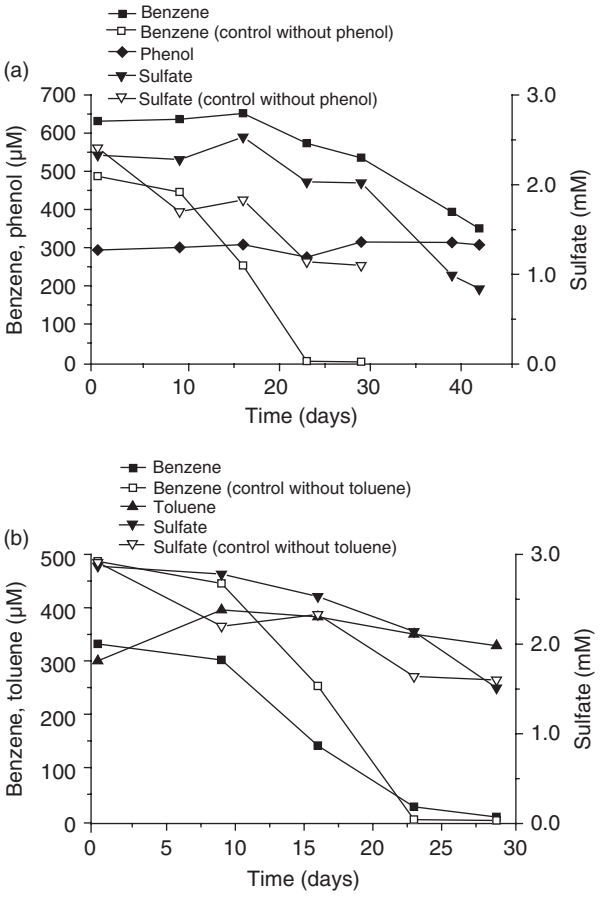

Fig. 2. Degradation of benzene by the enrichment culture BPL in the presence of (a) phenol and (b) toluene as co-substrates using sulfate as an electron acceptor. Data are the means of duplicate incubations.

Community fingerprinting and phylogenetic analysis

The bacterial community composition of the enrichment culture BPL grown on different substrates was assessed by T-RFLP analysis of 16S rRNA gene sequences. The T-RFLP profile of benzene-grown cells was clearly dominated by 16S rRNA gene sequences forming a 141-bp T-RF (Fig. 3a). *In silico* analysis considered the occurrence of an additional T-RF of 152 bp length to be a pseudo T-RF (Fig. 3a), which was verified by T-RFLP analysis of specific 16S rRNA gene clone sequences. Pseudo T-RFs are formed when the restriction enzyme failed to cut at the first recognition site, and thus a longer T-RF is formed by restriction at the second site (Egert & Friedrich, 2003). Thus, the T-RFs of 141 and 152 bp have derived from the same 16S rRNA gene sequence. The 141 bp T-RF could also be identified as a dominant peak in the biphenyl-grown culture together with an additional T-RF of 164 bp in length (Fig. 3b). A 164-bp T-RF was continuously present in all growth cultures (except lactate) tested for alternative electron donors (Fig. 3c–f).

To get insights into the phylogenetic affiliation of the microoorganims in the benzene-grown culture, we performed cloning and sequencing of almost full-length 16S rRNA gene sequences. The analysis clearly showed a dominance of 16S rRNA gene sequences (designated as BpP) that accounted for 30 out of 33 analyzed small-subunit sequences and that could be correlated with the 141-bp T-RF. These highly abundant sequences were affiliated with the genus *Pelotomaculum* within the Gram-positive family *Peptococcaceae* (Fig. 4) and grouped to a cluster that was related to *Pelotomaculum isophthalicum* JI (95% sequence similarity). Moreover, two closely related 16S rRNA gene sequences (BpC52 and BpC43; represented by the 305-bp T-RF) were detected that were affiliated to the family *Clostridiaceae*. The 16S rRNA gene sequence of the clone BpD108 was affiliated to the deltaproteobacterial family *Syntrophaceae*, exhibiting a similarity value of 93.3% to the dehalogenating bacterium *Desulfomonile tiedjei*.

Cell counts determined by FISH and DAPI staining

In order to demonstrate that bacteria with *Pelotomaculum*-related 16S rRNA gene sequences (BpP) are dominant and their cell numbers are increasing during the full time course

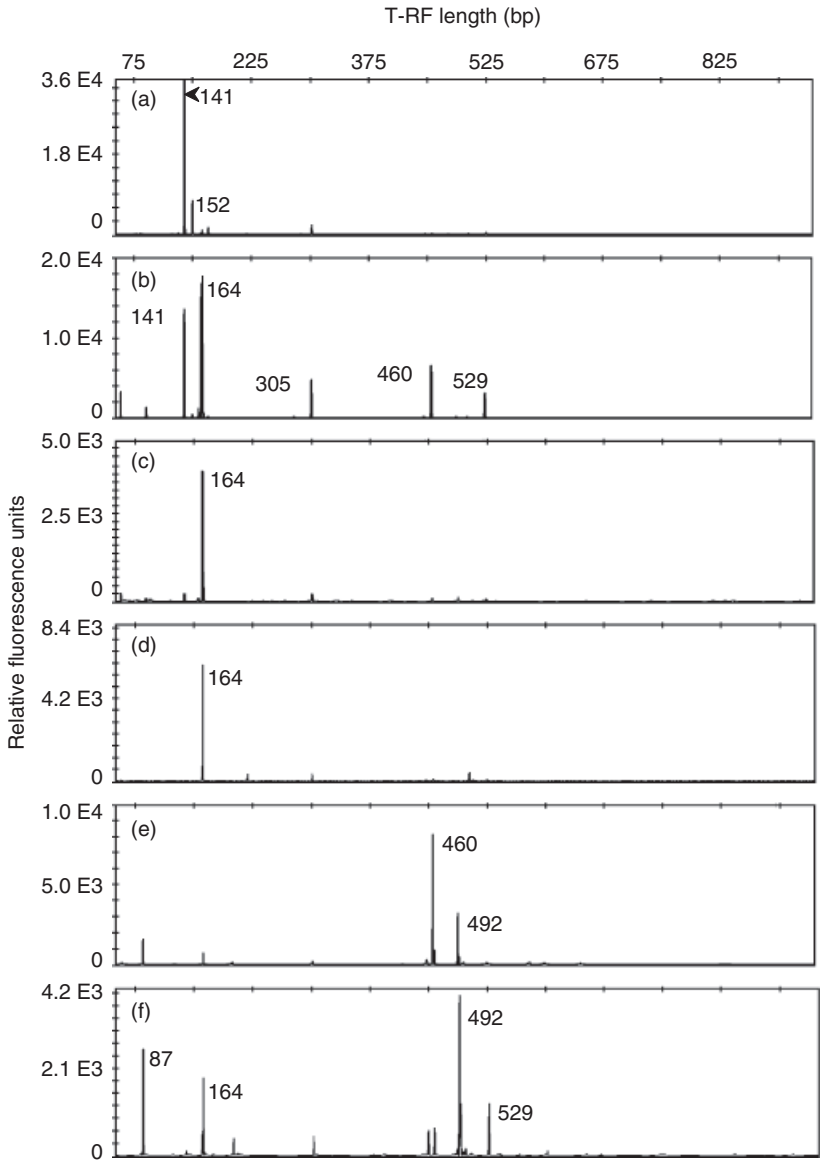

Fig. 3. T-RFLP fingerprinting profiles of 16S rRNA gene sequences of the enrichment culture BPL grown on (a) benzene, (b) biphenyl, (c) formate, (d) pyruvate, (e) lactate, and (f) H_2/CO_2 gas. Dominant base pairs are labelled with lengths (bp).

of benzene degradation, *in situ* hybridization with the probes DEM1164r, specific for *Desulfotomaculum* cluster I bacteria comprising *Pelotomaculum* sp. and EUB338 I-III mix (Bacteria) was performed (Fig. 5). No hybridization to 16S rRNA gene sequences of the enrichment culture BPL could be observed with the negative control probes BET42a and GAM42a. However, the probe BET42a but not DEM1164r hybridized to the control organism *T. thiophilus* D24TNT. During the time course of benzene degradation, the average percentage of DEM1164r-labelled cells was 94.5% ($n = 9$ time points) of the EUB338 I-III mix-positive cells (Fig. 5). The portion of the bacterial cells stained with DEM1164r and EUB338 I-III mix constituted 86.6% ($n = 9$) and 91.8% ($n = 9$) of the total DAPI-positive cells (Fig. 6).

Metabolite analysis

Metabolites were screened and quantified with LC-ESI-MS-MS and HPLC/UV fluorescence analysis during benzene degradation (54 days). Benzoate, 4-hydroxybenzoate, and phenol were detected in active as well as in autoclaved control cultures (Fig. 7). The phenol formation increased concomitant to benzene degradation in the active culture reaching a maximum concentration of 1.8 μM at day 28. Interestingly, the phenol concentration in the autoclaved control culture was comparably high, reaching the maximum value of 1.6 μM at day 47. The amount of benzoate in the active culture was highest at day 47 increasing to 0.4 μM, whereas its concentration in the autoclaved culture remained constantly low (0.06 μM). Additionally, at the end

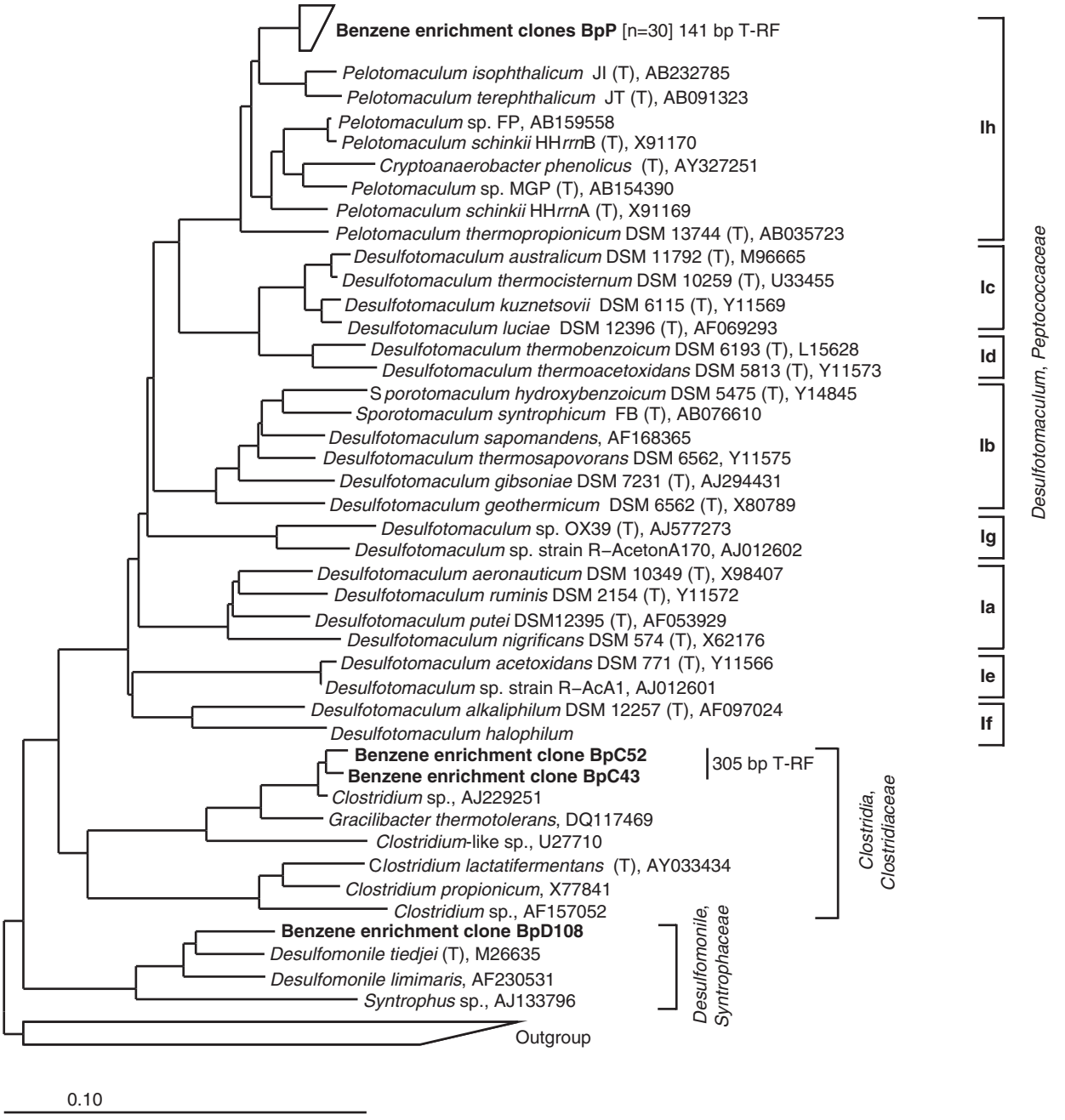

Fig. 4. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequences amplified from the enrichment culture BPL. 16S rRNA clone sequences from the enrichment culture BPL are shown in bold. Subdivisions Ia–Ih of the *Desulfotomaculum* cluster I are listed. An encompassing collection of organisms representing all major lineages of the *Archaea* and *Bacteria* was used as outgroup for tree calculations. The scale bar represents 10% sequence divergence.

of the time course of benzene degradation, 4-hydroxybenzoate was identified in the active culture at 0.14 μM. 2-Hydroxybenzoate was detected at maximum concentration of 2.9 nM from autoclaved control cultures and with a slightly higher concentration (7.9 nM) in the active culture (data not shown). Benzylsuccinate could not be identified.

Discussion

In the present study, we report on anaerobic benzene degradation by a sulfate-reducing enrichment culture obtained from soil at a former coal gasification site. Electron and carbon balances with $^{13}C_6$-benzene indicated a total

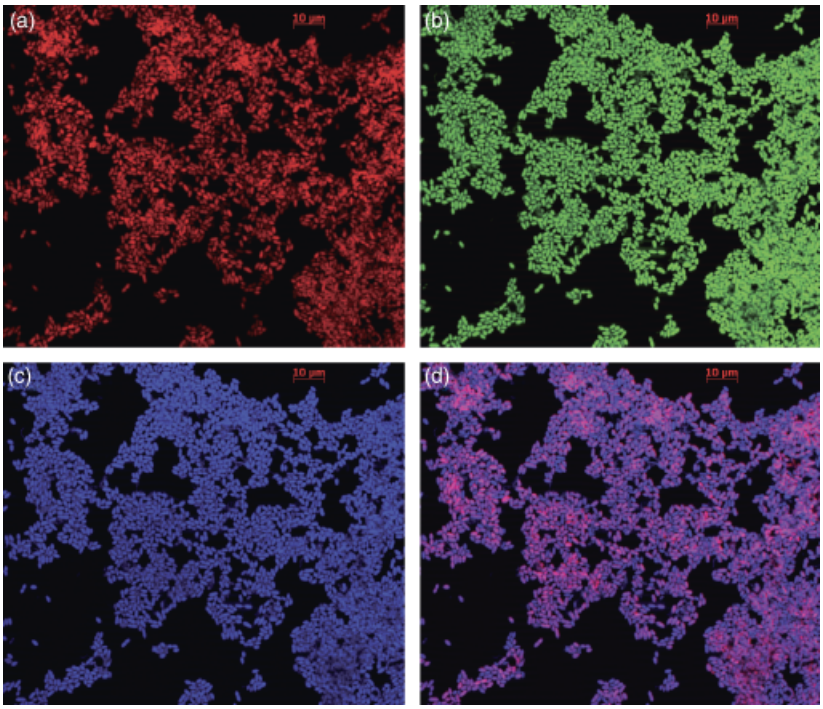

Fig. 5. Micrographs of cells from the culture BPL grown with benzene and sulfate as electron acceptor. (a) Specific labeling of *Desulfotomaculum* subcluster I cells with the Cy3-labeled probe DEM1164r, (b) general labeling with the Fluos-labeled probe mixture EUB338 I-III, (c) DAPI-staining of all bacteria, and (d) labeling of cells with the specific probe DEM1164r and DAPI resulting in overlays of the probe and DAPI signals; cells targeted by both labels appear in magenta and nontargeted cells in blue.

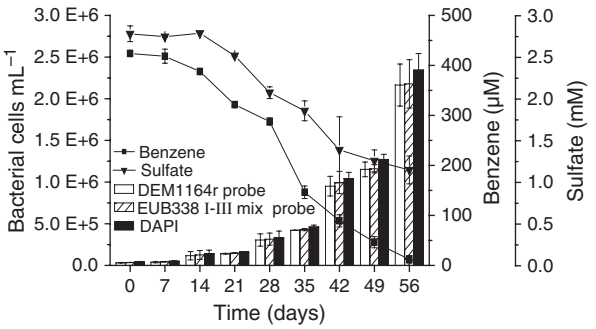

Fig. 6. Relative quantification of bacterial cells detected by *in situ* hybridization with the specific probe DEM1164r (*Desulfotomaculum* cluster I) or EUB338 I-III mix (bacteria) and DAPI staining during the full time course of benzene degradation by the enrichment culture BPL. Data are the means of duplicate incubations with two separate filter counting each and error bars represent SDs.

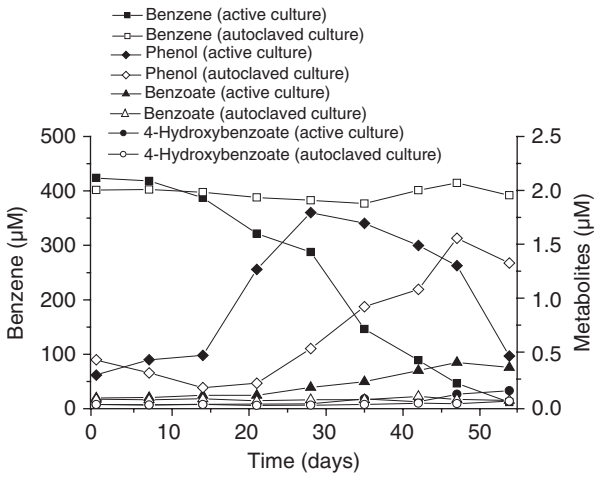

Fig. 7. Metabolite analysis from active and autoclaved control cultures under sulfate-reducing conditions during the full time course of benzene degradation. Data are the means of duplicate incubations.

oxidation of the aromatic hydrocarbon to CO_2 coupled to sulfate reduction.

Bacterial community structure of the benzene-degrading culture

Based on the quantitative determination of the putative benzene degrader by *in situ* hybridization and the analysis of 16S rRNA gene sequences, we have provided evidence leading to the hypothesis that organisms phylogenetically related to the Gram-positive genus *Pelotomaculum* play an important role in benzene degradation. However, the 16S rRNA gene-based sequence similarity to the next cultivated representative constitutes only 95%. Thus, these sequences could be clustered between the genera *Desulfotomaculum* and *Pelotomaculum*. *Pelotomaculum isophthalicum*-related 16S rRNA gene sequences were detected in benzene-contaminated groundwater and *in situ* reactor columns, degrading benzene under sulfate-reducing and denitrifying

conditions (Kasai *et al.*, 2007; Kleinsteuber *et al.*, 2008). Kleinsteuber *et al.* (2008) hypothesized that *Pelotomaculum* syntrophically ferments benzene to acetate under sulfate-reducing conditions. The similarity of the sequences of the *Pelotomaculum*-related phylotypes described by Kleinsteuber *et al.* (2008) to those identified in the present study range from 88.8% to 95.7% indicating that these phylotypes are totally different. Based on the results with our highly enriched culture, where only one dominant organism is present, we propose that bacteria with *Pelotomaculum*-related 16S rRNA gene sequences oxidize benzene directly with sulfate as electron acceptor. However, members of the genus *Pelotomaculum* were not reported to carry out dissimilatory sulfate reduction so far (Imachi *et al.*, 2002). Recently, Imachi *et al.* (2006) proposed that *Desulfotomaculum* subcluster Ih bacteria have lost their ability to reduce sulfate in an evolutionary process and thus are restricted to methanogenic environments for synthrophic oxidation of organic substrates. Such a synthrophic benzene degradation could be definitely excluded for the presented culture because neither methane nor methanogenic archaea have been detected (data not shown). Additionally, the *in situ* hybridization and T-RFLP analysis of the benzene-grown enrichment culture clearly showed the presence of only one dominant genotype. Thus, we can show here that bacteria that are phylogenetically affiliated to *Pelotomaculum* but physiologically strongly resemble *Desulfotomaculum* are utilizing benzene as sole carbon source with sulfate as electron acceptor. This hypothesis is supported by the identification of the γ subunit of the dissimilatory sulfite reductase (key enzyme of dissimilatory sulfate reduction) by proteomic analysis of the benzene-grown culture (data not shown). The identified peptide is closely related to the dissimilatory sulfite reductase of *Pelotomaculum thermopropionicum* (Imachi *et al.*, 2006).

Our knowledge of the role and importance of Gram-positive bacteria in degradation of aromatic hydrocarbons is quite limited. Recently, an iron-reducing enrichment culture was described where benzene degradation was performed by *Clostridia*-related bacteria in association with members of *Desulfobulbaceae* (Kunapuli *et al.*, 2007). In addition, few Gram-positive sulfate-reducers that degrade aromatics were successfully isolated in pure cultures (Cord-Ruwisch & Garcia, 1985; Tasaki *et al.*, 1991; Kuever *et al.*, 1999; Morasch *et al.*, 2004). Interestingly, the majority of these bacterial strains are phylogenetically restricted to the *Desulfotomaculum* cluster I. Morasch *et al.* (2004) isolated the strict anaerobic bacterium *Desulfotomaculum* sp. Ox39 that metabolizes the aromatic hydrocarbons toluene, *m*-xylene, and *o*-xylene. *Desulfotomaculum sapomandens* (Cord-Ruwisch & Garcia, 1985), *Desulfotomaculum thermobenzoicum* (Tasaki *et al.*, 1991), and *Desulfotomaculum gibsoniae* (Kuever *et al.*, 1999) exhibit a great versatility in the kind of aromatic electron donors they can use for growth, including benzoate, phenol, and 4-hydroxybenzoate. Molecular based investigations of benzene-degrading communities at contaminated sites and in enrichment cultures in the presence of different electron acceptors showed that they were mostly affiliated to *Beta*-, *Delta*-, and *Gammaproteobacteria* (Phelps *et al.*, 1998; Rooney-Varga *et al.*, 1999; Coates *et al.*, 2001; Musat & Widdel, 2008; Oka *et al.*, 2008). However, several studies identified *Firmicutes*-related 16S rRNA gene sequences that have so far not been brought into direct correlation with contaminant degradation (Phelps *et al.*, 1998; Ulrich & Edwards, 2003; Chang *et al.*, 2005). Nevertheless, the large diversity of the microbial communities was not enough to emphasize the key players and their role in benzene degradation. So far, only a limited number of sulfate-reducing benzene-degrading enrichment cultures have been purified to an extent that allowed the identification of the key degraders. Deltaproteobacterial phylotypes related to *Desulfobacterium* were found to be abundant in a benzene-degrading sulfate-reducing culture (Qiu *et al.*, 2006; Musat & Widdel, 2008). Furthermore, members of the family *Desulfobacteraceae* have been identified as significant bacteria in a sulfidogenic benzene-degrading culture by DNA stable isotope probing (Oka *et al.*, 2008). More recently, bacteria affiliated to the genera *Sulfurovum*, *Desulfovibrio*, and *Cryptanaerobacter/Pelotomaculum* have been characterized to be the most prominent bacteria in sand-filled columns percolated with groundwater from a benzene-contaminated aquifer whereas the proportion of the latter one increased after repeated benzene-spiking, indicating a role in benzene degradation (Kleinsteuber *et al.*, 2008). The cultivation of our *Pelotomaculum*-related bacterium further supports our hypothesis that Gram-positive microorganisms have been largely overlooked as being important in degradation of benzene (Kunapuli *et al.*, 2007, 2008). Especially Gram-positive sulfate-reducing bacteria may play an up to date underestimated role, because their ability of spore-formation allows this group to survive periods of environmental fluctuating conditions such as alternating oxic and anoxic conditions (Nielsen *et al.*, 2005).

Initial reaction mechanism of anaerobic benzene degradation

In previous studies, three possible mechanisms have been proposed for the initial reaction of anaerobic benzene degradation. Hydroxylation of benzene to phenol has been hypothesized as one putative pathway under methanogenic and denitrifying conditions (Vogel & Grbic-Galic, 1986; Weiner & Lovley, 1998; Caldwell & Suflita, 2000; Chakraborty & Coates, 2005). In the present study, phenol and the specific intermediate of anaerobic phenol degradation 4-hydroxybenzoate were detected in the active enrichment

culture. These results might indicate that benzene is initially hydroxylated to phenol. If the degradation would proceed via hydroxylation to phenol, the benzene-grown culture should have the ability to utilize phenol immediately. However, phenol as sole carbon source was not utilized by the enrichment culture BPL. In addition, benzene degradation was retarded when phenol was added as a cosubstrate. It seems that phenol decreased the activity of the benzene degraders or that the benzene degraders had to adapt to the presence of phenol, which may exhibit a toxic effect. This observation is congruent with results obtained from a sulfate-reducing culture enriched from marine sediment where the rate of benzene degradation was also slightly slower in the presence of phenol (Musat & Widdel, 2008).

In addition, 2-hydroxybenzoate has been identified as a putative metabolite in the benzene-grown cultures, which is not known to be a specific intermediate compound of anaerobic degradation of monoaromatic hydrocarbons, so far. Thus, it might indicate that the detected hydroxylated aromatic compounds are formed abiotically due to contact of reduced compounds with oxygen during sampling. This is strongly supported by the detection of phenol in our autoclaved control cultures. Kunapuli *et al.* (2008) showed that benzene is readily autohydroxylated by hydroxyl ($OH^{\bullet}$) radicals formed from oxygen during sampling, which might also have produced the hydroxylated monoaromatic compounds in our culture. Another evidence for this abiotic process is the formation of 4-hydroxybenzoate concomitant to benzoate evolution. The medium was supplemented with 3 mM of $FeCl_2$ to scavenge the produced sulfide. As a result of this reaction, reduced iron is produced that is considered to be a catalyst of Fenton's reaction. During sampling, ferrous ion gets in contact with oxygen, which results in the formation of highly reactive $OH^{\bullet}$ radicals (Kavith & Palanivelu, 2004; Mortazavi *et al.*, 2005). These $OH^{\bullet}$ radicals readily react with organic compounds such as benzene and benzoate forming hydroxylated products (Kunapuli *et al.*, 2008).

Methylation is proposed as an alternative mechanism for anaerobic degradation of nonsubstituted aromatic compounds (Ulrich *et al.*, 2005; Safinowski & Meckenstock, 2006). Benzylsuccinate as the specific metabolite of anaerobic toluene degradation was not detected in our benzene-grown culture. Similarly to recent studies (Musat & Widdel, 2008; Oka *et al.*, 2008), toluene could not be used as substrate by our culture and the presence of toluene in the medium had no effect on the rate of benzene degradation. This is in contrast to investigations where other authors clearly showed that toluene inhibits the rate of benzene degradation (Da Silva & Alvarez, 2007). Thus, methylation could probably be excluded as initial degradation mechanism in the investigated enrichment culture.

Another proposed pathway of anaerobic benzene degradation involves direct carboxylation of benzene to benzoate (Caldwell & Suflita, 2000). Previous studies with sulfate-reducing enrichment cultures using labeled benzene revealed the presence of ^{13}C-benzoate and d_5-benzoate as intermediate compounds and showed that the carboxyl group derived from benzene (Caldwell & Suflita, 2000; Phelps *et al.*, 2001). However, Kunapuli *et al.* (2008) showed for an iron-reducing culture that the carboxyl group of benzoate stems from the carbonate buffer. In the present study, benzoate was identified as putative intermediate compound, but we could not prove that the benzene-degrading bacteria have the capability to utilize benzoate as substrate. As benzoate cannot freely diffuse into cells, we assume that benzene degraders in our culture lack an active transport system for benzoate as it is reported for *Pseudomonas putida* (Thayer & Wheelis, 1982). Furthermore, it might be possible that benzoate could not be activated to benzoyl-Co-enzyme A due to the lack of a benzoate-CoA ligase (Barragan *et al.*, 2004). Nevertheless, our findings would tentatively favor a direct carboxylation of benzene as the initial activation reaction in the sulfate-reducing enrichment culture BPL.

Acknowledgements

The authors thank Holger Penning and Martin Elsner for help with GC/C/IRMS measurements. This project was supported by the DAAD-Helmholtz PhD exchange program through an operating grant (A/05/22356) awarded to N.A.L.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microb* **56**: 1919–1925.

Anderson RT & Lovley DR (1997) Ecology and biochemistry of *in situ* groundwater bioremediation. *Adv Microb Ecol* **15**: 289–350.

Anderson RT, Rooney-Varga JN, Gaw CV & Lovley DR (1998) Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ Sci Technol* **32**: 1222–1229.

Barragan M, Carmona M, Zamarro M, Thiele B, Boll M, Fuchs G, Garcia J & Diaz E (2004) The *bzd* gene cluster, coding for anaerobic benzoate catabolism, in *Azoarcus* sp. strain CIB. *J Bacteriol* **186**: 5762–5774.

Burland SM & Edwards EA (1999) Anaerobic benzene biodegradation linked to nitrate reduction. *Appl Environ Microb* **65**: 529–533.

Caldwell ME & Suflita JM (2000) Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. *Environ Sci Technol* **34**: 1216–1220.

Chakraborty R & Coates JD (2005) Hydroxylation and carboxylation – two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain RCB. *Appl Environ Microb* **71**: 5427–5432.

Chang W, Um Y & Pulliam Holoman T (2005) Molecular characterization of anaerobic microbial communities from benzene-degrading sediments under methanogenic conditions. *Biotechnol Progr* **21**: 1789–1794.

Christensen H, Kjeldsen P, Albrechtsen H, Heron G, Nielsen P, Bjerg P & Holm P (1994) Attenuation of landfill leachate pollutants in aquifers. *Crit Rev Env Sci Tech* **24**: 119–202.

Coates JD, Chakraborty R, Lack JG, O'Connor SM, Cole KA, Bender KS & Achenbach LA (2001) Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* **411**: 1039–1043.

Cord-Ruwisch R & Garcia JL (1985) Isolation and characterization of an anaerobic benzoate-degrading spore-forming sulfate-reducing bacterium, *Desulfotomaculum sapomandens* sp. nov. *FEMS Microbiol Lett* **29**: 325–330.

Daims H, Brühl A, Amann RI, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Da Silva M & Alvarez P (2007) Assessment of anaerobic benzene degradation potential using 16S rRNA gene-targeted real-time PCR. *Environ Microbiol* **9**: 72–80.

Dean BJ (1978) Genetic toxicology of benzene, toluene, xylenes and phenols. *Mut Res* **47**: 75–97.

Egert M & Friedrich MW (2003) Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl Environ Microb* **69**: 2555–2562.

Gibson DT & Parales RE (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* **11**: 236–243.

Grbic-Galic D & Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. *Appl Environ Microb* **53**: 254–260.

Imachi H, Sekiguchi Y, Kamagata Y, Hanada S, Ohashi A & Harada H (2002) *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. *Int J Syst Evol Micr* **52**: 1729–1735.

Imachi H, Sekiguchi Y, Kamagata Y *et al.* (2006) Non-sulfate-reducing, syntrophic bacteria affiliated with *Desulfotomaculum* cluster I are widely distributed in methanogenic environments. *Appl Environ Microb* **72**: 2080–2091.

Kasai Y, Takahata Y, Manefield M & Watanabe K (2006) RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Appl Environ Microb* **72**: 3586–3592.

Kasai Y, Kodama Y, Takahata Y, Hoaki T & Watanabe K (2007) Degradative capacities and bioaugmentation potential of an anaerobic benzene-degrading bacterium strain DN11. *Environ Sci Technol* **41**: 6222–6227.

Kavith V & Palanivelu K (2004) The role of ferrous ion in Fenton and photo-Fenton processes for degradation of phenol. *Chemosphere* **55**: 1235–1243.

Kazumi J, Caldwell ME, Suflita JM, Lovley DR & Young LY (1997) Anaerobic degradation of benzene in diverse anoxic environments. *Environ Sci Technol* **31**: 813–818.

Kellermann C & Griebler C (2009) *Thiobacillus thiophilus* D24TNT sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments. *Int J Syst Evol Micr* **59**: 583–588.

Kleinsteuber S, Schleinitz KM, Breitfeld J, Harms H, Richnow HH & Vogt C (2008) Molecular characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. *FEMS Microbiol Ecol* **66**: 143–157.

Kuever J, Rainey FA & Hippe H (1999) Description of *Desulfotomaculum* sp. Groll as *Desulfotomaculum gibsoniae* sp. nov. *Int J Syst Bacteriol* **49**: 1801–1808.

Kunapuli U, Lueders T & Meckenstock RU (2007) The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* **1**: 643–653.

Kunapuli U, Griebler C, Beller H & Meckenstock RU (2008) Identification of intermediates formed during anaerobic benzene degradation by an iron-reducing enrichment culture. *Environ Microbiol* **10**: 1703–1712.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogint ML & Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *P Natl Acad Sci USA* **82**: 6955–6959.

Lovley DR & Phillips EJ (1986) Availability of ferric iron for microbial reduction in bottom sediments of the freshwater tidal Potomac river. *Appl Environ Microb* **52**: 751–757.

Lovley DR, Coates JD, Woodward JC & Phillips EJ (1995) Benzene oxidation coupled to sulfate reduction. *Appl Environ Microb* **61**: 953–958.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acid Res* **32**: 1363–1371.

Lueders T, Kindler R, Miltner A, Friedrich MW & Kaestner M (2006) Identification of bacterial micropredators distinctively active in a soil microbial food web. *Appl Environ Microb* **72**: 5342–5348.

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Morasch B, Annweiler E, Warthmann RJ & Meckenstock RU (2001) The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. *J Microbiol Meth* **44**: 183–191.

Morasch B, Schink B, Tebbe CC & Meckenstock RU (2004) Degradation of *o*-xylene and *m*-xylene by a novel sulfate-

reducer belonging to the genus *Desulfotomaculum*. *Arch Microbiol* **181**: 407–417.

Mortazavi S, Sabzali A & Rezaee A (2005) Sequence-Fenton reaction for decreasing phenol formation during benzene chemical conversion in aqueous solutions. *Iranian J Env Health Sci Eng* **2**: 62–71.

Musat F & Widdel F (2008) Anaerobic degradation of benzene by a marine sulfate-reducing enrichment culture, and cell hybridization of the dominant phylotype. *Environ Microbiol* **10**: 10–19.

Nielsen L, Kadavy D, Rajagopal S, Drijber R & Nickerson K (2005) Survey of extreme solvent tolerance in Gram-positive Cocci: membrane fatty acid changes in *Staphylococcus haemolyticus* grown in toluene. *Appl Environ Microb* **71**: 5171–5176.

Ohlenbusch G, Zwiener C, Meckenstock RU & Frimmel FH (2002) Identification and quantification of polar naphthalene derivatives in contaminated groundwater of a former gas plant site by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* **967**: 201–207.

Oka AR, Phelps CD, McGuinness LM, Mumford A, Young LY & Kerkhof LJ (2008) Identification of critical members in a sulfidogenic benzene-degrading consortium by DNA stable isotope probing. *Appl Environ Microb* **74**: 6476–6480.

Peng J & Wan A (1997) Measurement of Henry's constants of high-volatility organic compounds using a headspace autosampler. *Environ Sci Technol* **31**: 2998–3003.

Phelps CD, Kerkhof LJ & Young LY (1998) Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. *FEMS Microbiol Ecol* **27**: 269–279.

Phelps CD, Zhang X & Young LY (2001) Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. *Environ Microbiol* **3**: 600–603.

Qiu Y, Sekiguchi Y, Hanada S, Imachi H, Tseng I, Cheng S, Ohashi A, Harada H & Kamagata Y (2006) *Pelotomaculum terephthalicum* sp. nov. and *Pelotomaculum isophthalicum* sp. nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. *Arch Microbiol* **185**: 172–182.

Rooney-Varga JN, Anderson RT, Fraga JL, Ringelberg D & Lovley DR (1999) Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. *Appl Environ Microb* **65**: 3056–3063.

Safinowski M & Meckenstock RU (2006) Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ Microbiol* **8**: 347–352.

Stubner S & Meuser K (2000) Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. *FEMS Microbiol Ecol* **34**: 73–80.

Tasaki M, Kamagata Y, Nakamura K & Mikami E (1991) Isolation and characterization of a thermophilic benzoate-degrading, sulfate-reducing bacterium, *Desulfotomaculum thermobenzoicum* sp. nov. *Arch Microbiol* **155**: 348–352.

Thayer JR & Wheelis ML (1982) Active transport of benzoate in *Pseudomonas putida*. *J Gen Microbiol* **128**: 213–217.

Ulrich AC & Edwards EA (2003) Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. *Environ Microbiol* **5**: 92–102.

Ulrich AC, Beller H & Edwards EA (2005) Metabolites detected during biodegradation of $^{13}C_6$-benzene in nitrate-reducing and methanogenic enrichment cultures. *Environ Sci Technol* **39**: 6681–6691.

USEPA. (1998) *Carcinogenic Effects of Benzene: An Update*. US Environmental Protection Agency, National Center for Environmental Assessment–Washington Office, Washington, DC, 97pp.

Vogel TM & Grbic-Galic D (1986) Incorporation of oxygen from water into toluene and benzene during anaerobic fermentative transformation. *Appl Environ Microb* **52**: 200–202.

Weiner JM & Lovley DR (1998) Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. *Appl Environ Microb* **64**: 1937–1939.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Widdel F & Bak F (1992) Gram-negative mesophilic sulfate-reducing bacteria. *The Prokaryotes* (Balows A, Trüper HG, Dworkin M, Harder W & Schleifer KH, eds), pp. 3352–3378. Springer-Verlag, New York.

Winderl C, Schaefer S & Lueders T (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. *Environ Microbiol* **9**: 1035–1046.