RESEARCH ARTICLE

Enrichment and characterization of a sulfate-reducing toluene-degrading microbial consortium by combining *in situ* microcosms and stable isotope probing techniques

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Abstract

A toluene-degrading microbial consortium was enriched directly in a BTEX-contaminated aquifer under sulfate-reducing conditions using *in situ* microcosms consisting of toluene-loaded activated carbon pellets. Degradation of toluene and concomitant sulfide production by the consortium was subsequently demonstrated in laboratory microcosms. The consortium was physiologically and phylogenetically characterized by isotope tracer experiments using nonlabeled toluene, [^{13}C]-α-toluene or [$^{13}C_7$]-toluene as growth substrates. Cells incubated with [^{13}C]-α-toluene or [$^{13}C_7$]-toluene incorporated 8–15 at.% ^{13}C and 51–57 at.% ^{13}C into total lipid fatty acids, respectively, indicating a lower specific incorporation of ^{13}C from [$^{13}C_7$]-toluene. In order to identify the toluene-assimilating bacteria, the incorporation of carbon from both [^{13}C]-α-toluene and [$^{13}C_7$]-toluene into rRNA was analyzed by stable isotope probing. Time and buoyant density-resolved 16S rRNA gene-based terminal restriction fragment length polymorphism profiles, combined with cloning and sequencing, revealed that an uncultured bacterium (99% sequence similarity) related to the genus *Desulfocapsa* was the main toluene-degrading organism in the consortium. The ratio of the respective terminal restriction fragments changed over time, indicating trophic interactions within this consortium.

Introduction

Petroleum hydrocarbons are among the most abundant groundwater contaminants, mostly as a result of improper processing and storing techniques. Aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene isomers (BTEX) are not only toxic but also mobile, because these compounds are more volatile and water-soluble than most other petroleum hydrocarbons. Therefore, the elimination of such pollutants is essential for safeguarding of drinking water resources and of groundwater systems. Previous studies have shown that BTEX hydrocarbons are amenable to microbial degradation under oxic as well as under anoxic conditions (Heider *et al.*, 1998; van Agteren *et al.*, 1998; Widdel & Rabus, 2001). However, the implementation of *in situ* biodegradation for remediating contaminated aquifers requires detailed knowledge of relevant microbial community members, including their physiology and ecology.

Although sulfate-reducing bacteria play a key role in the anaerobic mineralization of BTEX compounds, only a few cultures capable of toluene degradation under sulfate-reducing conditions have been isolated until now (Rabus *et al.*, 1993; Beller *et al.*, 1996; Harms *et al.*, 1999; Meckenstock, 1999; Morasch *et al.*, 2004). This fact might be due to the slow growth and/or the complex growth requirements of these bacteria. Traditionally, enrichment cultures are set up using either groundwater (e.g. Kasai *et al.*, 2006) or aquifer material (e.g. Beller *et al.*, 1996; Kleikemper *et al.*, 2002) as

inoculum for laboratory microcosms. In the present study, a specific field-based microcosm system facilitating the enrichment of indigenous groundwater microorganisms on site, as well as the subsequent analysis of their community structure and carbon assimilation patterns have been applied (Peacock *et al.*, 2004; Geyer *et al.*, 2005; Kästner *et al.*, 2006).

We have used these *in situ* microcosms to first enrich a novel sulfate-reducing consortium (ZBT 1) capable of catabolizing toluene in the field, and then functionally and phylogenetically characterize it in a laboratory experiment using ^{13}C-labeled toluene as a tracer. The net assimilation of toluene was quantified via ^{13}C-incorporation into total lipid fatty acids (TLFA). In addition, RNA-based stable isotope probing (SIP) was applied in order to identify the most active toluene-degrading members of the consortium and to shed light on possible trophic interactions within the consortium over time. By DNA or RNA-SIP, several degraders in anaerobic hydrocarbon-degrading bacterial communities have been identified recently (Manefield *et al.*, 2002; Kasai *et al.*, 2006; Whiteley *et al.*, 2006; Kunapuli *et al.*, 2007; Oka *et al.*, 2008; Herrmann *et al.*, 2009). We used [^{13}C]-α-toluene and [$^{13}C_7$]-toluene to elucidate the characteristics of carbon assimilation by the mixed culture and to reveal the extent of labeling required for TLFA- and RNA-SIP experiments. TLFA-SIP, in contrast to DNA- or rRNA-SIP, allows the detection of very small amounts of ^{13}C-label. The phylogenetic information obtainable via nucleic acid-SIP, however, is clearly superior.

Material and methods

Chemicals

Chemicals and solvents were obtained in p.A. quality from Merck unless otherwise stated. [^{13}C]-α-toluene and [$^{13}C_7$]-toluene were obtained from Sigma-Aldrich (St. Louis) with a chemical and isotope purity higher than 99%.

Enrichment of an anoxic toluene-degrading microbial consortium by *in situ* microcosms

The sulfate-reducing consortium named ZBT 1 was obtained from a BTEX-contaminated aquifer located in the area of a former hydrogenation plant close to the city of Zeitz (Saxony-Anhalt, Germany) (Vieth *et al.*, 2005; Schirmer *et al.*, 2006). The enrichment was performed using an *in situ* microcosm system (BACTRAP or BIOTRAP) consisting of Teflon® tubes of 5 cm length and 1 cm diameter with a perforation of 1 mm, which were filled with 0.5 g heated Bio-Sep® beads (kindly provided by Kerry Sublette, University of Tulsa, Tulsa). Both ends of the Teflon® tubes were plugged with glass wool to keep the beads inside the tubes. The beads are 2–3-mm spherical beads engineered from a composite of 75% powdered activated carbon and 25% aramid polymer (Nomex); they have a porosity of 74% and an internal surface area $> 600\,m^2\,g^{-1}$ bead. The outer pores are 1–10 μm wide. A detailed description of the microcosm system including its preparation is given elsewhere (Peacock *et al.*, 2004; Geyer *et al.*, 2005; Kästner *et al.*, 2006). In this study, four *in situ* microcosms were prepared. Following sterilization and hydration of the *in situ* microcosms by autoclaving at 121 °C, two of the microcosms were loaded with 130 μL toluene via the gas phase under reduced pressure as described previously (Geyer *et al.*, 2005). The other two microcosms without substrate loading served as controls. All microcosms were stored in anoxic water until their deployment in the monitoring well. The four *in situ* microcosms were fixed to a steel cable and deployed in the well Zz 53A/03 at a depth of 30 m. In March 2005, the groundwater of well Zz 53A/03 contained 1.05 mM sulfide, 0.088 mM benzene, as well as 0.07 μM toluene. After 70 days of incubation, the microcosms were recovered and immediately transferred to flasks completely filled with anoxic mineral medium as described elsewhere (Vogt *et al.*, 2007). On the same day, the beads of each microcosm were transferred inside an anaerobic glove box (gas atmosphere: 95% nitrogen, 5% hydrogen; Coy Laboratory Products Inc.) into 118-mL serum flasks subsequently filled with 100 mL of anoxic mineral medium (Vogt *et al.*, 2007) and closed gastight by crimped Teflon-coated butyl septa (ESWE Analysentechnik, Gera, Germany). Serum flasks containing the toluene-loaded beads were spiked with 2 μL neat toluene, whereas flasks filled with beads from control microcosms did not receive any substrate. The four flasks were incubated statically at 22 °C in the dark and regularly monitored for sulfide production. After 265 days of incubation, microorganisms colonizing the toluene-loaded beads were detached by gently shaking on a horizontal shaker. The resulting cell suspension was taken for inoculating bead-free mineral salt medium spiked with toluene as the sole carbon and energy source. This culture ('ZBT 1') was used as an inoculum for the SIP experiment.

Setup of the SIP experiment

Sterile 118-mL serum bottles were each filled with 90 mL of anoxic mineral medium and 110 beads (~1 g) that had previously been heated at 300 °C for 4 h in order to remove residual carbon compounds. Two laboratory microcosms were spiked with [^{12}C]-toluene, [^{13}C]-α-toluene or [$^{13}C_7$]-toluene to final concentrations of 4.7 mM, respectively, and immediately closed with Teflon-coated butyl septa. These microcosms were incubated for 24 h before inoculation, in order to allow uniform adsorption of the toluene to the bead material. Subsequently, all microcosms, including one control without toluene, were inoculated with 10 mL of the

enrichment culture ZBT 1 and incubated statically for 60 days at 20 °C in the dark. The microcosms were sampled twice per month for measurement of sulfide concentrations. The toluene concentrations of beads and liquid cultures were measured at the beginning and end of the experiment in order to determine the toluene turnover. For molecular analysis, 25 beads of each microcosm were taken anoxically after 20, 48 and 60 days of incubation and were frozen immediately and stored at −80 °C until analysis. Furthermore, 25 beads of each microcosm were taken after 60 days of incubation for fatty acid analysis. Samples were frozen and stored at −80 °C until analysis. All steps for preparing and sampling were performed in the anaerobic glove box.

Confocal laser scanning microscopy (CLSM)

The beads were first examined without staining in order to exclude autofluorescence. The beads were then carefully divided into halves and placed in a 5-cm Petri dish. After staining with a few drops of the nucleic acid-specific stain SYTO 60 (Molecular Probes, Eugene, OR), the Petri dish was flooded with tap water and immediately subjected to CLSM. The samples were examined with an upright microscope connected to a TCS SP1 (Leica, Heidelberg, Germany). The system was controlled by the LEICA CONFOCAL software, version 2.61 Build 1537174191. Samples were observed with a 63×0.9 NA water-immersible lens. Excitation was at 488 nm (reflection) and 633 nm (SYTO 60). Emission was recorded at 480–500 nm (reflection) and 650–750 nm (SYTO 60). Image series were projected as 3D isosurfaces using IMARIS version 6.0 (Bitplane, Zurich, Switzerland).

Toluene and sulfide analysis

Toluene was extracted from five beads with 1 mL dichloromethane overnight. Ten microliters of the dichloromethane–toluene mixture was transferred to a 20-mL glass vial and subsequently analyzed using a Varian 3800 gas chromatograph (Varian) as published elsewhere (Kleinsteuber *et al.*, 2008). The toluene concentration in the liquid culture was determined as also described by Kleinsteuber *et al.* (2008). Sulfide concentrations were determined spectrometrically as described by Herrmann *et al.* (2008).

Fatty acid and isotope analysis

Total lipids were extracted from beads as described by Geyer *et al.* (2005). The lipids were then transesterified to fatty acid methyl esters (FAMEs) by a mild alkaline methanolysis (Guckert *et al.*, 1985). The completely dried FAME fraction was dissolved in *n*-hexane containing 59 pmol μL^{-1} 21 : 0 FAME as the internal standard. For the identification and quantification of the FAMEs, a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass spectrometer (Agilent, Palo Alto) was used. The carboxylic acid fractions were separated on an HP5-5MSI column (30 m × 0.25 mm × 0.25 μm) by splitless injection of 1-μL samples with the following temperature program: initial temperature of 50 °C for 1 min, heat at 50 °C min^{-1} to 170 °C, heat at 4 °C min^{-1} to 300 °C, then heat at 40 °C min^{-1} to 320 °C and hold for 10 min. The fatty acids are designated in the form of *A*:*B*ω*C*, where *A* is the number of carbon atoms, *B* is the number of double bonds and *C* is the distance of the closest double bond (unsaturation) from the aliphatic end (ω-nomenclature) of the molecule. The prefix 'cy' indicates a cyclopropyl fatty acid. Absolute and relative amounts of TLFA in the samples were determined according to the concentration of the added internal FAME standard and were corrected for the methyl group added during derivatization (Boschker, 2004). Because the isotope composition of the highly enriched ^{13}C-fatty acids could not be analyzed accurately using GC-combustion-isotope ratio MS, MS analysis was used for the determination of isotope enrichment into fatty acids in the following way: the ^{13}C-incorporation into fatty acids causes the appearance of a series of isotopomers besides the natural molecular ion (M^+) (Annweiler *et al.*, 2000; Fang *et al.*, 2004). According to the distribution of these isotopomers, the percentage of labeled carbon (at.%) in the fatty acids can be described by:

$$\text{at.}\% = \Sigma(\text{ratio of } ^{13}\text{C isotopes incorporated}) \times (\text{frequency of respective isotopomer}) \quad (1)$$

The factors of the equation can be calculated by:

$$\text{ratio of } ^{13}\text{C isotopes incorporated} = J/N \quad (2)$$

and

$$\text{frequency of respective isotopomer} = A_{M+J}/A_T \quad (3)$$

where J is the number of ^{13}C isotopes, N is the number of carbon atoms in the TLFA and A_{M+J} is the abundance of the respective isotopomer. A_T is the total abundance of all isotopomers:

$$A_T = \sum_{J=0}^{N} A_{M+J} \quad (4)$$

Hence, the determination of the percentage of ^{13}C in the carbons of the fatty acids can be described by:

$$\text{at.}\% = \sum_{J=1}^{N} \frac{J}{N} \times \frac{A_{M+J}}{A_T} \quad (5)$$

RNA extraction

To ensure RNase-free conditions, all solutions were prepared with pyrocarbonate-treated distilled water, and glassware was baked at 200 °C for at least 4 h. RNA was extracted directly from beads using a protocol modified from Schmitt *et al.* (1990) and Lueders *et al.* (2004). From each sample, 25 beads were divided into four fractions and transferred to 2-mL bead-beating vials filled with 0.2 mL of 1 : 1 mixed 0.1 and 1.0 mm zirconia/silica beads. After crushing the Bio Sep® beads mechanically, 300 μL AE buffer (50 mM Na-acetate pH 5.3, 10 mM Na-EDTA), 200 μL of 200 mM NaH_2PO_4/Na_2HPO_4 buffer pH 5.6, 50 μL 20% sodium dodecyl sulfate solution and 450 μL AE-equilibrated phenol preheated to 65 °C were added to each vial. The suspension was mixed and incubated at 65 °C for 10 min. Subsequently, samples were lysed by bead beating at 6.5 m s^{-1} for 30 s (Fast Prep FP120; Qbiogene, Heidelberg, Germany) and then frozen at −80 °C for approximately 5 min. After briefly thawing, the samples were centrifuged for 5 min at 16 100 ***g*** and 4 °C. Four hundred microliters of the upper aqueous phase was transferred to a Phase Lock Gel Heavy tube (Eppendorf) and placed on ice. The extraction procedure was repeated by adding 200 μL of AE buffer to the crushed bead mixture. The mixture was mixed again and centrifuged for 5 min at 16 100 ***g***. Four hundred microliters of the upper phase was combined with the previously transferred aqueous phase and extracted subsequently with an equal volume of AE-equilibrated phenol/chloroform, followed by centrifugation at 16 100 ***g*** for 5 min. The aqueous phase was then transferred to a fresh microcentrifuge tube and RNA was precipitated by addition of 2 vol. PEG solution (30% w/v polyethylene glycol 6000 solved in 1.6 M NaCl), followed by centrifugation at 16 100 ***g*** for 30 min. After washing with 80% EtOH, the pellet was dried and resuspended in 40 μL RNase-free water. The aliquots were combined, followed by the digestion of coextracted DNA with DNA-freeTM Kit (Ambion) according to the manufacturer's instructions.

Isopycnic centrifugation

RNA was separated by equilibrium density gradient centrifugation and gradient fractionation using a modified protocol of Lueders *et al.* (2004). Seven hundred and fifty nanograms of RNA was loaded into a CsTFA gradient medium consisting of 4.65 mL CsTFA, up to 850 μL gradient buffer and 185 μL formamide. The gradients were centrifuged in an ultracentrifuge (OPTIMA™L-90 K) using a Near-Vertical Rotor (NVT 65.2, both Beckman Coulter) for 68 h at 39 000 r.p.m. (130 000 ***g***) and 20 °C. Twelve fractions were harvested from each gradient and further treated as described previously (Lueders *et al.*, 2004; Whiteley *et al.*, 2007).

16S rRNA gene reverse transcription, PCR and terminal restriction fragment length polymorphism (T-RFLP) analysis

Eleven microliters of RNA sample was reverse-transcribed using the RevertAidTM H Minus cDNA Synthesis Kit (Fermentas) by random priming with hexamers. For T-RFLP analysis, the cDNA was amplified with the 6′ carboxyfluorescin-labeled primer 27f and with primer 907r (Lane, 1991). Each 25-μL reaction (Taq PCR master mix, Qiagen) was performed with the following cycling conditions: 3 min of denaturation at 94 °C, 30 cycles of 45 s at 94 °C, 30 s at 52 °C, 1 min at 72 °C and a final extension for 10 min at 72 °C. The PCR products were purified using Wizard purification columns (Promega) and quantified by gel electrophoresis. Ten nanograms of the purified PCR product was digested with 10 U AluI (BioLabs, New England) in a total volume of 10 μL at 37 °C overnight. Digestion was stopped by heating samples at 65 °C for 20 min, followed by precipitation with 25 μL EtOH and 1 μL Na-acetate, pH 4.8, per sample. After purification of digests with 70% EtOH, the pellet was dried and mixed with 20 μL HiDi and 0.4 μL GeneScan-500 ROX Size Standard (Applied Biosystems). Each sample was then denaturated at 95 °C for 10 min and immediately transferred to ice, followed by loading on an ABI3100 genetic analyzer (Applied Biosystems). T-RFLP profiles were analyzed using GENEMAPPER software version 3.7 (Applied Biosystems); T-RFs smaller than 50 bp were excluded from further analysis.

Cloning and sequence analysis

A clone library was generated from the enrichment culture ZBT 1 using the primers 27f and 907r as described above. After purification of PCR products using the Wizard® SV Gel and PCR Clean-Up System (Promega), products were cloned into pCR 2.1 Vector (TA Cloning® Kit, Invitrogen). Inserts of 96 randomly selected clones were reamplified using vector-specific primers and purified PCR products were digested with AluI. After grouping of clones according to their restriction fragment length polymorphism, representative clones were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). For further phylogenetic analysis, we added the partial sequences obtained in this study to an alignment available in the ARB program package. New sequences were aligned using the aligning tool of this package (http://www.arb-home.de; Ludwig *et al.*, 2004). The alignment was based on primary and secondary structures, which improved the recognition of homologous positions. Where necessary, we included reference sequences not present in the ARB database from GenBank. The sequences were added to the ARB tree using the Quick Add Parsimony tool and

applying a specific filter as implemented in the same package. The alignment was verified by automatic alignment to the next relative sequences and corrected manually. Sequence data were deposited in GenBank under the accession numbers GQ121141–GQ121158.

Results and discussion

Enrichment of a toluene-degrading microbial consortium by *in situ* microcosms

In situ microcosms loaded with ^{12}C-toluene were exposed in the sulfidic zone of a BTEX-contaminated aquifer for 70 days in order to trap and enrich intrinsic toluene-degrading microorganisms. After retrieval of the microcosms from the well, the consortium attached to the beads was cultivated in laboratory microcosms. The sulfide concentration in this primary culture increased from 0.06 to 6.4 mM during 197 days of incubation, but showed no increase in the OD, indicating that toluene was consumed by microorganisms attached to the beads. Control microcosms without added toluene showed constant sulfide concentrations (~1 mM) over time. To further enrich the sulfate-reducing toluene degraders from the consortium, microorganisms were gently removed from the beads and transferred to a fresh mineral medium spiked with toluene (100 μM). Toluene was degraded to more than 90% within 32 days coupled to sulfide production. Aliquots of this enrichment culture, which was named 'ZBT 1,' were used for the following SIP experiment.

Characterization of the toluene-degrading consortium ZBT 1

Serum flasks filled with anoxic mineral medium and 110 beads were amended with [^{12}C]-toluene, [^{13}C]-α-toluene or [$^{13}C_7$]-toluene and subsequently inoculated with the enrichment culture ZBT 1. During the incubation period of 60 days, all toluene-spiked laboratory microcosms produced 2.5–2.7 mM sulfide, in contrast to the toluene-free control, in which the initial sulfide concentrations slowly decreased to below the detection limit. After 60 days, between 1.7 mM toluene ([^{12}C]-toluene, [$^{13}C_7$]-toluene) and 2.8 mM toluene ([^{13}C]-α-toluene) had been consumed on the beads, whereas the total toluene content in the liquid phase (0.05 ± 0.01 mM) had been consumed. A clear calculation of the stoichiometric relationship between the toluene consumed and the sulfide produced was not possible, because a part of the sulfide produced adsorbed to the bead material, as demonstrated by the decrease of sulfide concentrations during the first 15 days of incubation and the disappearance of sulfide in the toluene-free control (Supporting Information, Fig. S1). To examine the colonization of the beads by microorganisms, five beads were taken from each laboratory microcosm after 60 days of incubation and subjected to CLSM. All beads were densely colonized, mainly by microcolonies composed of rod-like bacteria (Fig. S2).

Microbial incorporation of toluene-derived carbon into TLFA

The biomass on the beads was quantified by fatty acid analysis, a method that also allowed the amount of ^{13}C incorporated from toluene to be determined. Total lipids extracted from the beads as well as liquid microcosms samples showed highly similar patterns, indicating that the inoculated microbial community did not split up into distinct surface-associated and planktonic subcommunities within the experiment, which has been described, for example for groundwater communities (Griebler & Lueders, 2008). However, it has to be considered that lipids provide only a low taxonomic resolution so that some differences may not be visible. Thus, only the results of the total lipids extracted from beads are shown and discussed here (Fig. 1). The total amount of fatty acids ranged from 5.6 nmol g^{-1} bead ([^{12}C]-toluene) to 8.4 nmol g^{-1} bead ([$^{13}C_7$]-toluene) ($n = 2$). The most abundant fatty acids detected were 16:0 and cyc17:0, a fatty acid that is typically found in sulfate-reducing bacteria (Kaur *et al.*, 2005). Smaller amounts of 16:1ω7, 18:1ω9 and 18:0 fatty acids were also detected. The total amounts of fatty acids retrieved from [^{13}C]-α- and [$^{13}C_7$]-toluene grown cultures were higher than those from [^{12}C]-cultures. This result is contrary to a recent study of Fang *et al.* (2004), who observed that four out of five tested aerobic toluene-metabolizing bacterial strains showed higher growth yields with nonlabeled toluene compared with [^{13}C-ring]-toluene. This different metabolic behavior regarding labeled or nonlabeled toluene species might be due to distinct physiological differences between the investigated bacteria. Our results imply, however, that the *in situ* microcosm system based on ^{13}C-loaded beads is not significantly influenced by artifacts caused by toxic effects of the labeled compound used.

In order to trace the flow of toluene-derived carbon into bacterial biomass, the ^{13}C-incorporation into TLFA was determined. After 60 days, all fatty acids extracted from beads or from liquid culture samples revealed significant labeling, except for the 18:0 fatty acid, which remained almost unlabeled (Fig. 1). Growth on [^{13}C]-α-toluene resulted in a ^{13}C-incorporation into TLFA between 8 and 15 at.%, while maximal labeling with [$^{13}C_7$]-toluene was between 51 and 57 at.%. Thus, [$^{13}C_7$]-toluene-grown microorganisms were labeled between three and six times higher than those grown on [^{13}C]-α-toluene. Interestingly, this indicates a lower ratio of incorporation of ^{13}C from [$^{13}C_7$]-toluene, because it is sevenfold more labeled than

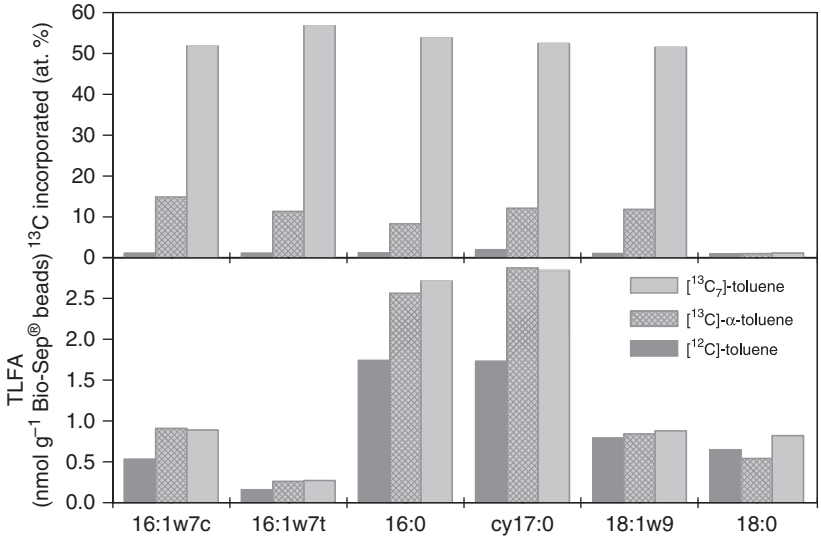

Fig. 1. Concentrations of each TLFA and ^{13}C incorporation into the TLFA as extracted from Bio-Sep® beads after 60 days of incubation with [^{12}C]-toluene, [^{13}C]-α-toluene or [$^{13}C_7$]-toluene, respectively. The data are means of duplicate microcosms. All SD were $< 20\%$ for the several fatty acids and $< 8\%$ for the isotope signatures.

[^{13}C]-α-toluene. The result also shows that the methyl moiety of toluene was assimilated in significant amounts by the degraders. The first step of the toluene degradation pathway under anoxic conditions is an addition of fumarate to the methyl moiety of toluene, catalyzed by the enzyme benzylsuccinate synthase (for a review, see Heider, 2007). The benzylsuccinate formed is further transformed into benzoyl-coenzyme A (benzoyl-CoA), bearing the former carbon of the methyl group in the thioester-linked carboxyl group. This functional group can end up in acetyl-CoA, which is a precursor for fatty acid biosynthesis in general. This may explain why the ratio of label incorporation from [^{13}C]-α-toluene appears significantly in the fatty acid pattern, in spite of the fact that the majority of formed acetyl-CoA is considered to be label-free.

Characterization and dynamics of toluene-assimilating bacteria

rRNA-SIP was performed in order to specifically identify the key degraders on the beads. Total RNA was extracted from beads after 20, 48 and 60 days of incubation. The RNA extracted from microcosms with nonlabeled toluene served as control to reconfirm the average buoyant density (BD) of $\sim$1.78 g mL^{-1} for unlabeled rRNA in CsTFA (Lueders *et al.*, 2004). A clear density shift of labeled vs. unlabeled rRNA was observed especially after 60 days. rRNA extracted from the [^{13}C]-α-toluene samples showed an average BD shift of $\sim$0.01 g mL^{-1}, while that of [$^{13}C_7$]-toluene samples was $\sim$0.02 g mL^{-1} (Fig. S3). Thus, even for the [^{13}C]-α-toluene incubations, labeled RNA could be successfully detected in equilibrium density centrifugation. Notably, the entire RNA extracted from [^{13}C]-α-toluene or [$^{13}C_7$]-toluene samples was heavier in comparison with the unlabeled RNA, indicating that all microorganisms within the consortium were incorporating carbon from the added toluene. However, secondary labeling effects via consumption of metabolic products or labeled cell material also cannot be excluded (Radajewski *et al.*, 2002), even though our experiment was performed in a time-resolved manner. The T-RFLP profiles performed from every RNA gradient showed no significant differences between the respective 'heavy' and 'light' rRNA fractions for each time point sampled. This supports our hypothesis that more or less all microorganisms within the consortium received carbon from toluene. However, microbial community structure showed an increasing diversity of T-RFs with time, indicating a microbial succession on the beads between 20 and 60 days of incubation (Fig. 2). After 20 days, a 151-bp T-RF clearly dominated the community on the beads. Over time, the 216- and 220-bp fragments became more abundant, but also other T-RFs (157, 234, 252, 295 and 438 bp) appeared in increased ratios after 60 days. RNA extraction after incubation times shorter than 20 days might have enabled to identify primary toluene-degraders more specifically. Here, however, we chose not to sacrifice any culture bottles at such an early stage of incubation due to the slow growth and the resulting low amounts of RNA within the cultures. Also, as repeatedly observed for rRNA-SIP in strictly anaerobic systems (Lueders *et al.*, 2006; Hatamoto *et al.*, 2007; Kovatcheva-Datchary *et al.*, 2009), we expected large amounts of intermediately labeled rRNA rather then small amounts of highly labeled rRNA; therefore, the use of labeled 'carrier' nucleic acids in ultracentrifugation (Gallagher *et al.*, 2005) would also not have improved label detection.

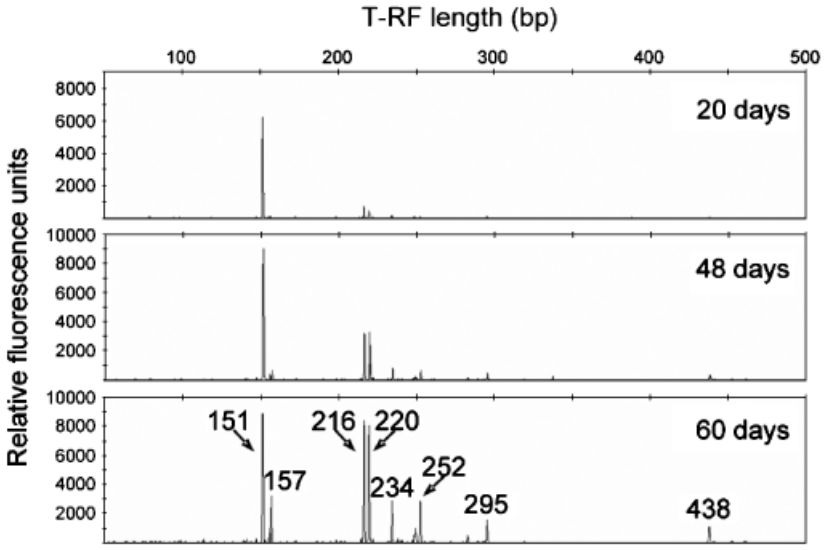

Fig. 2. Dynamics of community structure formed on Bio-Sep® beads during the incubation of the enrichment culture ZBT 1. 16S rRNA gene-based T-RFLP fingerprints of representative RNA gradient fractions generated from laboratory microcosms containing [$^{13}C_7$]-toluene after 20, 48 and 60 days of incubation are shown. Note that the 151-bp T-RF is out of the detection limit of 10 000 fluorescence units at day 48 and 60. T-RFLP profiles of microcosms containing [^{12}C]-toluene and [^{13}C]-α-toluene showed similar compositions (data not shown).

Reverse transcriptase-PCR and cloning of bacterial rRNA gene amplicons were performed to identify the key toluene degraders and secondary successors represented by the respective T-RFs. The majority of clones (89%) were closely related to a sulfate-reducing toluene-degrading isolate related to the genus *Desulfocapsa* described by Meckenstock (1999) (Fig. 3), and to a phylotype identified in a hydrocarbon- and chlorinated-solvent-contaminated aquifer (Dojka *et al.*, 1998). Phylotypes related to the genus *Desulfocapsa* were also recently found to be abundant in the sulfidic zone of a tar-oil-contaminated aquifer (Winderl *et al.*, 2008), providing hints to the important role of these organisms for aromatic hydrocarbon degradation under sulfate-reducing conditions in groundwater habitats. The dominance of this sequence in our library suggests that a *Desulfocapsa*-related phylotype is mainly responsible for toluene metabolism coupled to sulfate reduction in our consortium. This assumption is supported by the dominance of the respective T-RF (151 bp; Table S1), especially at the early time points (Fig. 2).

Some of the remaining clone sequences belonged to the genus *Desulfobacterium*, a genus that is known to comprise toluene-degraders (Harms *et al.*, 1999). Other sequences were related to the genus *Desulfovibrio* (Table S1). A recent study detected members of the genus *Desulfovibrio* in a toluene-degrading sulfate-reducing consortium, but no evidence of toluene degradation by these members was provided (Pelz *et al.*, 2001). Anaerobic toluene degraders occur in distinct phylogenetic lineages (Widdel & Rabus, 2001; Winderl *et al.*, 2007) and also the genes for the respective catabolic key-enzyme, the benzylsuccinate synthase (*bssA*), was shown to occur in a wide phylogenetic diversity in contaminated environments (Winderl *et al.*, 2007). Hence, we cannot rule out that those phylotypes found in low numbers in the clone library are able to catabolize toluene. This assumption is supported by the high abundance of the 216- and 220-bp T-RF, which represent clone sequences affiliated to the genus *Desulfobacterium* and unclassified *Deltaproteobacteria*. Those 216- and 220-bp T-RF observed in the profiles after a prolonged incubation time (especially after 60 days incubation), were, however, under-represented in the clone library, indicating cloning bias and general difficulties in obtaining reliable quantitative information from the PCR-based approach. Determining the presence and diversity of *bssA* or transcripts in the microcosms at different time points might have allowed elucidating how many different organisms in the consortium were actually involved in the initial attack on toluene. Nevertheless, we hypothesize that at least some of the less abundant T-RFs appearing later during the course of incubation do not attack toluene directly, but obtain carbon from metabolites, or even dead biomass of the key toluene degraders (Vogt *et al.*, 2005).

In summary, by combining *in situ* microcosms and SIP techniques, we were able to enrich and identify a novel sulfate-reducing consortium capable of metabolizing toluene. Because many other pollutants, for example benzene or MTBE compounds, can be incorporated into the beads (Kästner *et al.*, 2006), this approach is widely applicable to study microbial communities involved in the degradation of groundwater pollutants.

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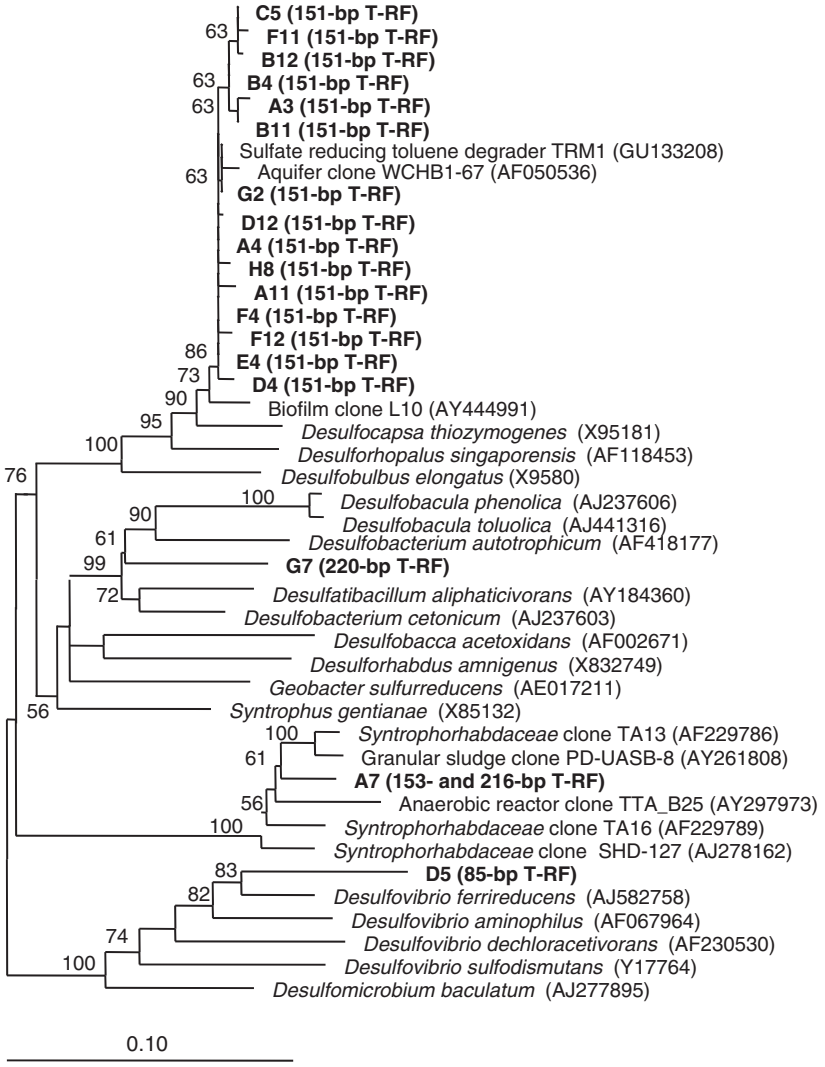

Fig. 3. Phylogenetic affiliation of the most abundant 16S rRNA gene clone sequences generated from enrichment culture ZBT 1. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences are given in parentheses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Sulfide production over time in individual laboratory microcosms established from the culture ZBT 1.
Fig. S2. CLSM images of microorganisms attached to Bio-Sep® beads after 60 days of microcosm incubation.
Fig. S3. Profiles of RNA-SIP gradients performed from individual laboratory microcosms after (a) 20 days, (b) 48 days and (c) 60 days of incubation with [^{12}C]-toluene, [^{13}C]-α-toluene or [$^{13}C_7$]-toluene.
Table S1. Number of 16S rRNA gene clones, phylogenetic affiliation and characteristic T-RFs of clones retrieved from the clone library generated from enrichment culture ZBT 1.

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