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ORIGINAL ARTICLE

DNA-SIP identifies sulfate-reducing *Clostridia* as important toluene degraders in tar-oil-contaminated aquifer sediment

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Global groundwater resources are constantly challenged by a multitude of contaminants such as aromatic hydrocarbons. Especially in anaerobic habitats, a large diversity of unrecognized microbial populations may be responsible for their degradation. Still, our present understanding of the respective microbiota and their ecophysiology is almost exclusively based on a small number of cultured organisms, mostly within the *Proteobacteria*. Here, by DNA-based stable isotope probing (SIP), we directly identified the most active sulfate-reducing toluene degraders in a diverse sedimentary microbial community originating from a tar-oil-contaminated aquifer at a former coal gasification plant. On incubation of fresh sediments with $^{13}C_7$-toluene, the production of both sulfide and $^{13}CO_2$ was clearly coupled to the ^{13}C-labeling of DNA of microbes related to *Desulfosporosinus* spp. within the *Peptococcaceae* (*Clostridia*). The screening of labeled DNA fractions also suggested a novel benzylsuccinate synthase alpha-subunit (*bssA*) sequence type previously only detected in the environment to be tentatively affiliated with these degraders. However, carbon flow from the contaminant into degrader DNA was only ~50%, pointing toward high ratios of heterotrophic CO_2-fixation during assimilation of acetyl-CoA originating from the contaminant by these degraders. These findings demonstrate that the importance of non-proteobacterial populations in anaerobic aromatics degradation, as well as their specific ecophysiology in the subsurface may still be largely ungrasped.

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Introduction

The identification of key microbial populations and the understanding of their ecophysiology with respect to specific functions in the environment is a major objective of modern day microbial ecology. Especially for hydrocarbon-contaminated groundwater systems, our present understanding of the diversity and ecology of relevant degraders is still in its infancy. At a former coal gasification plant in Germany with an underlying tar-oil-contaminated aquifer, the anaerobic degradation of benzene, toluene, ethylbenzene and xylenes (BTEX) has been reported to occur mostly under conditions of sulfate reduction (Griebler *et al.*, 2004). One of the few available Gram-positive BTEX-degrading sulfate reducers, *Desulfotomaculum* sp. strain Ox39 has been isolated from this site (Morasch *et al.*, 2004). However, it remains to be verified whether an importance of such non-proteobacterial populations for on-site aromatics degradation can be substantiated using cultivation-independent tools.

The potential of *in situ* microbiota to degrade a given substrate can be demonstrated, for example, by the detection of genes encoding key catabolic enzymes (Galvao *et al.*, 2005; Andreoni and Gianfreda, 2007). Accordingly, microbes potentially involved in on-site toluene degradation can be detected using the genes of benzylsuccinate synthase (Bss), the key enzyme of anaerobic toluene degradation (Boll *et al.*, 2002). At the investigated former coal gasification site, we have previously detected unidentified and deeply branching environmental homologs of benzylsuccinate synthase alpha-subunit (*bssA*), only distantly related to the known proteobacterial *bssA* sequences (Winderl *et al.*, 2007). However, mere PCR detection of *bssA* or related catabolic genes *in situ* did not allow identifying the microbes carrying these genes, and it also remained unanswered under which conditions they are active.

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In recent years, especially the use of stable isotopes in combination with molecular tools has enabled researchers to identify such key anaerobic contaminant degraders, not only in enrichments stemming from aquifers (Kasai *et al.*, 2006; Kunapuli *et al.*, 2007; Bombach *et al.*, 2010; Herrmann *et al.*, 2010), but also from a wide variety of other sediments and soils (Gallagher *et al.*, 2005; Kittelmann and Friedrich, 2008; Liou *et al.*, 2008; Oka *et al.*, 2008). Stable isotope probing (SIP) of nucleic acids allows for the specific identification of microorganisms catabolizing and assimilating carbon from a particular ^{13}C-labeled substrate, and if performed for DNA, also to identify and affiliate involved catabolic genes on labeled genomes (Jeon *et al.*, 2003; Leigh *et al.*, 2007).

The aim of this study was to identify, by means of DNA-SIP, the key microbes in toluene degradation under sulfate reduction in aquifer samples of the investigated former coal gasification plant. We wanted to identify involved *bssA* genes, and to elucidate whether an active role can be verified for unidentified *bssA* homologs previously detected at the site (Winderl *et al.*, 2007). Therefore, we exposed freshly sampled aquifer sediments containing a complex *in situ* microbial community to fully labeled $^{13}C_7$-toluene under close-to-*in situ* conditions. The key microbes in sulfate-dependent toluene degradation identified in these samples did not belong to the mostly deltaproteobacterial lineages described to date in this respect (Widdel *et al.*, 2006), but were affiliated to the *Peptococcaceae* (*Clostridia*). These findings provide novel evidence that the diversity of anaerobic BTEX degraders in the environment may still be largely underestimated.

Materials and methods

Sampling site and sample acquisition

Sampling was carried out at a former gasworks site (Testfeld Süd), which has been intensively studied and described elsewhere (Herfort *et al.*, 1998; Bockelmann *et al.*, 2001; Zamfirescu and Grathwohl, 2001; Griebler *et al.*, 2004). Aquifer sediment from the bottom of monitoring well B49 (at ~7.50 m depth, groundwater table at ~3.25 m) was retrieved in December 2006 with an aqua-sampler (Bürkle, Bad Bellingen, Germany). The sampling well was identical to the one previously screened for intrinsic *bssA* gene diversity (Winderl *et al.*, 2007). Sampled sediments and groundwater were immediately filled into sterile glass bottles without gaseous headspace to minimize oxygen exposure and transported to the laboratory under cooling.

Incubation of sediments

Replicates of ~8 g (wet weight) of freshly sampled sediment material were anoxically incubated in sterile 120-ml serum bottles containing 50 ml of low salt artificial groundwater medium under a headspace of N_2/CO_2 (80/20). The medium contained all components of the freshwater mineral medium described by Widdel and Bak (1992) in a 1:10 dilution, except for the bicarbonate buffer, which was added to 30 mM final concentration. The artificial groundwater medium was adjusted to pH 7. In addition, 1 mM Na_2S was used as reducing agent, 10 mM Na_2SO_4 was added as electron acceptor and 5 μM cAMP was added to stimulate activity (Bruns *et al.*, 2002). To warrant constantly low *in situ* concentrations of toluene during SIP incubation, 0.3 g of Amberlite XAD7 adsorber resin (Sigma-Aldrich, Munich, Germany) was added to each bottle (Morasch *et al.*, 2001). A 5 μl of either non-labeled (^{12}C) or fully ($^{13}C_7$) labeled toluene (Sigma-Aldrich) were injected through butyl rubber stoppers with a gastight syringe and allowed to adsorb to the carrier for 2 days. This resulted in a reservoir of ~0.96 mM toluene in each bottle. However, actual concentrations were maximally ~0.1 times as high due to sorption to XAD7. After sediment addition, the bottles were gassed with N_2/CO_2 (80:20 v/v), sealed anoxically with butyl stoppers and incubated statically for over 133 days at 16 °C in the dark. A total of 15 replicate bottles were prepared for each series (^{12}C and ^{13}C) for replicate biogeochemical measurements and successive time-dependent termination. Sediment-unamended bottles were also run as controls.

Process measurements

Liquid and gaseous samples were taken with syringes through the stoppers for monitoring toluene degradation on a weekly basis. Aqueous toluene concentrations were determined by headspace analysis of 1 ml subsamples on a Trace DSQ GC-MS (Thermo Electron, Dreieich, Germany) on a DB5 capillary column (J&W Scientific, Folson, CA, USA) as previously described (Anneser *et al.*, 2008). Oven temperature was 40 °C for 1 min, then ramped at a rate of 16 °C min^{-1} to 145 °C, then at a rate of 45 °C min^{-1} to 300 °C, and held for 1 min. The mass spectrometer (MS) was operated at 350 °C in the SIM scan mode for the masses 91, 92 (^{12}C-toluene), 98, 99 (^{13}C-toluene), and 96, 150 (fluorobenzene and 1, 4-dichlorobenzene-D4 of the EPA 524 internal standard mix, Dr. Ehrenstorfer GmbH, Augsburg, Germany). Sulfide concentrations in 200 μl liquid samples were monitored spectrophotometrically as described by Cline (1969) using a Cary 50 Bio UV-Vis photometer (Varian, Darmstadt, Germany) at a wavelength of 670 nm.

The formation of ^{13}C-labeled CO_2 was followed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Samples of 15 μl volume were taken from the headspace of each bottle and stable carbon isotope ratios of CO_2 were determined with the following setup: a Trace GC Ultra coupled to a Finnigan GC combustion III interface and a

Finnigan MAT 253 IRMS (all from Thermo Electron) through open split. The GC was equipped with a DB-5 column (0.25-μm film thickness, 0.25 mm in diameter, 30 m length; Agilent, Böblingen, Germany). Helium grade 5 was used as carrier gas with a constant flow rate of 1.4 ml min^{-1}. Injector split flow was set to 14 ml min^{-1}, that is, a split ratio of 1:10 and the temperature was held isothermally at 180 °C. The GC oven temperature was programmed as follows: 100 °C for 2.5 min, then ramped at 45 °C min^{-1} to 150 °C and held for 1.4 min). Carbon isotope signatures of CO_2 are reported in atom per cent (AT%) and were calibrated relative to Vienna PeeDee Belemnite as previously described (Kunapuli *et al.*, 2007). Calculations were carried out assuming that (1) the $^{12}CO_2$ pool was more or less constant, and (2) C-isotope fractionation between inorganic carbon in gaseous phase and liquid phase within the microcosms was negligible due to the strong ^{13}C-label. $^{13}CO_2$ values were measured within each microcosm at day 1 and were taken as t_0 for further measurements.

Nucleic acid extraction and ultracentrifugation
At selected time points, a pair of bottles (^{12}C and ^{13}C-toluene) was discarded. Sediment and biomass was collected by centrifugation at 4000 r.p.m. (3345 *g*) at 4 °C for 10 min with a Megafuge 1.0 R (Heraeus Instruments, Hanau, Germany). Pellets were frozen immediately at −20 °C until nucleic acid extraction. Total nucleic acids were extracted from ~2 ml of incubated sediment samples following a previously described protocol (Winderl *et al.*, 2007), but with a modified PTN extraction buffer (120 mM Na_2HPO_4, 125 mM Tris, 25 mM NaCl [pH 8]). In all, 5 μg of PicoGreen- (Invitrogen, Karlsruhe, Germany) quantified DNA extracts were loaded into a gradient medium of CsCl (Calbiochem, Merck, Darmstadt, Germany) of an average density of ~1.71 g ml^{-1} dissolved in gradient buffer (0.1 M Tris–HCl, pH 8; 0.1 M KCl; 1 mM EDTA) (Lueders *et al.*, 2004). Centrifugation was carried out in 5-ml polyallomer quick seal tubes in a VTI 65.2 vertical rotor (both from Beckman Coulter, Krefeld, Germany) using a Centrikon T-2190 ultra centrifuge (Kontron Instruments, Munich, Germany). Centrifugation runs were at 44 500 r.p.m. (180 000 *g*) at 20 °C over 65 h. In all, 13 fractions of each gradient were collected from 'heavy' to 'light' using a Perfusor V syringe pump (B. Braun, Melsungen, Germany). Refractometric measurement of fraction buoyant density (BD) and the recovery of DNA from gradient fractions were also carried out as described (Lueders *et al.*, 2004).

qPCR and T-RFLP fingerprinting
DNA collected from gradient fractions was quantified by bacterial 16S rRNA gene quantitative PCR (qPCR) in the presence of 0.1× SYBR Green as described in Kunapuli *et al.* (2007). From each gradient, 10 DNA fractions were selected for bacterial 16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. Fluorescent-labeled amplicons were generated as detailed elsewhere (Winderl *et al.*, 2008). Electrophoresis of *Msp*I-restricted fragments was carried out on an ABI 3730 DNA Analyzer (Applied Biosystems, Darmstadt, Germany), and data evaluation with the Gene Mapper 5.1 software, all as previously described (Lueders *et al.*, 2006).

In addition, non-density-resolved total DNA extracts from the inoculum and from the SIP microcosms were subjected to bacterial 16S rRNA and *bssA* gene fingerprinting. The latter was carried out with the 7772f/8546r-FAM primer pair (Winderl *et al.*, 2007), an annealing temperature of 52 °C and *Taq*I restriction of amplicons. Electrophoresis and data analysis were the same as for 16S fingerprints.

Cloning, sequencing and phylogenetic analysis
Amplicons generated with the primer sets Ba27f (Weisburg *et al.*, 1991)/907r (Muyzer *et al.*, 1995) (same as for T-RFLP) and 7772f/8546r (Winderl *et al.*, 2007) were cloned and sequenced (Winderl *et al.*, 2007, 2008) from selected gradient fractions (one 'light' and one 'heavy' 16S rRNA gene library, as well as one *bssA* gene library from a the same 'heavy' fraction). Selected terminal restriction fragments (T-RFs) predicted *in silico* for representative clones were verified *in vitro* (see Table 1). Chimeric clone sequences were identified using CHIMERA_CHECK 2.7 of RDP-II version 8.1 (http://rdp8.cme.msu.edu/html/) and by manual inspection of the alignment. From a total of 87 bacterial 16S rRNA gene clones, 3 were identified as chimeric and excluded from further analysis. Phylogenetic trees were reconstructed from sequence data as described (Winderl *et al.*, 2007, 2008). All sequences generated in this study have been deposited with GenBank and can be found under the accession numbers GU133208–GU133309.

Results

Exposure of aquifer sediments to ^{13}C-toluene
Freshly sampled aquifer sediments were incubated with $^{13}C_7$-toluene under close-to-*in situ* conditions (16 °C, < ~100 μM free toluene concentration, low-ionic strength groundwater medium). Within the first 57 days of incubation, concentrations of $^{13}CO_2$ or sulfide did not significantly increase (Figure 1), remaining at 1.1 AT% and ~0.5 mM, respectively, as initially contained in the groundwater medium. In between days 50 and 100 of incubation, the ^{13}C-ratio of CO_2 increased to ~10 AT%, concurring with pronounced sulfide production (up to 2.7 mM). Toluene was present in the system with a nominal total aqueous concentration of 960 μM. Yet, because of the XAD7 carrier resin, free aqueous concentrations varied between ~50 and ~100 μM during the

first 50 days of incubation, and it was not possible to quantitatively monitor toluene decrease during the incubation. However, it was possible to detect the final depletion of toluene below GC-MS detection limits (~0.3 μM) after day 121, at a time when sulfide production also ceased (Figure 1).

A similar time course of sulfide production was observed in the ^{12}C-toluene control incubations, whereas no depletion of toluene or increase of sulfide was observed in sediment-unamended controls (data not shown). Although BTEX measurements indicated a complete degradation of toluene with time, the ~10 AT% of $^{13}CO_2$ measured toward the end of degradation reached only ~72% of the maximally ~14 AT% expected, given that all carbon from the added toluene was oxidized to CO_2. In addition, given that the oxidation of 1 mmol of toluene yields 36 mmol of electrons, which can reduce 4.5 mmol of sulfate to sulfide, only ~50% of the electrons from the added toluene were recovered in sulfide.

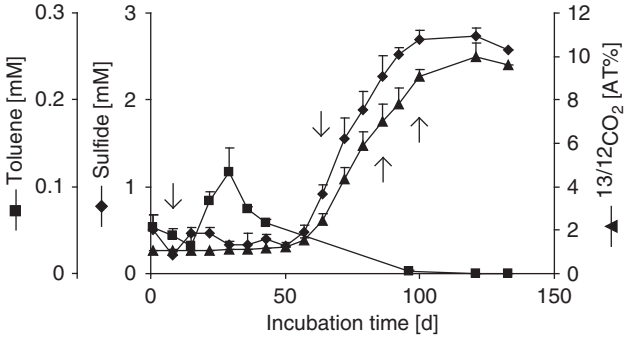

Figure 1 Production of sulfide and $^{13}CO_2$ during degradation of $^{13}C_7$-toluene in SIP microcosms. $^{13}C/^{12}C$ isotopic composition of CO_2 is indicated in atom per cent (AT%). Means of triplicate measurements are given + s.d. Arrows indicate successive time points selected for DNA-SIP analysis.

DNA gradient ultracentrifugation

On the basis of the process measurements, several successive time points of the experiment were selected for DNA extraction (Figure 1). These were just after the beginning of the experiment, at the apparent onset, midpoint and at the end of toluene-degradation activity (days 8, 64, 86, 100, respectively). Gradient centrifugation of all respective DNA extracts from ^{12}C- and ^{13}C-incubations was conducted. qPCR analysis of fractionated gradients detected maximal DNA banding in the 'light' fractions (at BDs between 1.68 and 1.69 g ml^{-1}) for all gradients. The quantitative distribution of bacterial DNA in gradients of two chosen time points (days 8 and 86) is shown in Figure 2. However, also for the 'heavy' fractions (that is, BDs >1.73 g ml^{-1}) of late ^{13}C-toluene gradients, qPCR did not detect substantially increased amounts of bacterial DNA. Instead, the appearance of a tailing of DNA into the partially labeled, 'intermediate' gradient fraction (1.70–1.71 g ml^{-1}) of ^{13}C-toluene gradients was observed (Figure 2b).

Unlike qPCR, bacterial 16S rRNA gene-targeted T-RFLP fingerprinting revealed clear labeling effects when comparing ^{12}C- and ^{13}C-toluene gradients over

Table 1 Composition of bacterial 16S rRNA gene clone libraries from ^{13}C-labeled (LA07Ba clones) and unlabeled fractions (LA10Ba clones) of DNA after 86 days of ^{13}C-toluene consumption

	Number of clones[a]		*T-RF (bp)*[b]	
Phylogenetic affiliation	*^{13}C-labeled 1.707*[c]	*Unlabeled 1.683*[c]	*Predicted*	*Measured*
Alphaproteobacteria	**4**	**1**	—	NA
Betaproteobacteria	**1**	**4**	—	NA
Thauera related		3	490	490
Gammaproteobacteria	**1**	**1**	490	490
Deltaproteobacteria	**6**	**6**	—	NA
Uncultured *Desulfobulbaceae*	3	1	162	159
Uncultured *Desulfobacteriaceae*	2	4	166, 513	163, 511
Epsilonproteobacteria	—	**2**	468	469
Mollicutes	—	**2**	—	NA
Clostridia	**11**	**2**	—	NA
Desulfosporosinus related	10	—	148, 179	146, 178
Actinobacteria	**7**	**3**	—	NA
Chloroflexi	**5**	**12**	—	NA
Uncultured I	2	2	372, 373	373
Uncultured II	3	7	518, 519	520
Uncultured III	—	3	579	579
OP5[d]	**4**	—	—	NA
Other diverse uncultured	**6**	**4**	—	NA

Abbreviations: NA, not applicable; T-RF, terminal restriction fragment.
[a]Class-level clone counts (given in bold) include genus- or lineage-specific clone numbers (nonbold).
[b]Characteristic T-RFs (bp) predicted for all or a major fraction of clones of given affiliation are indicated together with T-RFs actually measured in fingerprints. Values separated by a comma indicate more than one characteristic T-RF for a lineage.
[c]The CsCl buoyant density (g ml^{-1}) for the respective gradient fractions is given.
[d]Phyla without cultivated representatives are named in accordance with Rappé and Giovannoni (2003).

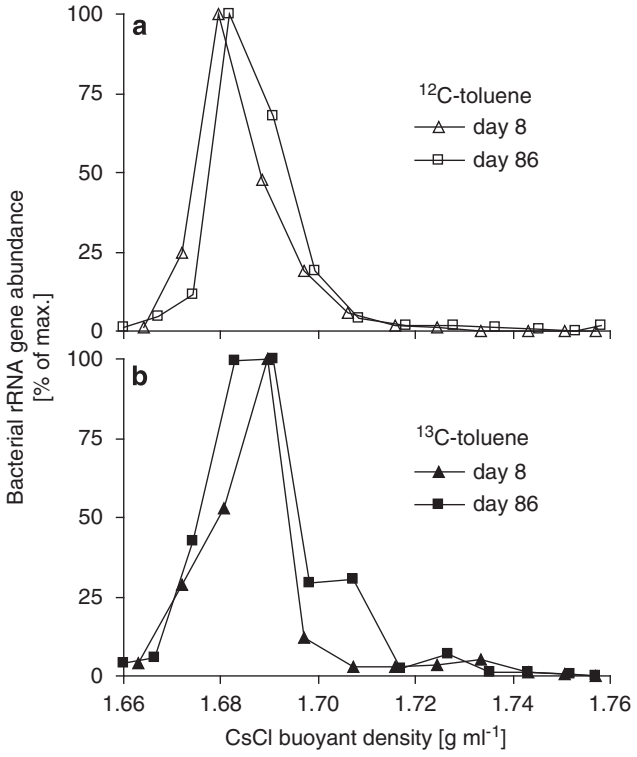

Figure 2 Quantitative profiles of DNA distribution in SIP gradients after 8 and 86 days of incubation with either unlabeled (**a**) or $^{13}C_7$-labeled toluene (**b**). Distribution of bacterial 16S rRNA genes was measured by qPCR of DNA from gradient fractions.

time (Figure 3). After 64 days, we observed strong labeling-dependent community shifts within the 'intermediate' gradient fractions, and also within the 'heavy' DNA fractions ($\sim$1.74 mg l^{-1}) after 86 and 100 days of incubation. Such distinctions were not observed for the corresponding (BD) fractions of the ^{12}C control gradients, and also the 'light' fractions from ^{12}C- and ^{13}C-DNA gradients remained highly similar over time. Both criteria have been defined as strong arguments to delineate true label incorporation into selected microbes in SIP (Neufeld *et al.*, 2007; Lueders, 2009).

As identified by T-RFLP, especially the 146, 159 and 178 bp T-RFs appeared selectively enriched in the labeled DNA fractions (Figure 3). In contrast, the 89, 141, 470, 486 and 490 bp T-RFs were abundant especially in the 'light' fractions. To further resolve specific label allocation to defined community members (that is, T-RFs), we plotted relativized T-RF abundances over comparative gradient fractions (Figure 4). As previously demonstrated (Kunapuli *et al.*, 2007), this facilitates a semiquantitative comparison of the distribution of specific T-RFs between ^{12}C- and ^{13}C-gradients. This interpretation (Figure 4) clearly shows that the peak abundance of especially the 146 and 178 bp T-RFs shifted from 'light' to labeled fractions under ^{13}C-labeling. The shift of $\sim$0.02 g ml^{-1} in BD corresponds to 50% of the shift of $\sim$0.04 g ml^{-1} expected for full ^{13}C-labeling (Buckley *et al.*, 2007; Lueders, 2009). To a lesser extent, also the 159 bp T-RF shifted in BD, but was distributed with two maxima, one at 'light' and one at 'intermediate' BD. All other T-RFs did not show labeling-dependent shifts of distribution maxima, as exemplified for the 490 bp T-RFs in Figure 4.

Comparative direct fingerprinting analysis of non-density-resolved DNA from the inoculum (Figure 3) indicated that especially the 141, 146, 159 and 490 bp T-RFs were already initially present within the on-site microbial community, all at notable relative T-RF abundances of 9, 7, 7 and 17%, respectively.

Identification of labeled 16S rRNA genes

The specific microbial community members represented by the detected T-RFs were identified by cloning and sequencing. Two 16S rRNA gene-targeted clone libraries were established, one from a 'light' (BD = 1.683 g ml^{-1}) and one from a partially labeled (1.707 g ml^{-1}) DNA fraction of the 86-day ^{13}C-toluene incubation (see Figure 3 for fingerprints of the respective fractions). In all, 37 and 45 non-chimeric clones were sequenced, respectively (Table 1). Members of the *Chloroflexi* (32%), *Deltaproteobacteria* (16%) and *Betaproteobacteria* (11%) were dominating the 'light' library, whereas *Clostridia* (24%), *Actinobacteria* (16%), *Deltaproteobacteria* (13%) and *Chloroflexi* (11%) were found to dominate within the library of the partially labeled fraction. Among the first, a cluster of uncultured relatives of the Gram-positive genus *Desulfosporosinus* were represented by the 146 and 178 bp T-RFs (Figure 5), which we identified to incorporate $\sim$50% ^{13}C-label.

In addition, other important T-RFs could be identified using the clone libraries (Table 1, Figure 5). These were uncultured *Desulfobulbaceae* related to the toluene-degrading sulfate reducer TRM1 (Meckenstock, 1999) (159 bp T-RF), the 16S rRNA sequence of which has also been deposited in the course of this study (GU133208), uncultured *Desulfobacteriaceae* (163 and 511 bp), uncultured *Chloroflexi* (373 & 520 bp), as well as *Thauera*-related *Betaproteobacteria* (490 bp, together with a member of the *Gammaproteobacteria*).

bssA *genes detected in labeled DNA fractions*

The partially labeled DNA fraction (1.707 g ml^{-1}) from the ^{13}C-incubated microcosm (86 days) dominated by *Desulfosporosinus*-related degraders was also used to generate a *bssA*-targeted clone library. In this study, 19 putative *bssA* clones were sequenced (Figure 6). The library was dominated by a cluster of sequences (79% of all clones) closely related to the previously described clone D12-31 detected at another gasworks site, which has been tentatively named as 'F2'-lineage of unaffiliated environmental *bssA* genes (Winderl *et al.*, 2007). Three further clones were *Thauera*-related *bssA*

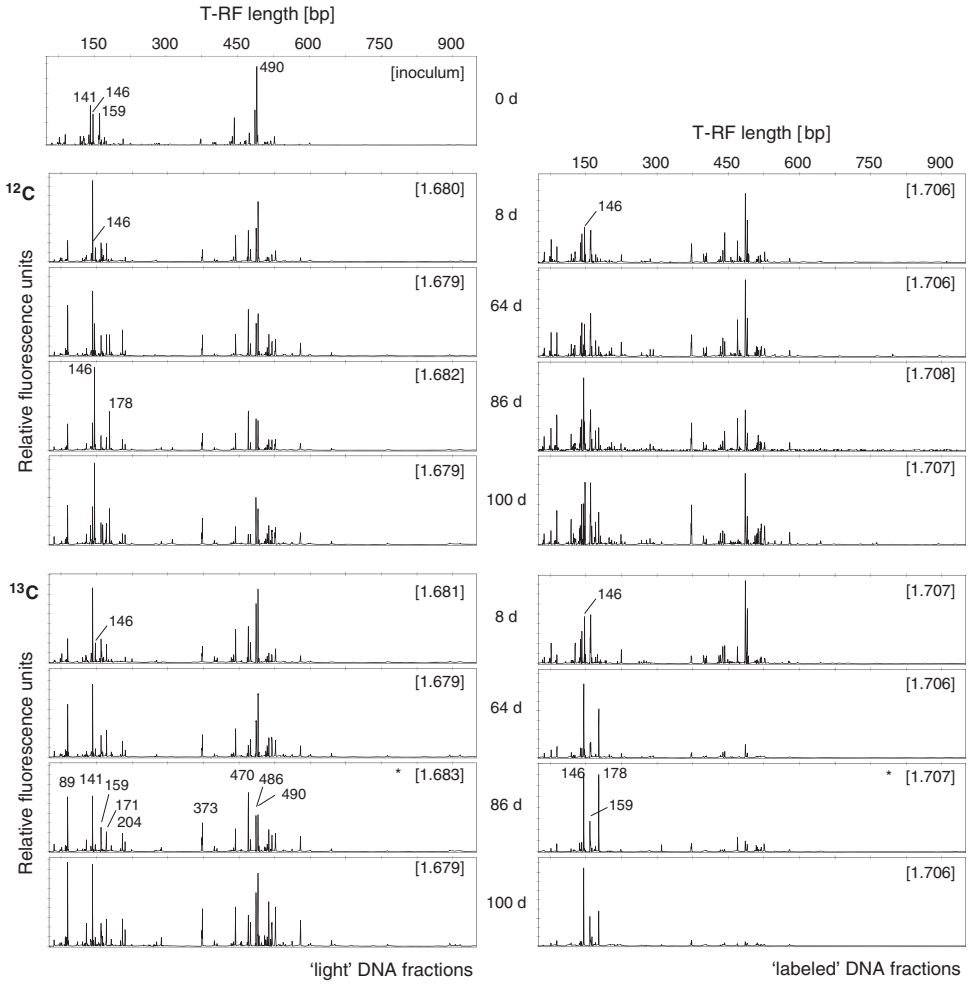

Figure 3 Bacterial 16S rRNA gene T-RFLP fingerprints of sedimentary DNA of the inoculum and of density-resolved SIP gradient fractions after 8, 64, 86 and 100 days of incubation with unlabeled (^{12}C) or $^{13}C_7$-labeled toluene. The lengths (bp) of selected T-RFs are given. Numbers in parentheses are CsCl BDs (g ml^{-1}) of gradient fractions. Fractions selected for cloning are marked with an asterisk.

sequence types, and one was distantly related to the *bssA* gene of strain TRM1 (Winderl *et al.*, 2007). Although *bssA*-targeted T-RFLP analysis of gradient fractions was not performed, a conserved 53 bp T-RF was predicted for the 15 retrieved clones related to D12-31. Direct *bssA*-targeted T-RFLP fingerprinting of inoculum DNA (data not shown) indicated that this 53 bp fragment was readily detectable within the on-site degradation gene pool at a relative T-RF abundance of ~17%.

Discussion

*SIP under close-to-*in situ *conditions*

One strategy of this study was to closely mimic *in situ* conditions for the sampled degrader community during SIP incubation. The microcosms were incubated at 16 °C in the dark, which reasonably reflects on-site groundwater temperatures of ~12–14 °C in average. All components of the used freshwater medium (Widdel and Bak, 1992) were used in 1:10 dilution (except for the bicarbonate buffer) to mimic groundwater low ionic strength. To ensure more or less constant substrate supply over a long period at low concentrations, XAD7 adsorber resin was used, which lowered initial toluene concentrations in the cosms from ~1 mM to an average of ~66 μM over the first 50 days of incubation. This was only twofold of the maximal toluene concentrations of ~33 μM reported close to the investigated well (Griebler *et al.*, 2004). In addition, the added 10 mM of sulfate were in a similar relation to reported *in situ* concentrations of up to ~5 mM (Griebler *et al.*, 2004). Most relevantly, we used freshly sampled aquifer sediment material containing a complex *in situ* microbial community for our experiment. This contrasts the present study from related recent studies, in which SIP has been used as a tool to functionally dissect previously enriched, less complex laboratory cultures of anaerobic BTEX degraders (Kunapuli *et al.*, 2007; Oka *et al.*, 2008; Bombach *et al.*, 2010; Herrmann *et al.*, 2010).

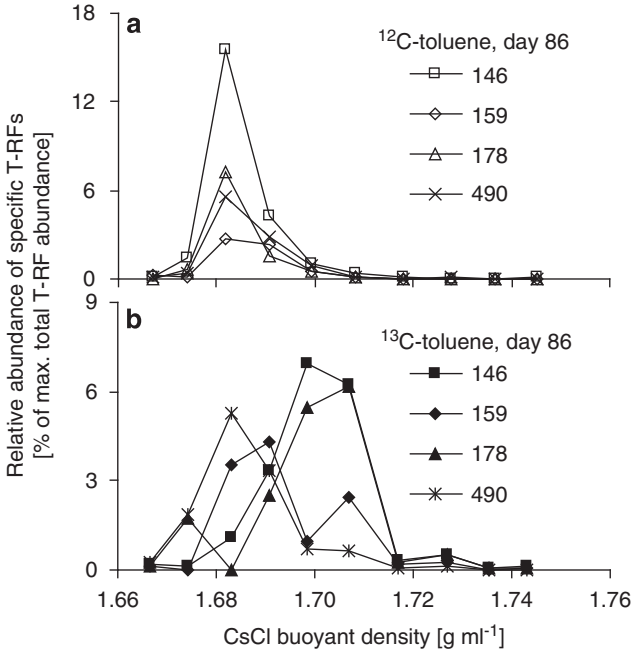

Figure 4 Comparative abundance distribution profiles of selected T-RFs in ^{12}C- (**a**) and $^{13}C_7$-toluene (**b**) SIP gradients after 86 days. Abundances were calculated by multiplying total gene abundances (Figure 2) by specific T-RF peak height ratios for all fractions of given gradients (Kunapuli *et al*., 2007).

Despite this close-to-*in situ* setup, degradation activity in the microcosms required a considerable time span to become detectable (over 50 days). This may indicate that indigenous toluene-degrading communities had to undergo certain lag and adaptation phases after sampling and transfer to the SIP microcosms. In addition, SIP requires substantial ^{13}C-substrate oxidation and assimilation activities to obtain the degree of nucleic acid labeling essential for successful gradient resolution. Here, DNA-SIP is less sensitive and requires longer incubation times for high ^{13}C incorporation into newly synthesized genomes than rRNA-SIP (Manefield *et al*., 2007). Unfortunately, sufficient amounts of high-quality rRNA for RNA-SIP were not extractable from our microcosms. However, considering the long incubation time for degradation activity to become noticeable, it seems unlikely that rRNA-SIP would have provided additional insights into a system as low in temporal and functional dynamics as the one investigated.

Gram-positive key populations in toluene degradation

We demonstrate that ^{13}C-toluene was quantitatively degraded and converted to CO_2 coupled to sulfate reduction within our microcosms. In parallel,

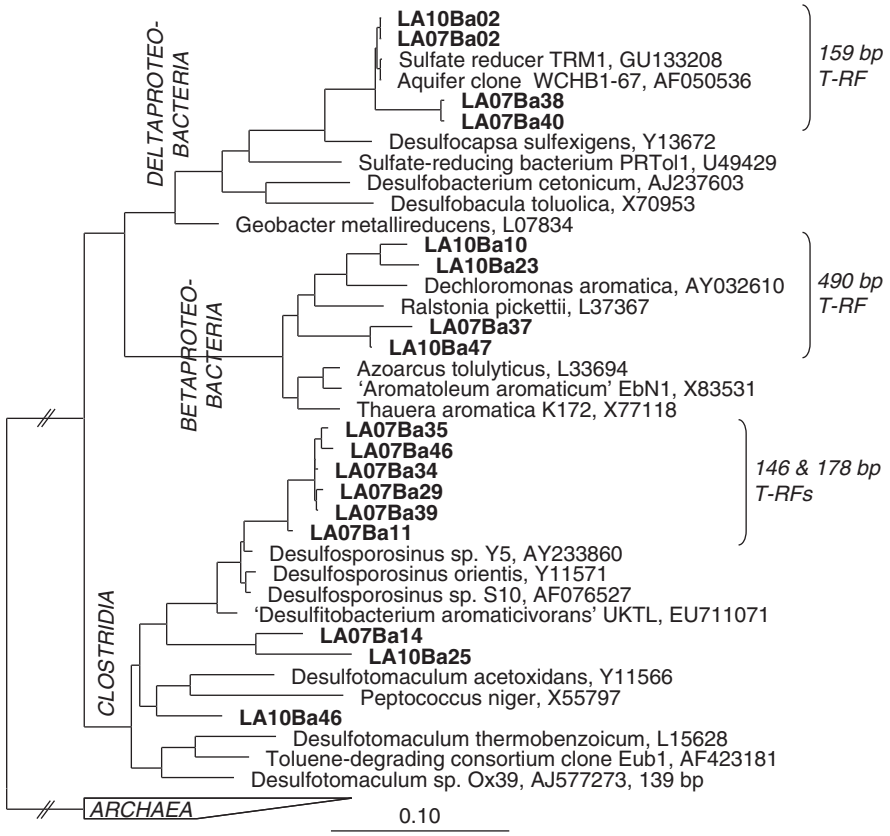

Figure 5 Maximum likelihood tree of the phylogenetic affiliation of representative bacterial 16S rRNA gene clones from day 86 ^{13}C-toluene SIP gradient fractions. Clones from ^{13}C-labeled DNA (LA07Ba) and 'light' DNA (LA10Ba) are in bold, GenBank accession numbers are indicated. The scale bar represents 10% sequence divergence, branch lengths to the outgroup have been scaled down to 25%.

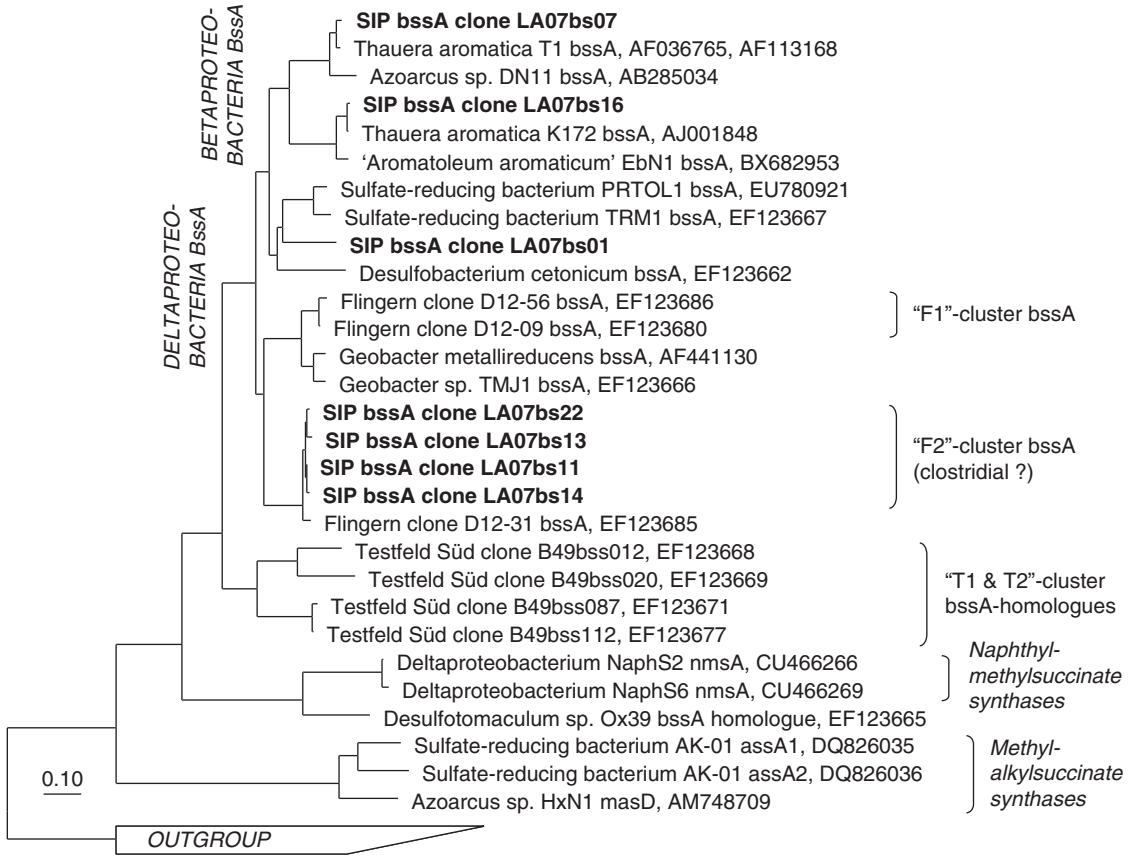

Figure 6 FITCH distance matrix tree showing the phylogenetic affiliation of *bssA* gene clones from ^{13}C-labeled DNA of the day 86 ^{13}C-toluene SIP gradient. Clones from this study are in bold, GenBank accession numbers are indicated. The scale bar represents 10% sequence divergence. Naming of environmental *bssA* lineages is in accordance with Winderl *et al.* (2007). The outgroup consists of related non-*Bss* pyruvate formiate lyases (NCBI protein identifiers: CAJ67970, AAM54728, ZP_00099983, ZP_00130812).

we observed a clear incorporation of ^{13}C-label (~50 AT%) into the DNA of the most active toluene degraders within the *in situ* microbiota. These were uncultured relatives of spore-forming *Desulfosporosinus* and *Desulfotomaculum* spp., represented by the 146 and 178 bp T-RFs. From our results it is impossible, however, to define whether two closely related, but functionally identical sub-populations were represented by the two T-RFs, or whether it was one population carrying two minimally differing (~98% sequence similarity) *rrn* operons (Pei *et al.*, 2009).

Members of the *Peptococcaceae h*ave been previously described as important constituents of microbial communities in contaminated aquifers (Robertson *et al.*, 2001; Franzmann *et al.*, 2002), and two respective isolates have been described to oxidize toluene (Liu *et al.*, 2004; Morasch *et al.*, 2004). More recently, related microbes have also been identified to be involved in the anaerobic degradation of benzene (Kunapuli *et al.*, 2007; Abu Laban *et al.*, 2009; Herrmann *et al.*, 2010). Taken together, this evidence strengthens the hypothesis that members of the *Peptococcaceae* do not only represent important microbial community constituents in deep, oligotrophic subsurface environments (Chivian *et al.*, 2008), but also in shallow, contaminated aquifers (Franzmann *et al.*, 2002; Kunapuli *et al.*, 2007). This is supported by the fact that the T-RFs representing these *Desulfosporosinus* relatives were already detectable within the *in situ* community at the beginning of our experiment. Especially the 146 bp T-RF was clearly visible in the inoculum and in all 'light' gradient fractions on day 8 (Figure 3), before any potential stimulatory or enrichment effects of the SIP incubation.

If such Gram positives are more relevant in subsurface contaminant degradation under strictly anoxic conditions than currently recognized, this may have some interesting implications for the microbial ecology of pollutant breakdown at such sites. First, spore-forming degrader populations may be capable of enhanced survival under unfavorable environmental conditions, such as geochemical or redox changes imposed by dynamics of the groundwater table, temporal electron acceptor depletion or toxicity caused by transiently high contaminant loads.

Second, our electron balance revealed that only ~50% of the electrons from the added toluene were

traceable to produced sulfide. This electron recovery was similarly low as in a recent SIP study of a benzene-degrading, sulfate-reducing enrichment culture (Herrmann *et al.*, 2010), irrespective of the fact that we were working with fresh sediment samples containing a complex aquifer microbiota. Part of the produced sulfide could have been missed due to precipitation with sedimentary ferrous iron; however, a delayed appearance of sulfide with respect to $^{13}CO_2$ production was not observed. Therefore, it cannot be excluded that the electrons stemming from toluene were not exclusively used for sulfate reduction. Besides sulfate, members or relatives of the genus *Desulfosporosinus* have also been reported to use alternative electron acceptors, such as sulfur, Mn(IV) or Fe(III) (Liu *et al.*, 2004; Spring and Rosenzweig, 2006), all of which could have been present in the inoculated aquifer sediments, and which would have been thermodynamically more attractive.

Third, the bulk of identified degrader DNA was only ~50 AT% ^{13}C-labeled. Thus, degrader anabolism must have substantially diluted the incorporation of ^{13}C from the contaminant with an unlabeled carbon source. Again, this low labeling efficiency coincides with labeling ratios observed in recent SIP studies of BTEX-degrading, iron- or sulfate-reducing enrichments (Kunapuli *et al.*, 2007; Bombach *et al.*, 2010; Herrmann *et al.*, 2010). Besides medium carbonate, no other C source was added to the microcosms. Still, for example, acetate could have been produced by other degradation and fermentation processes intrinsically occurring in the sampled aquifer material. Unfortunately, we did not quantify acetate (or other organic carbon) during our SIP incubation. Although the two described related toluene-oxidizing *Clostridia* do not grow on acetate (Liu *et al.*, 2004; Morasch *et al.*, 2004), the utilization of acetate for assimilatory purposes cannot be excluded. However, if abundant acetate or other organic carbon had been available during the incubation, sulfate reduction would have been expected independent of toluene degradation, which was clearly not observed (Figure 1).

Acetate could also have been formed by the toluene-degrading sulfate reducers themselves, which would occur on incomplete oxidation of toluene. Although both complete and incomplete oxidizers of organic substrates can be found within the genus *Desulfotomaculum* (Widdel, 2006), *Desulfosporosinus* spp. are typically considered as incomplete oxidizers (Spring and Rosenzweig, 2006). The incomplete oxidation of 1 mol of toluene to 1 mol of bicarbonate and 3 mol of acetate would still be exergonic ($\Delta G^{\circ\prime} = -70.5$ kJ per mol of toluene) for the primary degrader. However, this model would require a secondary sulfate reducer consuming the produced acetate to become equally or even more strongly labeled than the primary degrader over time, which we did not observe. Moreover, the two isolated hydrocarbon-degrading *Desulfosporosinus* and *Desulfotomaculum* relatives described to date were both found to completely oxidize toluene to CO_2 (Liu *et al.*, 2004; Morasch *et al.*, 2004).

Rather, a further mechanism seems much more probable to explain the observed low labeling efficiency: the assimilation of acetyl-CoA derived from toluene in denitrifying, facultatively anaerobic BTEX degraders proceeds through the glyoxylate cycle (Heider and Fuchs, 1997; Rabus, 2005), allowing biomass buildup exclusively based on carbon from the labeled substrate. In accordance, abundant highly ^{13}C-labeled degrader DNA was observed in preliminary SIP incubations of the same inoculum using nitrate as electron acceptor (data not shown). However, strictly anaerobic sulfate reducers and other *Peptococcaceae* typically lack enzymes of the glyoxylate cycle (Kosaka *et al.*, 2006; Chivian *et al.*, 2008), and assimilate acetyl-CoA through reductive carboxylation to pyruvate by the pyruvate: ferredoxin oxidoreductase (Heider and Fuchs, 1997). Given that unlabeled CO_2 is used for carboxylation, this lowers maximal biomass labeling to ~66%, which will be further reduced by additional carboxylation reactions in downstream anabolism. In essence, the observed high ratios of heterotrophic CO_2 assimilation and the resulting low assimilation efficiency for carbon from the contaminant by the key degraders is certainly a relevant input parameter for researchers modeling carbon flow and degrader biomass yields in contaminated subsurface environments (Thullner *et al.*, 2007).

Other microbes involved in toluene degradation

The only further T-RF indicating label incorporation into degraders other than these Gram positives was the 159 bp fragment, representing relatives of the toluene-degrading sulfate reducer TRM1 (Meckenstock, 1999). This strain yet awaits valid description, but it may represent a new BTEX degrader related to *Desulfocapsa* within the *Desulfobulbaceae*. Previously, we had already sequenced the *bssA* gene of this strain (Winderl *et al.*, 2007), but now have also deposited the corresponding 16S rRNA sequence (GU133208). However, from the comparably high abundance of the 159 bp T-RF remaining in 'light' fractions also under ^{13}C-labeling (Figure 4b), we conclude that these *Deltaproteobacteria* consumed only minor amounts of labeled toluene and were by far not as important for quantitative toluene turnover as their clostridial counterparts under the conditions of our SIP incubation. Secondary cross-feeding of label to microbes other than these primary degraders can be excluded, as no fragments other than the 146, 178 and 159 bp T-RFs were identified to appear enriched in labeled DNA.

Members of the *Desulfobulbaceae* were also identified as degraders in the recent SIP investigation of a toluene-degrading enrichment culture

(Bombach *et al.*, 2010), and we have also found such microbes to dominate in zones of high degradation activity directly at another field site (Winderl *et al.*, 2008). Up to now, TRM1 is the only reported BTEX-degrading strain within the *Desulfobulbaceae*. This may be a fine example of how cultivation-dependent and -independent approaches can go hand in hand to unravel the relevance of previously unrecognized degraders in environmental processes (Nichols, 2007).

Catabolic genes of clostridial populations

Our SIP data indicate *Desulfosporosinus* relatives to be most active in toluene degradation in our microcosms, and that their DNA was ~50 AT% labeled after ^{13}C-incubation. Thus, it is a fair assumption that also the affiliated *bssA* genes of these Gram positives can be found enriched in the respective gradient fractions. The cloning and sequencing of *bssA* amplicons from this DNA fraction revealed a clear dominance (15 of 19 clones) of one *bssA* sequence type closely related to the previously detected 'F2'-lineage of unidentified *bssA* sequences (Winderl *et al.*, 2007). This indicates that this *bssA* lineage may actually belong to the identified *Desulfosporosinus* relatives. So far, the placement of putative clostridial degradation genes in *bssA* phylogeny has not been answered. However, as long as genes from a pure culture isolate or at least a (meta-)genomic sequence linking both 16S and *bssA* genes are not available, this affiliation is merely tentative.

At the same time, the function and affiliation of the more deeply branching 'T'-cluster *bssA* homologs previously detected in Testfeld Süd sediments (see Figure 6) remains enigmatic. These genes may well be active in the degradation of hydrocarbons other than toluene, which can also involve fumarate addition (Callaghan *et al.*, 2008; Grundmann *et al.*, 2008; Musat *et al.*, 2009). Alternatively, it could indicate that intrinsic toluene-degrading microbial populations at the site have changed between the two time points of sampling (August 2004 and December 2006), which is supported by the fact that in 2004 we did not detect any 'F2'-lineage *bssA* genes at the site, whereas direct *bssA*-targeted fingerprinting of DNA from our SIP inoculum, taken from the same well as in 2004, did.

Furthermore, three of the cloned *bssA* sequences were related to betaproteobacterial *Thauera* and *Azoarcus* spp. *bssA* genes and one to TRM1 *bssA* (Winderl *et al.*, 2007). Although a minor contribution of TRM1 relatives to toluene degradation seems likely (see above), this can clearly be excluded for denitrifying *Thauera* relatives. Nitrate was neither present in our microcosms, nor is it an important electron acceptor at the site. An increased abundance of the respective rRNA gene clones and the 490 bp T-RF was not inferable for labeled DNA (Table 1, Figure 4). Thus, although the PCR assays we used did detect the presence of these degraders, SIP data clearly excluded any functional importance in toluene degradation under the investigated geochemical settings.

In summary, we have identified the most active sulfate-reducing toluene degraders within a diverse aquifer microbial community to be related to *Desulfosporosinus* spp. within the *Peptococcaceae*. The functional assignment of these novel degraders, along with their tentatively associated *bssA* sequence type as well as the observed high ratios of heterotrophic CO_2-fixation during assimilation of carbon from the contaminant by these degraders will foster our understanding of the biodiversity and ecophysiology of microbial populations in natural attenuation.

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