RESEARCH ARTICLE

Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP

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Abstract

Bioavailability of electron acceptors is probably the most limiting factor in the restoration of anoxic, contaminated environments. The oxidation of contaminants such as aromatic hydrocarbons, particularly in aquifers, often depends on the reduction of ferric iron or sulphate. We have previously detected a highly active fringe zone beneath a toluene plume at a tar-oil-contaminated aquifer in Germany, where a specialized community of contaminant degraders codominated by *Desulfobulbaceae* and *Geobacteraceae* had established. Although on-site geochemistry links degradation to sulphidogenic processes, dominating catabolic (benzylsuccinate synthase α-subunit, *bssA*) genes detected *in situ* appeared to be more related to those of *Geobacter* spp. Therefore, a stable isotope probing (SIP) incubation of sediment samples with $^{13}C_7$-toluene and comparative electron acceptor amendment was performed. We introduce pyrosequencing of templates from SIP microcosms as a powerful new strategy in SIP gradient interpretation (Pyro-SIP). Our results reveal the central role of *Desulfobulbaceae* in sulphidogenic toluene degradation *in situ*, and affiliate the detected *bssA* genes to this lineage. This and the absence of ^{13}C-labelled DNA of *Geobacter* spp. in SIP gradients preclude their relevance as toluene degraders *in situ*. In contrast, *Betaproteobacteria* related to *Georgfuchsia* spp. became labelled under iron-reducing conditions. Furthermore, secondary toluene degraders belonging to the *Peptococcaceae* detected in both treatments suggest the possibility of functional redundancy among anaerobic toluene degraders on site.

Introduction

Groundwater, a primary source of drinking water, is subject to contamination by a huge variety of anthropogenic compounds. Among them, petroleum derivates, especially aromatic ones, are significantly persistent. Therefore, degradation of compounds such as benzene, toluene, ethylbenzene and xylenes (BTEX) by intrinsic aquifer microbial communities, specifically under anaerobic conditions, is of particular interest for the comprehension and prediction of natural attenuation. Anaerobic BTEX degradation is known to occur under different terminal electron-accepting processes such as nitrate, iron or sulphate reduction, and methanogenesis (Anderson & Lovley, 1997; Christensen *et al.*, 2000). However, the co-occurrence of or competition between different degrader guilds *in situ* is not well understood. Clearly, the dominance of a given degrader lineage is assumed to be dictated by electron acceptor availability.

Previous evidence from a tar-oil-contaminated aquifer site in Germany has shown the establishment of a highly active anaerobic toluene-degrading microbial community at the lower fringe of a BTEX plume (Winderl *et al.*, 2008; Anneser *et al.*, 2010). Even though toluene degradation at the site is presumed to be driven by sulphate reduction (Anneser *et al.*, 2008; Winderl *et al.*, 2008), this community is codominated by microorganisms affiliated to the *Desulfobulbaceae* and the *Geobacteraceae*. Both deltaproteobacterial lineages are known to harbour anaerobic toluene degraders, but these are either sulphate- or iron-reducers

(Meckenstock, 1999; Kane *et al.*, 2002). At the same time, the dominating anaerobic toluene degradation genes (benzylsuccinate synthase α-subunit, *bssA*) detected at the site were more closely related to *Geobacter*-affiliated *bssA* than to other known sulphate-reducing degraders (Winderl *et al.*, 2007). However, the utility of the *bssA* gene as a phylogenetic marker is not robust, as cases of lateral gene transfer of this gene have been reported (Shinoda *et al.*, 2005; Winderl *et al.*, 2007). Moreover, recent studies indicate that certain members of the *Peptococcaceae* (*Clostridia*), also relevant constituents of the respective lower plume fringe community (Winderl *et al.*, 2008; Anneser *et al.*, 2010), carry a previously unrecognized capacity for toluene degradation under iron- (Kunapuli *et al.*, 2010) and sulphate-reducing conditions (Winderl *et al.*, 2010).

To resolve this dilemma and to assign phylogenetically key toluene-degrading functionality and genes at this site, we conducted a stable isotope probing (SIP) experiment with fresh sediment samples taken *in situ* from the degradation-active lower plume fringe. Sediments were incubated in microcosms under amendment of $^{13}C_7$-toluene and either sulphate or ferric iron as an electron acceptor. SIP allows the direct identification of environmental microorganisms degrading and assimilating carbon from a ^{13}C-labelled hydrocarbon and, if conducted for DNA, affiliation of respective degradation genes (Lueders, 2010). We hypothesize that if both *Desulfobulbaceae*- and *Geobacteraceae*-related degraders are important in toluene degradation *in situ*, both will become labelled in the respective SIP incubations. However, if only one of the two lineages is important in the breakdown of toluene *in situ*, only this will be labelled while respiring the appropriate electron acceptor. In contrast, a different degrader lineage, not dominating (or important) *in situ*, could become detectable using the other oxidant. We test this hypothesis using a new combination of DNA-SIP and high-throughput pyrosequencing of amplicons from SIP incubations to identify the key toluene-degrading lineage in these sediments.

Materials and methods

Sampling site and sample acquisition

Sampling was performed within a well-studied (Anneser *et al.*, 2008, 2010; Winderl *et al.*, 2008) tar-oil-contaminated aquifer in Düsseldorf-Flingern, Germany, in September 2008. Sediments ranging between 6.9 and 7.75 m below the ground surface were sampled from intact direct-push liners and immediately dispensed into 1-L glass bottles full of anoxically autoclaved (under N_2/CO_2) deionized water. Bottles were directly closed without gaseous headspace to minimize oxygen exposure of sediments and transported to the laboratory under cooling (4 °C).

Incubation of sediments

Replicates (~8 g wet weight) of homogeneously mixed sediment material were transferred (in an anoxic tent) into sterile 120-mL serum bottles containing 50 mL of artificial anoxic groundwater medium as described previously (Winderl *et al.*, 2010). To ensure constantly low *in situ*-like concentrations of toluene during SIP incubation, 0.3 g of Amberlite XAD7 absorber resin in each microcosm was loaded with a total of 1 mM of either nonlabelled (^{12}C) or fully labelled $^{13}C_7$ toluene (Sigma-Aldrich, Munich, Germany) (Winderl *et al.*, 2010). Parallel incubation series were amended with either 10 mM of sodium sulphate or 40 mM of amorphous Fe(III) oxyhydroxide prepared by titration of a solution of $FeCl_3$ to a pH of 7 with NaOH (Lovley & Phillips, 1986). Twelve replicate microcosms for each treatment were set up. Abiotic control bottles (autoclaved three times), amended with each electron acceptor and unlabelled toluene, were also prepared to exclude the occurrence of abiotic redox reactions. The bottles were incubated statically for over 4 months in the dark at 16 °C, which was close to *in situ* aquifer temperatures of 14–16 °C.

Process measurements

Liquid and gaseous samples were taken from replicate ^{12}C- and ^{13}C-toluene and control bottles to monitor toluene degradation weekly. Aqueous toluene and sulphide concentrations were determined as described previously (Winderl *et al.*, 2010). Fe(II) was monitored from 100 μL liquid subsamples also as described previously (Bosch *et al.*, 2010), using a Cary 50 Bio UV-Vis photometer (Varian, Darmstadt, Germany) at a wavelength of 508 nm. The formation of $^{13}CO_2$ was followed via GC combustion-isotope ratio MS (GC-C-IRMS) on 15 μL samples taken from the head space of each bottle, as described previously (Winderl *et al.*, 2010).

Nucleic acid extraction and ultracentrifugation

At selected time points, a pair of bottles (^{12}C and ^{13}C) for each electron acceptor series (sulphate, ferric iron) was sacrificed for DNA-SIP analyses. Sediment, XAD7 and biomass were collected by centrifugation at 3350 ***g*** at 4 °C for 10 min using a Megafuge 1.0 R (Heraeus Instruments, Hanau, Germany). Pellets were frozen immediately at − 20 °C and DNA extracted as described previously (Winderl *et al.*, 2008), with minor modifications (~12 h of DNA precipitation at 4 °C instead of ~4 h). For each single extract, replicate extractions were pooled in maximum 100 μL of elution buffer (Qiagen, Hilden, Germany) and stored frozen (− 20 °C) for downstream analyses.

Approximately 1 μg of PicoGreen-quantified (Invitrogen, Darmstadt, Germany) DNA extract was loaded onto a

gradient medium of CsCl (average density 1.71 g mL^{-1}, Calbiochem, Merck, Darmstadt, Germany) in gradient buffer (0.1 M Tris-HCl at pH 8, 0.1 M KCl, 1 mM EDTA) and centrifuged (180 000 ***g***, ~65 h) as described elsewhere in detail (Lueders, 2010; Winderl *et al.*, 2010). Thirteen fractions from each gradient were collected from 'heavy' to 'light' using a Perfusor V syringe pump (Braun, Melsungen, Germany). Refractometric measurement of fraction buoyant density (BD) and the recovery of DNA from gradient fractions were also performed as published (Lueders, 2010).

Analyses of density-resolved DNA fractions

Recovered DNA from CsCl gradient fractions was analysed by bacterial 16S rRNA gene-targeted qPCR in the presence of 0.1 × SYBR Green as described (Kunapuli *et al.*, 2007). Most fractions from each gradient were selected for bacterial 16S rRNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. FAM-labelled amplicons were generated with the primers Ba27f (5′FAM-aga gtt tga tcm tgg ctc ag-3′) and 907r (5′-ccg tca att cct ttg agt tt-3′) in a Mastercycler ep gradient (Eppendorf, Hamburg, Germany) with the following cycling conditions: initial denaturation (94 °C, 5 min), followed by 24 or 28 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and elongation (70 °C, 60 s). Each 50 μL PCR reaction contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1.25 U recombinant Taq polymerase (all from Fermentas, St. Leon-Rot, Germany), 0.2 μg μL^{-1} bovine serum albumin (BSA) (Roche, Penzberg, Germany), 0.5 μM of each primer (Biomers, Ulm, Germany) and 1 μL of template DNA. Amplicons were restricted using MspI and separated by capillary electrophoresis as reported (Lueders *et al.*, 2006). Electropherograms were evaluated as described (Winderl *et al.*, 2008). In addition, density-resolved DNA was also subjected to bacterial *bssA* gene fingerprinting. This was conducted with the 7772f/8546r-FAM primer pair as described (Winderl *et al.*, 2010), but with a PCR annealing temperature of 58 °C (Winderl *et al.*, 2007). Amplicons were digested with TaqI, while PCR chemistry and the remaining procedures were as for 16S fingerprints.

Amplicon pyrosequencing from SIP microcosms

Amplicon pyrosequencing was performed on unfractionated total DNA extracted from ^{13}C-microcosms. Barcoded amplicons for multiplexing were prepared with the primers Ba27f (5′-aga gtt tga tcm tgg ctc ag-3′) and Ba519r (5′-tat tac cgc ggc kgc tg-3′) (Lane, 1991) extended as amplicon fusion primers with respective primer A or B adapters, key sequence and multiplex identifiers (MID) as recommended by 454/Roche (http://454.com/products-solutions/experimental-design-options/amplicon-sequencing.asp). Amplicons were generated under the same cycling conditions as described above for T-RFLP. Each 50 μL PCR reaction contained 1 × PCR buffer, 1.44 mM $MgCl_2$, 0.1 mM dNTPs, 1% dimethyl sulphoxide, 1.25 U Taq polymerase (all from FastStart High Fidelity Taq DNA Polymerase kit, Roche), 0.32 μg μL^{-1} BSA (Roche), 0.3 μM of each MID-primer (Biomers) and 1 μL of template DNA. Amplicons were purified and pooled as specified by the manufacturer. Emulsion PCR, emulsion breaking and sequencing were performed applying the GS FLX Titanium chemistry following protocols and using a 454 GS FLX pyrosequencer (Roche) as recommended by the developer. For this study, two amplicons were sequenced in a pool of 14 mixed amplicons on 1/8th of an FLX picotitre plate. Quality filtering of the pyrosequencing reads was performed using the automatic amplicon pipeline of the GS Run Processor (Roche), with a modification of the valley filter (vfScanAllFlows false instead of TiOnly) to extract sequences.

Afterwards, reads were further quality-trimmed using the TRIM function of GREENGENES (DeSantis *et al.*, 2006) with the following settings: good-quality score 20, window size 40 bp and window threshold 90%. Subsequently, reads were batched per sample based on MID-identifiers with BIOEDIT (Hall, 1999) and reads with inferior read length (< 250 bp) were excluded from further analysis. The total community composition was classified via read affiliation using the RDP classifier (Wang *et al.*, 2007) at a confidence threshold of 70%. Read abundance percentage of classified lineages was recorded.

For downstream analysis of sequenced amplicon pools (T-RF prediction, phylogenetic inference), matching sequences from forward- and reverse-reads were assembled into contigs with the SEQMAN II software (DNAStar) using assembly thresholds of at least 98% sequence similarity over a 50-bp match window. Contigs without at least one forward and one reverse read were not considered for further analysis. Selected dominating amplicon contigs were then integrated into an ARB (Ludwig *et al.*, 2004) database (version SSURef-95, July 2008). Sequences were aligned using automated aligners in the ARB_EDIT4 editor. For phylogenetic affiliation, a 'backbone' tree based on selected full-length (> 1200 bp) reference sequences of cultivated and uncultivated taxa was constructed using quartet puzzling as described previously (Kunapuli *et al.*, 2010). Subsequently, shorter sequences including the selected amplicon contigs were added to the tree using the ARB parsimony tool, thereby maintaining the overall tree topology.

T-RFs of amplicon contigs were predicted using ARB_EDIT4. Possible deviations between predicted and measured T-RFs were handled by referring to our previous thorough verification of lineage-specific T-RFs by direct T-RFLP analysis of cloned amplicons generated from the same contaminated site (Winderl *et al.*, 2008). In summary, this

new bidirectional amplicon pyrosequencing approach allows the integrated evaluation of pyrosequencing results from the phylogenetic placement of representative assembled dominating amplicon sequences to T-RF matching. All raw and trimmed reads generated in this study have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE25449. The selected amplicon contigs used for phylogenetic analysis have been deposited at GenBank under the accession numbers HQ625653–HQ625672.

Results

Exposure of aquifer sediments to ^{13}C-toluene

Figure 1 shows the evolution of major redox species during SIP incubation. Increasing concentrations of sulphide, with a rapid increase up to 2.8 mM between the 10th and the 12th week, were paralleled by a steep increase in $^{13}CO_2$ (up to ~12 atom percent, AT%) in sulphate-amended ^{13}C-toluene microcosms. In contrast, iron(III)-amended microcosms showed a primary increase of Fe^{2+} up to ~9 mM during the first 6 weeks, followed by a secondary, slower increase to up to ~15 mM until the end of the experiment. Only this secondary increase of Fe^{2+} was connected to an increase in $^{13}CO_2$ concentrations, albeit to much lower ratios (only ~4 AT%) than under sulphate amendment. ^{12}C-toluene microcosms showed comparable dynamics of reduced electron acceptors.

The time course of toluene consumption was not detectable due to the presence of the XAD7 carrier resin, as described before (Winderl *et al.*, 2010). Although toluene was present with a nominal total concentration of ~1 mM, desorbed free aqueous concentrations varied around ~50 μM for most of the incubation time. Complete toluene depletion was observed in the sulphate-reducing incubations after the 13th week, while ~35 μM remained detectable in the iron-reducing incubations by the end of the experiment (15th week). Autoclaved controls did not show significant abiotic reactions, with sulphide concentration not exceeding ~0.1 mM and iron(II) concentration not exceeding ~4.6 mM. As observed previously for electron balances in similar SIP microcosms (Winderl *et al.*, 2010), sulphate microcosms ended with ~60% of the electrons from the added toluene recovered in sulphide and ~85% of the added labelled carbon recovered as $^{13}CO_2$. In iron incubations, only ~42% of the added electrons from toluene were recaptured in ferrous iron, and < 30% of the carbon from ^{13}C-toluene was recovered as $^{13}CO_2$ (both assuming complete toluene oxidation).

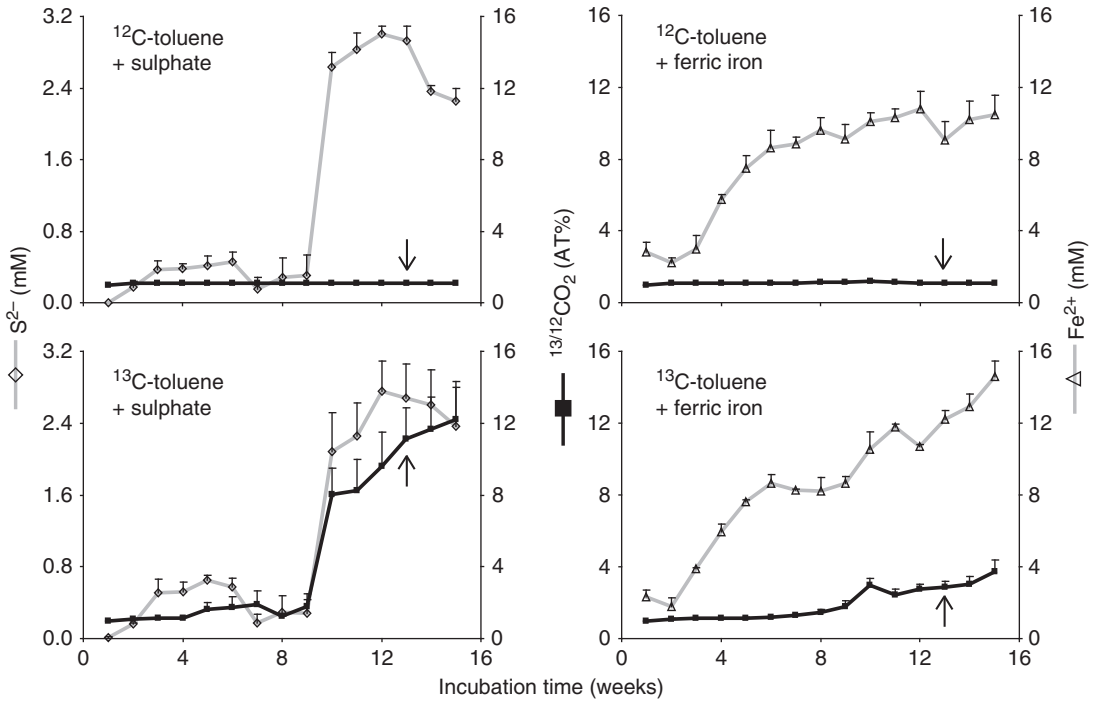

Fig. 1. Production of sulphide, $^{13}CO_2$ and ferrous iron during the degradation of unlabelled (^{12}C) or $^{13}C_7$-toluene in comparative SIP microcosms. $^{13}C/^{12}C$ isotopic composition of CO_2 is indicated in atom percent (At%). Means of triplicate microcosms are given +SE. Arrows indicate the time point chosen for DNA-SIP analyses.

Detection and identification of ^{13}C-labelled degraders

One late time point, when substantial respiration and mineralization activities had been observed in both SIP incubation series (after 13 weeks), was selected for the detection of labelled DNA by isopycnic ultracentrifugation. This choice was driven mainly by the iron-reducing microcosms, where significant shifts in $^{13}CO_2$ were not detectable earlier. Nevertheless, clear BD shifts compared with the respective ^{12}C-control gradients and enrichment of bacterial DNA, especially in 'intermediate' gradient fractions, were appreciable in SIP gradients from both incubation series (Fig. 2).

It was possible to detect clear distinctions between 'light' and 'heavy' DNA fractions of ^{13}C-gradients (Fig. 3) in bacterial community fingerprints. Thus, 'intermediate' and 'heavy' fractions of DNA from ^{13}C-toluene sediments showed a dominance of especially the 159 bp, but also the 146- and 177-bp T-RFs. At the same time, 'light' fractions showed selection for the 163- and 469-bp T-RFs. In contrast, all major T-RFs were detected in all gradient fractions from the sulphate-amended ^{12}C-toluene control. In the ferric iron SIP microcosms, bulk DNA from ^{12}C-control gradients was dominated mainly by two T-RFs: the 159- and 430-bp fragments. Also, other fragments, such as the 288-bp T-RF, were detected in 'lighter', low GC-content DNA fractions (Fig. 3). In contrast, while 'light' bulk DNA in ^{13}C-gradients was still dominated by the 159-bp T-RF, the 288- and 430-bp fragments were of greater relative abundance in 'intermediate' and 'heavy' gradient fractions. Therefore, especially the latter seemed to represent microorganisms involved in ferric iron-dependent toluene degradation.

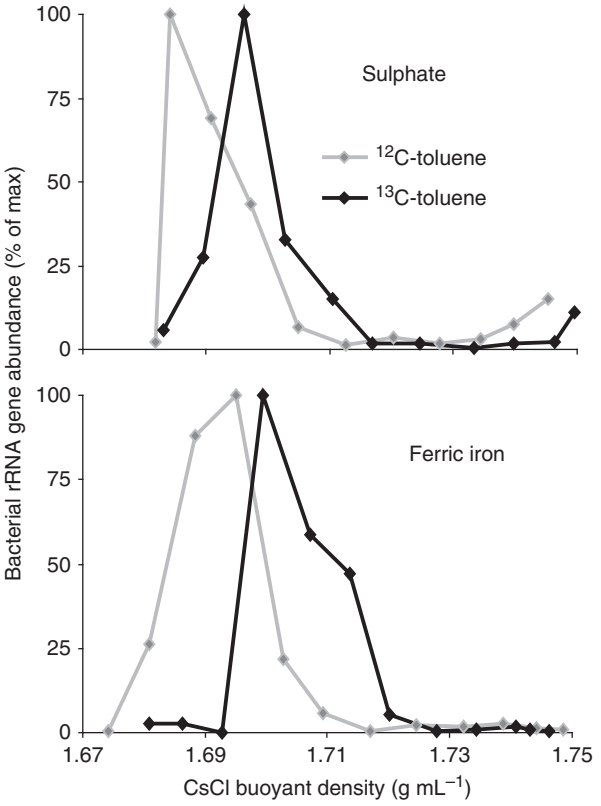

Fig. 2. Quantitative profiles of DNA distribution, measured via 16S rRNA gene qPCR, in comparative SIP gradients of DNA from microcosms amended with unlabelled (^{12}C) toluene or $^{13}C_7$-toluene and sulphate or ferric iron.

To identify the bacterial lineages represented by T-RFs from density-resolved SIP gradient fractions, barcoded amplicon pyrosequencing (Miller *et al*., 2009; Quince *et al*., 2009) of two representative bulk DNA extracts was performed. Table 1 summarizes the total pyrosequencing read, trimming and contig assembly statistics for the two amplicon pools, while Table 2 illustrates the comprehensive affiliation of reads and that of deduced T-RFs. SIP microcosm communities were mainly composed of reads within the *Delta-*, *Beta-* and *Epsilonproteobacteria*, and also the *Clostridia*. Sulphate-amended samples showed high relative abundances of sequences within the *Desulfobulbaceae* (29%), *Desulfosporosinus* spp. (21%) and *Sulfuricurvum* spp. (13%). In contrast, abundant reads affiliated to *Thermincola* spp. (16%), unclassified *Desulfobacterales* (9%), the *Rhodocyclaceae* (7%), *Comamonadaceae* (13%) and *Actinobacteria* (4%) constituted clear community distinctions detectable in the ferric iron SIP incubations.

The phylogenetic placement of important amplicon contigs assembled from dominating pyrosequencing reads is illustrated in Fig. 4. In combination with T-RFs previously predicted and measured for cloned bacterial rRNA genes from the same site (Winderl *et al*., 2008), a comprehensive affiliation of T-RFs detected in density-resolved DNA gradient fractions to defined lineages was possible. Almost all the peaks present in the electropherograms could actually be matched to pyrosequencing read data. Thus, the 159-, 146- and 177-bp T-RFs identified as ^{13}C-labelled under sulphate amendment represented amplicons originating from members of the *Desulfobulbaceae* and *Desulfosporosinus* spp. (Table 2). In contrast, the 163- and 469-bp T-RFs dominating in 'light' fractions were affiliated to *Sulfuricurvum* spp. The 288- and 430-bp fragments with increased abundance in 'heavy' ferric iron SIP DNA represented amplicons related to *Thermincola* spp. and within the *Rhodocyclaceae*. In contrast, the 159-bp T-RF dominating in 'light' fractions of the same treatments was affiliated to a second cluster of unclassified *Desulfobulbaceae*, which was distinct from the labelled sequence types from the same family found under sulphate reduction. However, the primary *Desulfobulbaceae*-related sequence type dominating in sulphate-reducing SIP microcosms was also detected in iron-reducing incubations, albeit much less abundant (Table 2, Fig. 4).

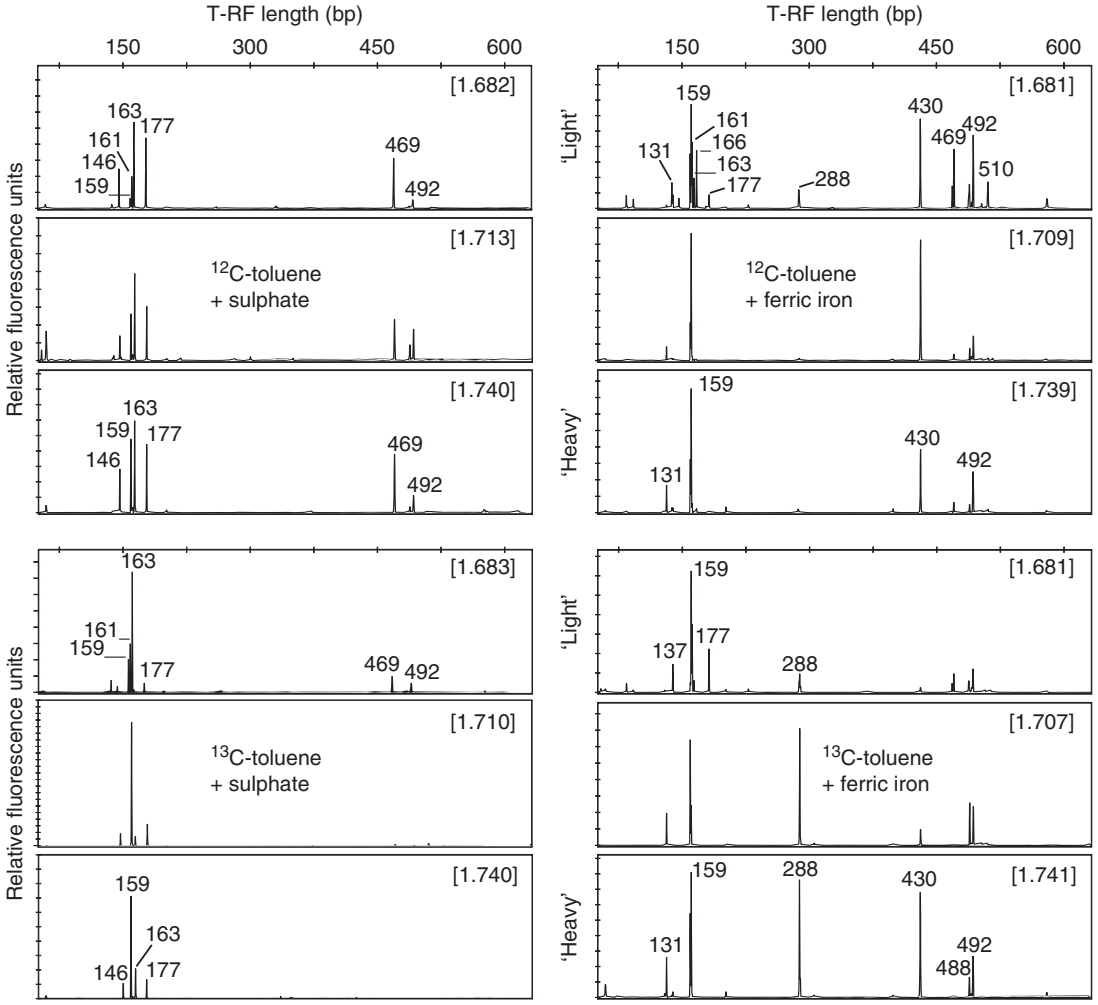

Fig. 3. Bacterial 16S rRNA gene T-RFLP fingerprints from density-resolved SIP gradient fractions of DNA from microcosms amended with unlabelled (^{12}C) or $^{13}C_7$-toluene and comparative electron acceptors. The lengths (bp) of selected T-RFs are given. Numbers in parentheses are CsCl BDs (g mL^{-1}) of gradient fractions.

Table 1. Number of reads and average read lengths produced in bidirectional pyrosequencing of ~520-bp bacterial 16S rRNA gene fragments from comparative SIP microcosm DNA

Electron acceptor	Sulphate	Ferric iron
Total forward reads*	1981	2441
Total reverse reads*	1781	2053
Average length total reads (bp)	496	492
Trimmed forward reads, > 250 bp	1653	1827
Trimmed reverse reads, > 250 bp	1169	1428
Average length trimmed reads (bp)	355	358
Amplicon contigs†	67	64

*The two samples were sequenced among 14 distinct MID-barcoded amplicons on 1/8 FLX picotiter plate.

†Criteria for the assembly of full-length (~520 bp) amplicon contigs are described in the text.

bssA genes detected in SIP gradient fractions

Finally, we aimed to affiliate the *bssA* genes previously detected *in situ*, tentatively named the 'F1-lineage' (Winderl *et al.*, 2007), to either key sulphate- or iron-reducing toluene degraders detected in SIP incubations. For this, we performed *bssA*-targeted T-RFLP fingerprinting of density-resolved DNA gradient fractions, to screen for and identify *bssA* sequence types via T-RFs. For the hitherto unidentified 'F1-lineage' of *bssA* genes, *Taq*I restriction of amplicons produces a 478-bp T-RF, which was found to dominate the fractions of density-resolved DNA from sulphate- and iron-reducing SIP microcosms (Fig. 5). Additionally, an as yet unidentified 248-bp T-RF was found in DNA fractions from sulphate treatments and a further unidentified 77-bp T-RF in fractions from iron treatments.

Table 2. Phylogenetic affiliation of trimmed reads produced in pyrosequencing of bacterial 16S rRNA gene fragments retrieved from comparative SIP microcosm DNA

	Sulphate		Ferric iron		
Phylogenetic affiliation*	Reads	%	Reads	%	T-RF (bp)†
Bacteria	2822		3254		
Unclassified *Bacteria*	143	**5.1**	169	**5.2**	NA
Proteobacteria	1869	**66.2**	2034	**62.5**	
Unclassified *Proteobacteria*	19	**0.7**	12	**0.4**	NA
Alphaproteobacteria	3	**0.1**	20	**0.6**	NA
Betaproteobacteria	30	**1.1**	959	**29.5**	NA
Unclassified *Betaproteobacteria*	10	0.4	294	9.0	NA
Rhodocyclaceae	1	0.0	231	7.1	430
Comamonadaceae	17	0.6	414	12.7	ND
Curvibacter spp.	0	0.0	130	4.0	488
Rhodoferax spp.	1	0.0	115	3.5	492
Gammaproteobacteria	52	**1.8**	87	**2.7**	NA
Deltaproteobacteria	925	**32.8**	852	**26.2**	NA
Unclassified *Deltaproteobacteria*	8	0.3	262	8.1	NA
Desulfobulbaceae	817	29.0	233	7.2	159
Unclassified *Desulfobacterales*	9	0.3	289	8.9	159
Desulfovibrionaceae	32	1.1	37	1.1	ND
Geobacteraceae	6	0.2	11	0.3	160
Epsilonproteobacteria	840	**29.8**	104	**3.2**	NA
Helicobacteraceae	838	29.7	97	3.0	NA
Sulfuricurvum spp.	375	13.3	35	1.1	163, 469
Unclassified *Helicobacteraceae*	454	16.1	61	1.9	ND
Firmicutes	662	**23.5**	867	**26.6**	NA
Clostridia	654	**23.2**	861	**26.5**	NA
Unclassified *Clostridales*	28	1.0	140	4.3	NA
Thermincola spp.	0	0.0	512	15.7	288
Desulfosporosinus spp.	589	20.9	41	1.3	146, 177
Unclassified *Peptococcaceae*	1	0.0	156	4.8	ND
Acidobacteria	3	**0.1**	3	**0.1**	NA
Actinobacteria	19	**0.7**	143	**4.4**	131
Bacteroidetes	20	**0.7**	14	**0.4**	NA
Chloroflexi	92	**3.3**	20	**0.6**	NA
Spirochaetes	10	**0.4**	4	**0.1**	NA

*Phylum- or division-level read abundances (bold) include genus- or lineage-specific read abundances (nonbold).

†Characteristic T-RFs were predicted for important lineages via assembled amplicon contigs, but are given as T-RFs actually observed in the electropherograms and as verified in (Winderl *et al.*, 2008). Commas separate more than one characteristic T-RF for a lineage. NA, not applicable for this level; ND, not detected for this lineage.

Discussion

The aim of this study was to unravel whether *Desulfobulbaceae*- or *Geobacteraceae*-related putative toluene degraders detected at the highly active lower fringe of a contaminant plume (Winderl *et al.*, 2008) are actually important in onsite toluene degradation, and to affiliate the previously detected 'F1-lineage' of *bssA* genes (Winderl *et al.*, 2007) to one of these lineages. This objective was pursued using a novel combination of DNA-SIP and high-throughput pyrosequencing of amplicons from SIP microcosms. For the first time, this strategy was used in combination with T-RFLP fingerprinting of SIP gradient DNA to identify relevant degraders. We used freshly sampled sediments containing a diverse *in situ* aquifer degrader community as inoculum, which contrasts our study with other recent reports, where SIP has been applied to less complex laboratory cultures of anaerobic BTEX degraders (Kunapuli *et al.*, 2007; Oka *et al.*, 2008; Bombach *et al.*, 2010; Herrmann *et al.*, 2010). Both parallel SIP incubations required considerable lag phases before biodegradation became noticeable, as observed previously (Winderl *et al.*, 2010), and attributed to transfer and adaptation among degraders during SIP incubation. In addition, the lack of recovered CO_2 and electrons from the added toluene may indicate the parallel involvement of fermenters and methanogens in toluene breakdown, which cannot be excluded, but seems unlikely, because sulphate and ferric iron were added in nonlimiting quantities for toluene oxidation.

Once activated, the selective role of electron acceptor availability was especially apparent for sulphate-reducing degraders, where high sulphate reduction and toluene oxidation rates were observed over a short period of time. SIP in combination with amplicon pyrosequencing revealed that degraders within the *Desulfobulbaceae*, a lineage well known to dominate at the lower plume fringe *in situ* (Winderl *et al.*, 2008; Anneser *et al.*, 2010), and for which cultivated toluene-degrading isolates (Meckenstock, 1999) and enrichments (Bombach *et al.*, 2010) have been described, were highly represented in the 'heavy' DNA fractions from sulphate-reducing microcosms. Thus, their central role in sulphidogenic toluene degradation *in situ* is unambiguously demonstrated. Moreover, the low abundance of *Geobacter*-related sequences (0.2%) in amplicon libraries from these incubations clearly precludes an affiliation of the 'F1' *bssA* lineage to degraders within the *Geobacteraceae*. Therefore, an affiliation of the 'F1' *bssA* lineage to the detected degraders within the *Desulfobulbaceae* seems warranted, even though a phylogenetically distinct *bssA* sequence type has been observed previously for other degraders within the *Desulfobulbaceae* (Winderl *et al.*, 2007; Jehmlich *et al.*, 2010). This strongly indicates another example of lateral gene transfer for this catabolic marker (Shinoda *et al.*, 2005; Winderl *et al.*, 2007), which clearly limits its identification potential for unknown catabolic homologues detected in environmental gene libraries. However, this affiliation must be interpreted with caution before isolation of a pure culture of these new *Desulfobulbaceae*-affiliated degraders, or at least until (meta-) genomic sequence data linking both markers are obtained.

A secondary population of sulphate-reducing toluene degraders identified in SIP gradients was related to *Desulfosporosinus* spp. within the *Peptococcaceae*. Although detected, but not abundant in clone libraries previously generated

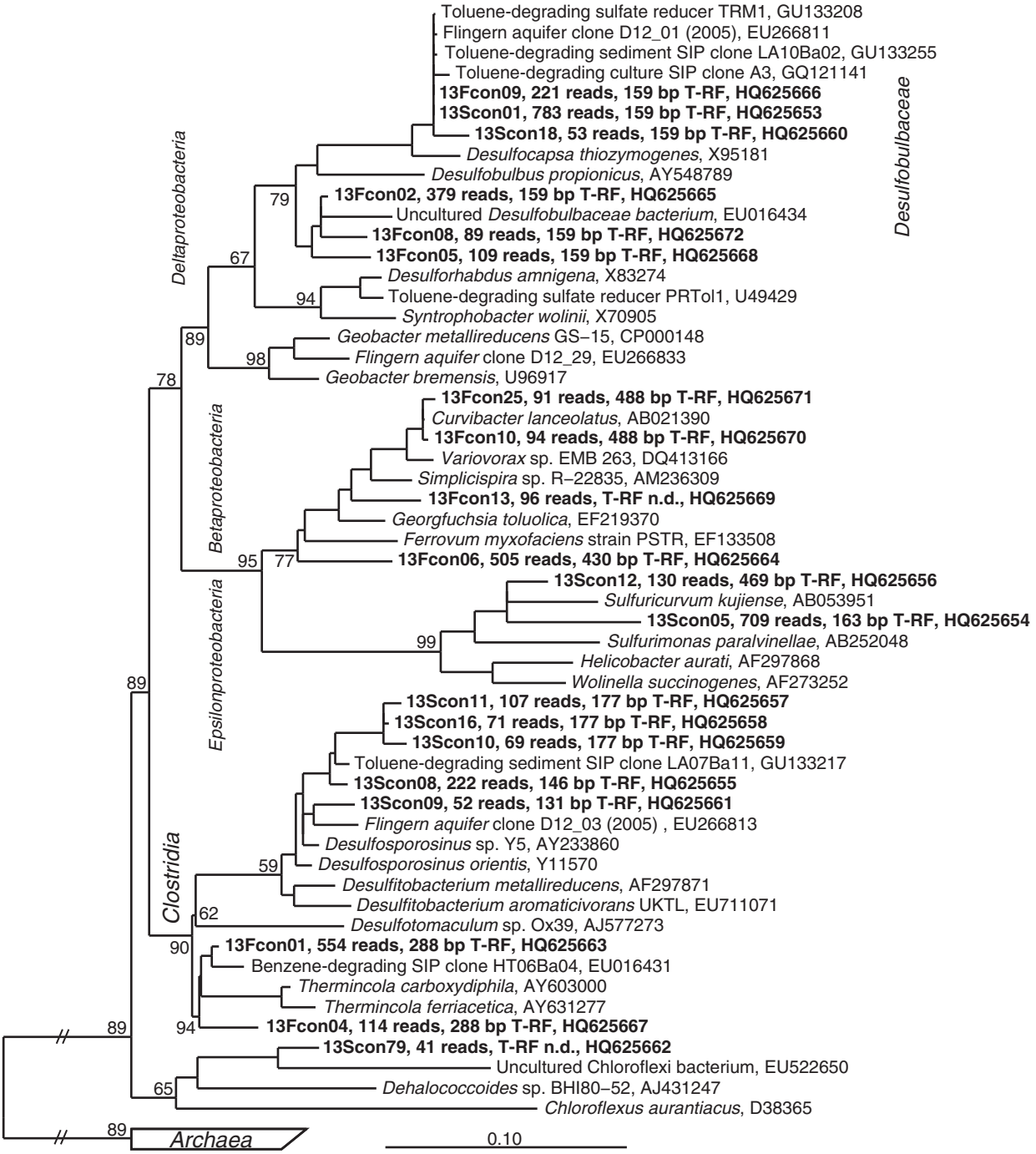

Fig. 4. Phylogenetic affiliation of selected assembled pyrosequencing contigs (given in bold) of ~520-bp bacterial 16S rRNA gene amplicons from comparative SIP microcosms. Contig naming indicates treatment (e.g. 13F, 13S) as well as comprised total reads and predicted T-RFs (bp). T-RFs were predicted from sequence data, but are given as T-RFs actually measured in electropherograms and as verified in (Winderl *et al.*, 2008). ND, no MspI restriction site in amplicon contig. The tree was reconstructed with the quartet puzzling and the subsequent addition of shorter sequences. Percentages at nodes show branching confidence values deduced from 10 000 intermediate trees. GenBank accession numbers are indicated. Scale bar, 10% sequence divergence, branch lengths to outgroup have been scaled down to 25%.

from *in situ* sediment samples (Winderl *et al.*, 2008), the capacity of members of this genus for anaerobic toluene degradation has already been demonstrated (Liu *et al.*, 2004; Winderl *et al.*, 2010). Their detection as secondary degraders in SIP suggests the possibility of functional redundancy among sulphate-reducing toluene degraders *in situ*.

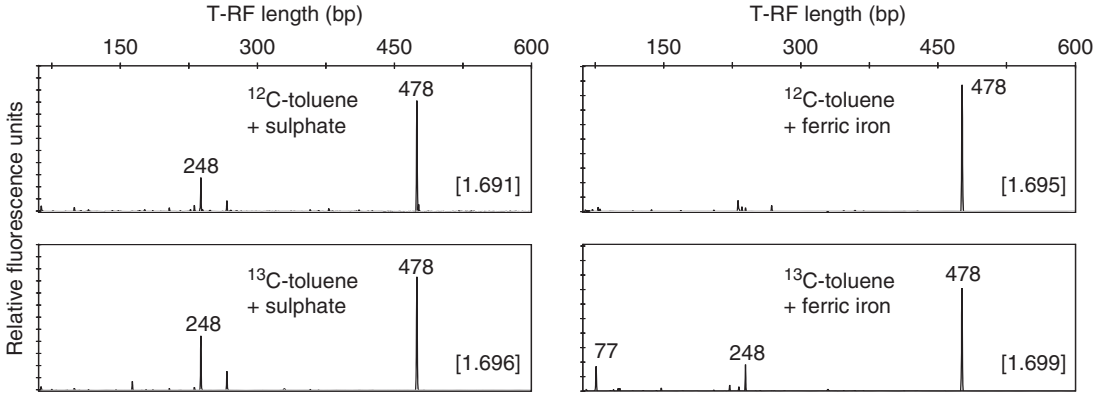

Fig. 5. Bacterial *bssA* gene-based T-RFLP fingerprints of representative density-resolved SIP gradient fractions. The lengths (bp) of selected T-RFs are given. Numbers in parentheses are CsCl BDs (g mL^{-1}) of gradient fractions.

An important unlabelled lineage detected in the sulphate-reducing microcosms was *Sulfuricurvum* spp. within the *Epsilonproteobacteria*. These microorganisms are normally capable of sulphide oxidation coupled to reduction of nitrate or oxygen (Kodama & Watanabe, 2004; Campbell *et al*., 2006). Although traces of nitrate could have been present in our microcosms, they would not have sustained abundant sulphide-oxidizing populations, as indicated by pyrosequencing reads. Intriguingly, similar *Epsilonproteobacteria* have been detected recently in sulphate-reducing, toluene- and benzene-degrading enrichment cultures (Kleinsteuber *et al*., 2008; Müller *et al*., 2009), where their function also remained unclear (Herrmann *et al*., 2010).

In iron-reducing SIP microcosms, specific toluene degradation activities and electron recoveries appeared much lower, unsurprisingly, as added toluene was apparently not completely oxidized during incubation. The dominating labelled phylotype of iron-reducing toluene degraders detected in SIP was related to the genus *Thermincola* within the *Peptococcaceae* (288-bp T-RF). Relatives of this lineage have also been identified recently as iron-reducing benzene degraders (Kunapuli *et al*., 2007), but their involvement in toluene degradation has not been described to date. Another labelled phylotype detected in our DNA gradients was within the *Rhodocyclaceae*. Recently, a novel iron-reducing betaproteobacterial toluene degrader, *Georgfuchsia toluolica*, has been isolated (Weelink *et al*., 2009) from a BTEX-contaminated aquifer. The sequence type represented by the 430-bp T-RF clustered close to *Ferrovum myxofaciens* (Hallberg *et al*., 2006) and *G. toluolica* within the *Rhodocyclaceae*. Therefore, the diversity of iron-reducing BTEX degraders within the *Betaproteobacteria* may also be larger than recognized previously.

Because the signatures of both *Georgfuchsia*- and *Thermincola*-related degraders (the 430- and 288-bp T-RFs) were hardly detectable in the respective aquifer sediments (Winderl *et al*., 2008), this illustrates their enrichment as iron-reducing toluene degraders during our SIP incubation. In contrast, the *Geobacteraceae* shown to be dominant *in situ* (Winderl *et al*., 2008; Anneser *et al*., 2010), and that were also abundant (~15% relative T-RF abundance at the lower plume fringe) in the samples used for SIP incubation (data not shown), do not appear to be directly involved in on-site toluene breakdown. Likely, these abundant *Geobacteraceae* catabolize some of the other electron donors potentially provided by the plume (e.g. hydrocarbons other than toluene, fatty acids). Also at the BTEX-contaminated aquifer from which *G. toluolica* was isolated (Weelink *et al*., 2009), members of the *Geobacteraceae* are widespread and abundant, but not directly involved in toluene breakdown (Staats *et al*., 2011).

If *Geobacter* populations are not involved in toluene oxidation *in situ*, this again supports linking the 'F1-lineage' of *bssA* genes (Winderl *et al*., 2008) to the *Desulfobulbaceae* as key toluene degraders in the Flingern sediments. As mentioned above, these *Desulfobulbaceae*-related degraders were also found (albeit unlabelled) in the iron-reducing incubations. Therefore, the detection of the 'F1-lineage' sequence type in both DNA gradients seems warranted. At the same time, no known *bssA* sequence types were inferable from DNA gradients for the clostridial and betaproteobacterial toluene degraders detected in surplus. Here, we cannot be sure that the primer pair utilized for *bssA*-fingerprinting is actually capable of detecting all relevant fumarate-adding gene lineages potentially present in our SIP microcosms. Although the primer set has been demonstrated to detect readily the *bssA* of most known iron- and sulphate-reducing toluene degraders (Winderl *et al*., 2007), primer development and optimization for the detection of fumarate-adding catabolic genes is still an ongoing process.

In summary, our results unambiguously reveal the central role of *Desulfobulbaceae* in toluene degradation at the lower plume fringe at the Flingern contaminated aquifer. Second, we substantiate the absence of true iron-reducing toluene-

degrading populations in the same zone, because only degraders not previously detected or abundant *in situ* were identified in SIP. Interestingly, secondary toluene degraders within the *Peptococcaceae* detected under both sulphate- and iron-reducing conditions give rise to speculations about niche differentiation or functional redundancy among degradative potentials on site. Finally, we show that new technologies such as amplicon pyrosequencing can be of substantial benefit for the assignment of catabolic capacities to defined lineages in SIP. Hence, amplicon pyrosequencing in combination with fingerprinting of density-resolved nucleic acids was established as a powerful new strategy in SIP gradient interpretation.

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