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ORIGINAL PAPER

Detection of monochlorobenzene metabolizing bacteria under anoxic conditions by DNA-stable isotope probing

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Abstract Cultivation-independent analyses were applied to study the structural diversity of the bacterial community which developed in groundwater inoculated microcosms actively metabolizing monochlorobenzene (MCB) under anaerobic conditions. Addition of ^{13}C-labelled MCB demonstrated that the community produced $^{13}CO_2$ as a metabolite at slightly increasing rates over a period of 1,051 days while no ^{13}C-methane evolved. Genetic profiles of partial 16S rRNA genes generated with the single-strand conformation polymorphism (SSCP) technique by PCR from directly extracted total DNA revealed that, despite the long incubation period, six replicate microcosms were characterized by almost the same microbial members. Nine distinguishable contributors to the SSCP-profiles were characterized by DNA sequencing, revealing the presence of different members from the phyla *Proteobacteria*, *Fibrobacteres* and from the candidate division OD1. DNA-stable isotope probing (SIP) was applied to distinguish the actual MCB metabolizing bacteria from the other community members. This study reveals for the first time the structural diversity of an anaerobic MCB metabolizing bacterial community. However, it also demonstrates the limitations of SIP to detect bacteria slowly metabolizing carbon sources under anaerobic conditions.

Keywords Biodegradation · Contamination · Chlorobenzenes · Bitterfeld · Stable isotope probing

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Introduction

Monochlorobenzene (MCB) is a highly recalcitrant groundwater contaminant forming large plumes in contaminated aquifers worldwide. MCB is well degraded under aerobic conditions and this process has been studied extensively [for a review see van Agteren et al. (1998)], but yet little is known about the degradation of MCB under anoxic conditions. Previously, Nowak et al. described the anaerobic dehalogenation of MCB to benzene in microcosms

but only when additionally provided with higher chlorinated benzenes (Nowak et al. 1996). Recently, Fung et al. described the reductive dechlorination of dichlorobenzenes and MCB to benzene in anaerobic microcosm studies (Fung et al. 2009). During the last years, our laboratory provided evidence for the anaerobic degradation of MCB under anoxic conditions at two different field sites (Kaschl et al. 2005; Braeckevelt et al. 2007; Nijenhuis et al. 2007; Stelzer et al. 2009). Compound specific isotope analysis of MCB indicated degradation under anoxic conditions in the aquifer. In additon, stable isotope tracer studies using in situ microcosms supported these findings and showed that MCB was used as a carbon source for microbial biomass formation in situ. Parallel laboratory microcosm studies using stable isotope labelled MCB provided evidence for the mineralization of MCB under anoxic conditions.

Thus far, no information is available regarding the diversity of microorganisms involved in anaerobic MCB degradation and also actual degradation pathway remains to be revealed. Two main types of hypothetical degradation pathways could be proposed (1) reductive dehalogenation of MCB producing benzene which could then be further degraded (Nowak et al. 1996; Adrian and Gorisch 2002; Fung et al. 2009) or (2) initial modification of the benzene ring forming for example a chlorinated benzoate, phenol or chlorotoluene as proposed for benzene (Edwards and Grbić-Galić 1992; Anderson et al. 1998; Lovley 2000; Phelps et al. 2001; Coates et al. 2002). No evidence exists proving strong support for either pathway although benzene is found as a usual co-contaminant with MCB (Nowak et al. 1996; Kaschl et al. 2005; Nijenhuis et al. 2007; Stelzer et al. 2009).

In our previously described microcosms prepared from MCB contaminated, sulfate rich (>1 g/l) groundwater (Nijenhuis et al. 2007), production of $^{13}CO_2$ indicated mineralization of MCB derived carbon and the incorporation of MCB derived carbon was supported by the observation of ^{13}C-labelled total lipid fatty acids (TLFA) in these microcosms. The amount of MCB degradation was very low (<10% of the added MCB) after more then 1,050 days and the link between the observed degradation and a terminal electron accepting process could not be substantiated (Nijenhuis et al. 2007).

In this study, we characterized the diversity of the microbial community from actively metabolizing microcosms (enrichment cultures) inoculated with groundwater and incubated in presence of MCB for 1,051 days. DNA was extracted directly from the microcosms and subjected to PCR amplification of partial 16S rRNA genes using primers hybridizing to highly conserved regions of the gene. The diversity of PCR products was visualized by genetic profiling using electrophoretic separation by single strand conformation polymorphism (SSCP) (Schwieger and Tebbe 1998; Dohrmann and Tebbe 2005). Contributors to the profiles were characterized by subsequent DNA-sequencing of the PCR products. In order to directly detect and identify actively MCB metabolizing microorganisms an attempt was made using ^{13}C-MCB and stable isotope probing (SIP) of total DNA. In context of other substrates, SIP has been proven to be potentially applicable for such purposes (Neufeld et al. 2007b), e.g., link the microbial community structure with function in benzene degrading communities (Kasai et al. 2006; Kunapuli et al. 2007; Oka et al. 2008).

Materials and methods

Chemicals

The [$^{13}C_6$] chlorobenzene was purchased from Chemotrade Leipzig (Germany) with chemical and isotopic purity of >99%. The [$^{13}C_6$] chlorobenzene preparation further contained an impurity of 0.02% [$^{13}C_6$]-benzene. All other chemicals were obtained in p.a. quality or higher.

Microcosms

Microcosms were prepared as described previously under anoxic conditions (Nijenhuis et al. 2007). Briefly, MCB contaminated groundwater from well SAF11 at the field site in Bitterfeld was taken and after transport to the laboratory, microcosms were prepared in 120 ml vials with 100 ml liquid under anoxic conditions using an anaerobic glovebox (98–96% N_2/2–4%H_2). The vials were closed with Teflon coated grey butyl rubber stoppers and crimped with aluminum crimps. In total, 11 microcosms were prepared. Of this set, two microcosms were autoclaved for 40 min on three consecutive days to prepare sterile controls. Then, three non-sterilized

and the three sterilized microcosms were amended with natural abundance (n.a.) MCB and the other three with [$^{13}C_6$]-MCB to a concentration of approx. 100 μmol l^{-1}. The microcosms were incubated at 20°C as static cultures (without shaking) and the carbon isotope composition of methane and CO_2 was analyzed frequently over time using a gas chromatography-combustion-isotope-ratio-monitoring-mass-spectrometer system (GC-C-IRMS) as described previously (Nijenhuis et al. 2007).

DNA extraction and CsCl gradient centrifugation

Six bottles of the incubation experiments were sacrificed, three amended with [$^{13}C_6$]-MCB and three with n.a. MCB, after 1,051 days of incubation. The cells were collected using a filtration unit (Sartorious, Germany) and polyethersulfone filters of a pore of 0.2 μm (Pall Corporation, USA). The filters were cut in small pieces and distributed in 2 ml screw-cap tubes containing Lysing matrix B (Qbiogene, MP biomedicals, USA) and 250 μl TNS buffer (500 mM Tris–HCl pH 8.0, 100 mM NaCl, 10% SDS) and 750 μl of $NaPO_4$ buffer. The mixture was homogenized on a FastPrep FP120 instrument (Savant Instruments, USA) at 6.5 m/s for 45 s. After centrifugation DNA was extracted according to the protocol of Lueders et al. (2004) with an additional bead-beating step for cell lysis. For stable isotope probing (SIP), CsCl gradient centrifugation was performed in 4.9 ml polyallomer Optiseal tubes and using a VTI 65.2 vertical rotor (Beckman Coulter). The centrifuge used in the study was a Sorvall OTD combi and centrifugation was done at 20°C for 39 h at 43,000 rpm. The preparation of gradient buffer and CsCl centrifugation media with an average density of $\sim$1.72 g l^{-1} was done as described previously (Lueders et al. 2004). Approximately 100 ng of total DNA was loaded for each gradient and centrifuged gradients were fractionated into 11 aliquots, each with a volume of approx. 300 μl. Fractions were collected from bottom to top by displacement with water from the top using a peristaltic pump. Density of each gradient fraction was determined using an AR200 digital refractometer (Reichert Inc., Depew, NY, USA). DNA was retrieved from each gradient fraction with polyethylene glycol precipitation, washed in 70% ethanol and dissolved in 30 μl elution buffer (Qiagen, Hilden, Germany).

PCR amplification and SSCP

For microbial community analyses, genetic profiles were generated by single strand conformation polymorphism (SSCP) based on PCR amplified partial 16S rRNA gene sequences. DNA extracted from the enrichment cultures (microcosms) and, for SIP analyses, DNA extracted from the CsCl gradient centrifugation were used as a template. The 16S rRNA gene sequences were amplified using universal primers for bacteria (Com1: 5′-CAGCAGCCGCGGTAATAC-3′, and Com2-Ph: 5′-CCGTCAATTCCTTTGAGTTT-3′) (Schwieger and Tebbe 1998). These primers amplify a 400 bp fragment which includes the two variable regions, V4 and V5. The Com2-Ph primer was phosphorylated at the 5′end for the following single-strand digestion (Schwieger and Tebbe 1998). A reaction mixture of 50 μl contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.5 μM each primer, 1.75 U HotStar *Taq* DNA Polymerase (QIAGEN, Hilden, Germany), and 1 μl of template (dilutions of the extracted DNA in 10 mM Tris–HCl, pH 8.0). The PCR program included an initial denaturation for 15 min at 95°C, and 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 70 s extension; followed by a final elongation step of 5 min at 72°C. PCR products size was verified by gel electrophoresis on 1.2% agarose gels. To avoid possible biases during individual PCR amplification, three independent PCR reactions were performed for each template DNA in parallel. Subsequently the PCR products of the three parallels were combined, purified (QIAquick spin columns QIAGEN) and quantified with PicoGreen reagent for double-stranded DNA (Molecular Probes, Eugene, Oreg.). Single-strand digestion and SSCP profiles were performed as described previously (Dohrmann and Tebbe 2005). In the case of DNA from the CsCl gradient fractions, equal volumes were digested rather than equal quantities of DNA. The processing of the SSCP profile gels was done using the GelCompar software (Applied Maths NV, Sint-Martens-Latem, Belgium).

Cloning and sequencing of partial 16S rRNA genes

Dominant bands of SSCP profiles were cut out from dried gels to obtain the sequences of 16S rRNA

genes. The single strand DNA was recovered by the crush and soak procedure (Sambrook et al. 1989). The DNA was then amplified by PCR with the primers Com1 and Com2-Ph, and purified as described above. The PCR products were then cloned into competent cells of *Escherichia coli* JM109 using the pGEM-T Vector system from Promega (Mannheim, Germany). The clones were further amplified with the Com1 and Com2-Ph primers. The resulting PCR products were subjected to another SSCP procedure to compare their electrophoretic mobility with the bands from the original community profiles. Only cloned PCR products in the expected positions were further sequenced. The obtained sequences were compared to public databases using blastn to identify closely related sequences (http://www.ncbi.nlm.nih.gov/blast/) and analyzed using the Classifier tool provided by the Ribosomal Database Project (http://rdp.cme.msu.edu/).

Phylogenetic tree construction

A Maximum-likelihood tree of 16S rRNA sequences was calculated with the ARB software. The ARB database (ssu_jan04_corr_opt) was updated for the closest related gene sequences of the new sequences found by database searches and by sequences of the candidate division OD1. Sequences of >1,000 nucleotides were selected to build a tree applying the AxML algorithm and the included filter "bacteria_rrn_dec04". Shorter sequences were added by the "parsimony interactive" tool by application of individual group filters. Individual group filters of 50% conservation were calculated for each sequence on >30 closely related sequences to the new sequences of >1,300 nucleotides length. Nucleotide sequences are accessible at the EMBL database under accession numbers FN868322–FN868332.

Data analysis

All statistical analyses were carried out using the R software (R Development Core Team, 2008; Version 2.6.2). A non-metric multidimensional scaling (nm-MDS), based on the binary numerical matrix (including the presence or absence of SSCP bands) converted to a dissimilarity matrix was used to evaluate the changes in bacterial community structures among microcosms.

Results

Microcosms

Anaerobic microcosms prepared with groundwater from the contaminated plume in Bitterfeld/Wolfen region and spiked with ^{13}C-MCB were analyzed after 1,051 days of incubation [see also (Nijenhuis et al. 2007)]. As a control, sterilized microcosms as well as microcosms spiked with ^{12}C-MCB were incubated in parallel. Activity was analysed using the carbon isotope signature of CO_2 and methane in the microcosms. MCB mineralisation was only indicated with the non-sterilized microcosms, demonstrating that the biological activity was crucial. Progressive enrichment of $^{13}CO_2$ over time was observed with a maximum δ value of +553 [‰] (+330; 553; and 545‰ respectively for three parallel microcosms used) corresponding to about 4.5–7.3% (or 0.45–0.73 µmol) of the added MCB. In contrast, there was no significant enrichment in $^{13}CH_4$ during the 1,051 days (see Fig. 1), indicating that methanogens were not involved in the degradation.

Microbial community analysis

A total of six microcosms was analyzed for the diversity of 16S rRNA genes amplified from the consortia which developed during the 1,051 days. Band from the individual SSCP profiles of each

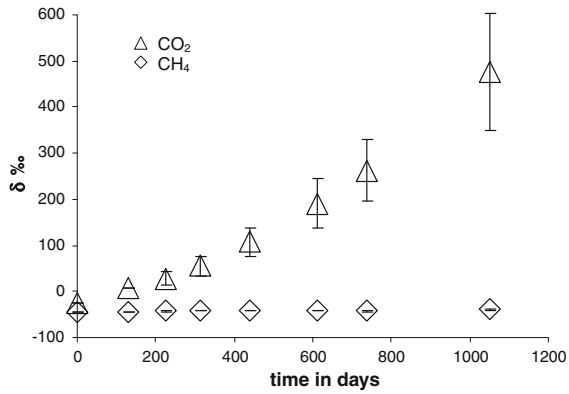

Fig. 1 Carbon isotope signature of CO_2 (*open triangle*) and methane (*open diamonds*) in the microcosms prepared with groundwater from SAF11. The average for the three microcosms used for this study is shown. *Error bars* depict standard deviation obtained from three replicate microcosms. If the *error bars* are not visible, they are smaller then the *symbols*

microcosm varied in their intensities, but consistently nine different bands could be detected (Fig. 2). However, slight variability between the different microcosms could be observed probably due to the long incubation times of the microcosms. Despite these differences, there was an indication that the bacterial communities generally consisted of the same members, which was confirmed by statistical analysis of the community profiles (dissimilarity indexes Jaccard <0.8; NMDS scales <1).

In order to identify the bacteria contributing to the profiles, nine different bands, indicated in Fig. 2 as a to i, were characterized by DNA sequencing. Phylogenetic assignment of the obtained sequences indicated that bacteria originated from three phyla, i.e., *Proteobacteria* (subclasses beta, delta and epsilon), *Fibrobacteres* and candidate division OD1 (Fig. 3).

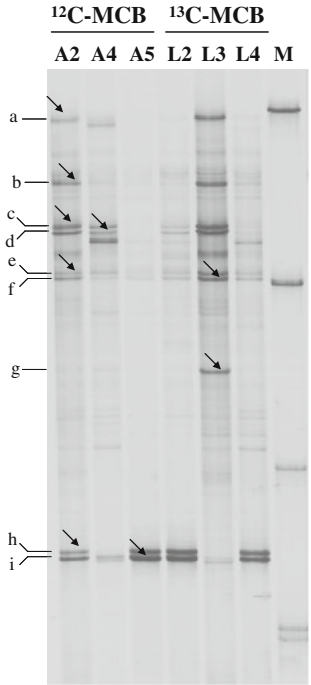

Fig. 2 Analysis of 16S rRNA gene fragments from DNA extracted from anoxic microcosms metabolizing monochlorobenzene (MCB) by SSCP. Migration patterns obtained with single stranded PCR products of 16S rRNA genes amplified from anaerobic microcosms amended with ^{12}C-MCB (*A2*, *A4*, *A5*) and ^{13}C-MCB (*L2*, *L3*, *L4*) bacterial community. The sequenced bands are indicated with an *arrow*. *M* SSCP migration marker, each band represent PCR-amplified rRNA genes from pure cultures of (from *top* to *bottom*) *Bacillus licheniformis*, *Rhizobium trifolii*, *Flavobacterium johnsoniae*, and *Rhizobium radiobacter* (*double band*)

Most of the sequences retrieved from the gel bands (except for a, h and i) were most closely related to sequences from yet uncultured bacteria (see Table 1) which were detected in samples from anoxic and/or contaminated environments, for example, anaerobic fluidized bed bioreactors treating 2,4-dinitroanisole (band b) and hydrocarbon contaminated aquifer (band c, d and g). The sequence retrieved from band a (MC_L3.3a) was identical to the sequence of a strain of the *Acidovorax* genus. The sequences representing band h and i (MC_L4.1a and MC_L4.4a, respectively), were most closely related to *Sulfuricurvum kujiense*, which is a facultative sulphur-oxidizing bacterium (Kodama and Watanabe 2004). Band e and f were closely related to *Desulfocapsa thiozymogenes*. This organism was described as a sulfate reducing bacterium capable to disproportionate inorganic sulphur compounds (Janssen et al. 1996). The sequences represented by bands c and d (MC_L3.6a and MC_A2.7a) clustered closely with an uncultured bacterium from the *Hydrogenophilus* genus. Members of this genus are capable of oxidizing hydrogen and sulfur compounds (Miyake et al. 2007).

The phylogenetic assignment did not allow inferring the exact physiological roles of the different bacterial community members and their metabolic interactions. Surprisingly, only one of the sequences obtained was related to sulfate reducing bacteria although sulfate constitutes the main electron acceptor in the media.

SIP analysis

In order to elucidate further the role of the individual bacterial community members, SIP analyses were conducted. Due to the low biomass of the individual microcosms it was necessary to combine the DNA extracted from three microcosms which had been amended with ^{12}C-MCB and ^{13}C-MCB, respectively. For identification of PCR-amplified partial 16S rRNA genes specifically obtained from ^{13}C enriched fractions, comparisons were made to gradients run with DNA obtained from replicate microcosms fed with ^{12}C-MCB. Each density fraction of the gradients was analysed for their presence of partial 16S rRNA genes by PCR-SSCP (Fig. 4). In fact, there were two bands present in the heavy fractions from the labelled samples that did not appear in the unlabeled samples.

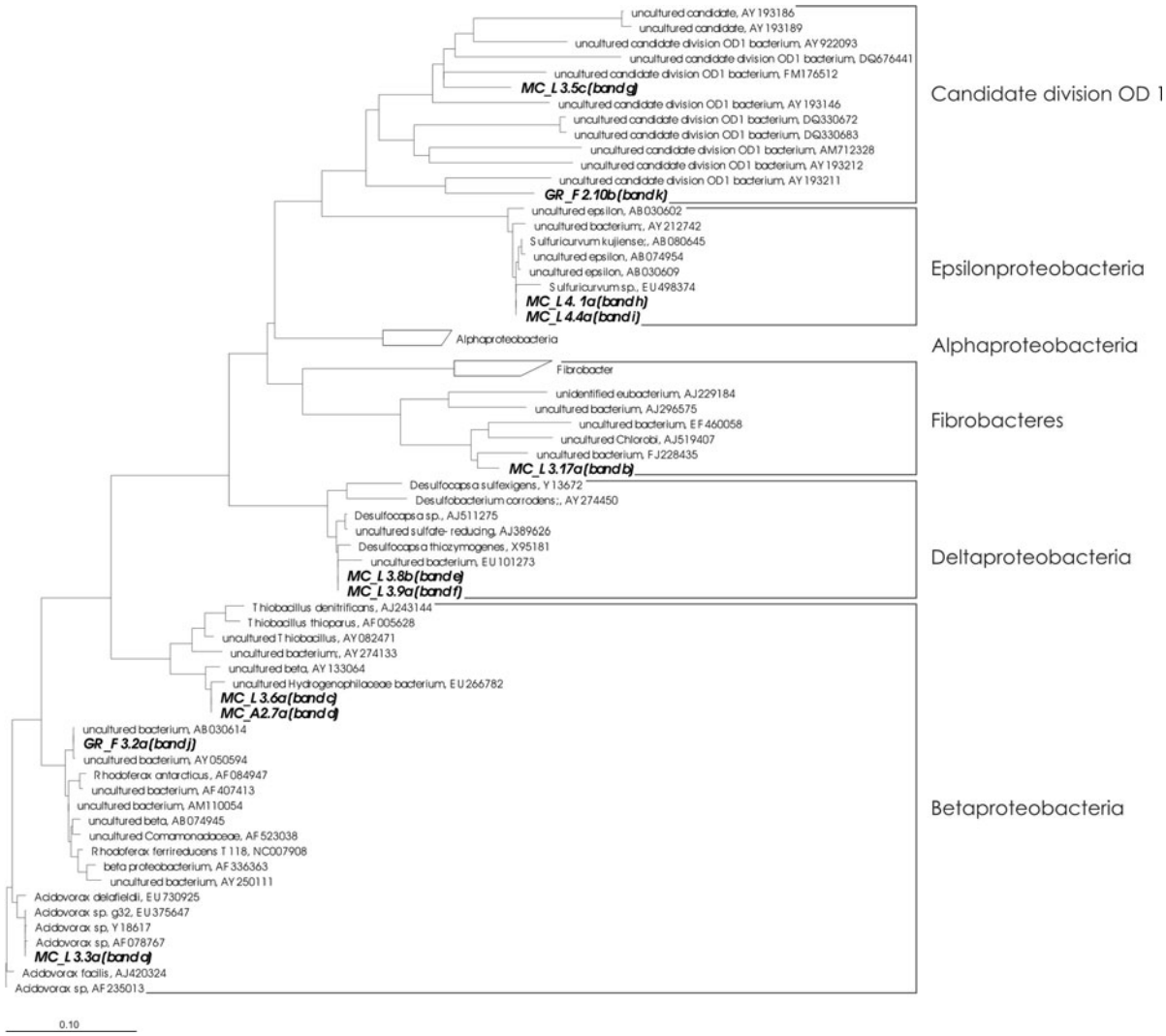

Fig. 3 Phylogenetic tree of 16S rRNA sequences recovered from PCR-SSCP bands indicated with *bold letters* and designated according to the sample origin as (MC) when coming from microcosms and as (GR) when bands were retrieved from the gradient SSCP-profile. The sequences obtained were aligned with sequences from the ARB database, updated with new sequences found by database searches and with sequences of the candidate division OD1. The maximum-likelihood tree of 16S rRNA sequences was calculated using the ARB software

These two bands were candidates to represent microorganisms contributing to the MCB degradation. Sequence GR_F3.2a (Fig. 4, band j) which was found in the heavy fraction was identical to a sequence from *β-Proteobacteria* (AY050594) obtained in another study from a reactor system treating the MCB contaminated aquifer in Bitterfeld (Alfreider et al. 2002). The second sequence (GR_F2.10b, band k) belonged to the candidate division OD1, most closely related to a sequence retrieved from sulphur river filaments (AY193211).

Because of the presence of two bands in all lanes of the SSCP-gel, with a similar migration to bands h and i, a second gradient was run using pooled DNA of the fractions. The two bands clearly moved to the light fractions, but they could be still detected in the heavy fractions from both labelled and unlabeled samples (data not shown). These facts suggested that these sequences were likely not derived from organisms degrading MCB, but probably correspond to a post-run contamination. However, the presence of these two bands did not alter the original pattern.

Table 1 Identity of 16S rRNA gene sequences of bands retrieved from SSCP profiles from anoxic microcosms growing on MCB

Phylum or class[a]	Band	Description, GeneBank accession number[b]	Identity (%)[b]	Source[b]
β-Proteobacteria	a	*Acidovorax delafieldii* strain NBGD35, HQ003420	100	Gurudongmar Lake, India
	c, d	Uncultured *Hydrogenophilaceae* bacterium clone D10_09, EU 266782	100	Tar-oil contaminated aquifer sediments
	j	Uncultured bacterium clone GOUTB7, AY050594	100	Reactor system treating MCB contaminated groundwater
δ-Proteobacteria	e, f	Uncultured bacterium clone RS06101_B83, EU101273	100	Sulfidic cave stream biofilm
ε-Proteobacteria	h, i	*Sulfuricurvum kujiense*, AB080645	99	Sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions
OD1	g	Uncultured candidate division OD1 bacterium, AY193186	92	Contaminated aquifer
	k	Uncultured candidate division OD1 bacterium, AY193211	98	Sulfur river filaments
Unclassified bacteria	b	Uncultured bacterium clone UC-20, FJ228435	93	Anaerobic fluidized bed bioreactors treating 2,4-dinitroanisole and N-methyl-4-nitroaniline

[a] Classifier Tool, Ribosomal database project II (Naive Bayesian rRNA Classifier Version 2.0) (http://rdp.cme.msu.edu)

[b] Nucleotide Blast tool, NCBI BLAST (http://www.ncbi.nlm.nih.gov/)

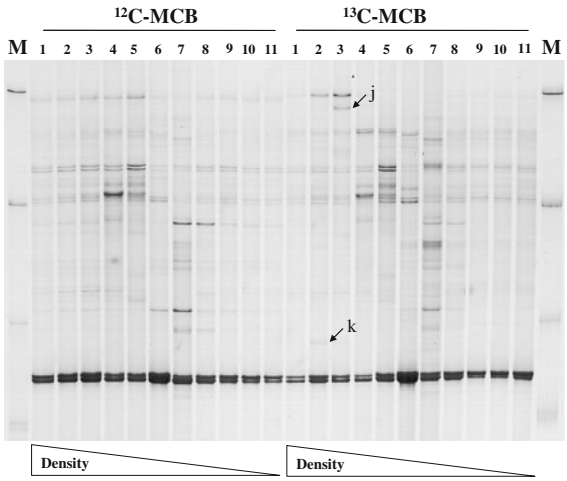

Fig. 4 SSCP profiles of 16S rRNA gene fragments of density resolved fractions obtained from DNA-Stable isotope probing gradients of ^{12}C-MCB and ^{13}C-MCB amended groundwater microcosms. The bands possibly representing microorganisms involved in MCB degradation are indicated by an *arrow*. *M* SSCP migration marker

Discussion

The SSCP-profiles and DNA sequence analyses of this study revealed that during a period of more than three years of incubation in presence of MCB and absence of oxygen, a bacterial community of approximately nine different members (phylotypes) prevailed. Most sequences were related to *Proteobacteria* and the most dominant bands belonged to the Class *Epsilon-Proteobacteria*. Surprisingly, the sequences retrieved from only two bands (f, e), which apparently came from the same organism, were close relatives to a sulfate-reducing bacterium (SRB) *Desulfocapsa thiozymogenes*. The groundwater was rich in sulfate, but the active community seems not to be specialised in using this electron acceptor. Another alternative electron acceptor could be nitrate which was found, in very low levels (approx. 3 μM), in the groundwater used as inoculum for the microcosms. The presence of a facultative aerobic organism related to *Acidovorax* (MC_L3.3a) was surprising since the microcosms were anoxic. However, previously an *Acidovorax facilis* strain UFZ B517 was isolated from the aquifer in Bitterfeld which was capable of aerobic MCB degradation but it could grow with acetate or succinate under nitrate reducing conditions (Vogt et al. 2004). This suggests a possible involvement of these organisms in degradation of MCB or its products in oxic zones of the aquifer. Since it did not appear in the

labelled community, these organisms seem not to be active in our microcosms. Other sequences were related to *Sulfuricurvum* (h, i) which is a facultative anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium (Kodama and Watanabe 2004). Also sequences related to *Hydrogenophilus* (band c and d) could be obtained. Because of the large range of putative metabolic processes associated with the detected organisms, it is difficult to establish the type of interactions between the community members.

After the density gradient centrifugation, only two sequences could be identified in the heavy fractions which were potentially related to MCB degradation. This could be due to the low extent of mineralisation of MCB in the microcosms or a high specialisation of the microbial community for MCB degradation, resulting in low amounts of ^{13}C-labelled DNA and not visible bands. It has been shown that the ^{13}C incorporation into RNA is more efficient compared to that into DNA (Manefield et al. 2002). However, during RNA-SIP experiments it is also necessary that the ^{13}C tracer is assimilated by only a fraction of the total microbiota in order to identify the active community members (Whiteley et al. 2006). Thus, long incubation times would lead to major cross-feeding effects in the system studied here. Due to the low percentage of mineralization and low biomass obtained during shorter incubation times, RNA-based SIP was not considered as an option to apply on our microcosms. One of the sequences obtained (GR_F3.2a) was previously observed in groundwater from the same aquifer as the microcosms were derived from (Alfreider et al. 2002), and according to the phylogenetic analyses it affiliates to the family *Comamonadaceae*. This family contains members capable of aerobic as well as anaerobic, nitrate reducing, metabolism (Schmalenberger et al. 2008; Weelink et al. 2008; Sun et al. 2009; Yagi et al. 2009) and some of them have been previously detected in aerobic and anaerobic benzene microcosms (Fahy et al. 2006). The other sequence present in the heavy fractions (GR_F2.10b) was associated with the OD1 cluster, which corresponds to a new candidate division close related to the candidate division OP11 (Harris et al. 2004). Sequences belonging to OD1 division have been found in many environments. However, since no cultivated members are available for this division there is no clear role of them in the nature, even some hypothesis linked them to sulphur cycle according to their distribution in the environment (Harris et al. 2004).

If biodegradation would go via an initial dechlorination, these dehalogenating microorganisms were most likely excluded from the analysis since they would have used carbon sources likely available in the groundwater to build their biomass or only would become labelled secondary after using products from MCB degradation. The observed sequences may then only reflect a community responsible for anaerobic benzene degradation. However, previous studies using DNA-SIP have suggested microorganisms belonging to family *Desulfobacteraceae* as dominant benzene degrading organism (Musat and Widdel 2008; Oka et al. 2008) which were not detected among the enriched organisms in the microcosms of this study but only in the overall community.

The results obtained in this study have to be analysed with care since several factors could be considered as sub-optimal conditions to perform DNA-SIP. First of all, the SIP experiment was performed with relatively low amount of DNA, approx. 100 ng DNA compared to previously described studies (Neufeld et al. 2007a). Second, the identification of the actively biodegrading microbial population would require an incorporation of more than 20% ^{13}C in the DNA for separation by isopycnic centrifugation for sufficiently resolving ^{13}C-DNA or RNA from ^{12}C-DNA or RNA (Radajewski et al. 2003). But, to prevent isolation of high G+C content ^{12}C-DNA in the ^{13}C-labelled fraction, a >50 atom% ^{13}C-incorporation may be required (Radajewski et al. 2003). TLFA analysis of our microcosms suggested the incorporation of max. 5% ^{13}C in the microbial biomass, but specific community members could contain a higher enrichment (Nijenhuis et al. 2007). It was not possible to link the degradation with a specific electron accepting process due to the high background concentration of sulfate and the low amount of MCB degraded (<10% of added MCB) (Nijenhuis et al. 2007). In additon, DNA-SIP would be limited by the fact that the microcosms were incubated for a very long time (>1,050 days) which could have resulted in cross feeding although this effect may have been reduced due to the fact that MCB was very slowly degraded (Radajewski et al. 2003). In any case, though, primary consumers would be expected to have the highest incorporation into the biomass of the carbon stable isotope tracer. Nevertheless, labelled

DNA could be isolated and sequenced providing information on the microbial community actively incorporating the carbon of MCB into its biomass. In general, it appears that despite these limitations, at least a first hint could be obtained of the microorganisms involved in MCB degradation following the approach used in this study. This suggests that SIP approaches may be more sensitive than suggested previously (Radajewski et al. 2003; Whitby et al. 2005; Neufeld et al. 2007a) and functions even with low degradation rates and resulting low quantities of ^{13}C-labelled DNA.

In conclusion this study revealed the structural diversity of the bacterial community evolving over more then three years of enrichment with monochorobenzene as a sole carbon source. The low total biomass and the slow mineralisation by the community members were not ideal for SIP experimental conditions to univocally reveal the metabolically active members of the community. It will be interesting to see the bacterial diversity of other enrichments under similar conditions in order to elucidate the functional contribution of the single bacterial members in the anaerobic degradation of MCB.

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