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Identification of *tertiary* butyl alcohol (TBA)-utilizing organisms in BioGAC reactors using ^{13}C-DNA stable isotope probing

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Abstract Biodegradation of the gasoline oxygenates methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE) can cause *tertiary* butyl alcohol (TBA) to accumulate in gasoline-impacted environments. One remediation option for TBA-contaminated groundwater involves oxygenated granulated activated carbon (GAC) reactors that have been self-inoculated by indigenous TBA-degrading microorganisms in ground water extracted from contaminated aquifers. Identification of these organisms is important for understanding the range of TBA-metabolizing organisms in nature and for determining whether self-inoculation of similar reactors is likely to occur at other sites. In this study ^{13}C-DNA-stable isotope probing (SIP) was used to identify TBA-utilizing organisms in samples of self-inoculated BioGAC reactors operated at sites in New York and California. Based on 16S rRNA nucleotide sequences, all TBA-utilizing organisms identified were members of the *Burkholderiales* order of the *β-proteobacteria.* Organisms similar to *Cupriavidus* and *Methylibium* were observed in both reactor samples while organisms similar to *Polaromonas* and *Rhodoferax* were unique to the reactor sample from New York. Organisms similar to *Hydrogenophaga* and *Paucibacter* strains were only detected in the reactor sample from California. We also analyzed our samples for the presence of several genes previously implicated in TBA oxidation by pure cultures of bacteria. Genes Mpe_B0532, B0541, B0555, and B0561 were all detected in ^{13}C-metagenomic DNA from both reactors and deduced amino acid sequences suggested these genes all encode highly conserved enzymes. One gene (Mpe_B0555) encodes a putative phthalate dioxygenase-like enzyme that may be particularly appropriate for determining the potential for TBA oxidation in contaminated environmental samples.

Keywords *Tertiary* butyl alcohol · Stable isotope probing · *Polaromonas* · *Methylibium*

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Introduction

Tertiary-butyl alcohol (TBA) is widely used as an industrial solvent (Clark 2002). However, environmental contamination by this compound occurs primarily as a result of biodegradation of gasoline oxygenates including methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE). Anaerobic biodegradation of MTBE can generate

TBA and, as anaerobic conditions are frequently encountered at gasoline spill sites, TBA concentrations can often exceed those of MTBE (Schmidt et al. 2004). Accumulation of TBA in ground water sources of drinking water is of concern as this compound is potentially more toxic than its parent, MTBE (Cirvello et al. 1995).

Although TBA is often only slowly biodegraded under anaerobic conditions (Wilson et al. 2005), rapid aerobic TBA biodegradation has been demonstrated in microcosm studies using stream bed sediments (Bradley et al. 1999), surface water sediments (Bradley et al. 2002) and various cultures (Deeb et al. 2003; Fortin et al. 2001; François et al. 2002; Hanson et al. 1999; Hatzinger et al. 2001; Kharoune et al. 2001; Mo et al. 1997; Piveteau et al. 2001; Salanitro et al. 1994; Steffan et al. 2000). Several pure cultures of aerobic TBA-utilizing bacteria have also been isolated and characterized. These strains include, among others, *Methylibium petroleiphilum* PM1 (Hanson et al. 1999), *Aquincola tertiaricarbonis* L108 (Müller et al. 2008), and *Hydrogenophaga flava* ENV 735 (Steffan et al. 2000). Aerobic TBA biodegradation also occurs through a cometabolic process in which some alkane-oxidizing bacteria fortuitously convert TBA to products such as 2-methyl-1,2-propanediol and 2-hydroxyisobutyric acid (Steffan et al. 1997).

Removal of TBA from contaminated ground water is problematic as this compound is fully miscible with water. Commonly used water purification approaches involving sorption of trace organics by granulated activated carbon (GAC) are also inefficient due to the limited capacity of these matrixes for TBA. Treatment of TBA contamination by conventional GAC is therefore expensive due to the need for frequent carbon replacement. However, many microorganisms can colonize GAC beds and these materials can then be used as effective biological water treatment systems if the environmental and physiological requirements of the attached microorganisms can be met and maintained.

Beyers et al. described a GAC-containing packed bed reactor for the aerobic treatment of water contaminated with alkyl ethers and TBA (Beyers et al. 2001). This reactor received oxygen and nutrients to support and sustain microbial activity. Operational versions of these systems have since become known as BioGAC reactors. To decrease reactor start up times, some BioGAC reactors have been bioaugmented with commercially available MTBE- and TBA-degrading cultures (Sun et al. 2003). In other instances the reactors have been allowed to self-inoculate using indigenous organisms present in ground water extracted from MTBE- and TBA-contaminated aquifers. The commercial cultures used to bioaugment BioGAC reactors have not been extensively characterized but, in the case of the BioRemedy product, the cultures were derived from mixed culture BC-1 originally described by Salanitro et al. (1994). *Rhodococcus aetherivorans* is a significant component of this culture (Goodfellow et al. 2004). The only previous microbiological study of organisms in a self-inoculated BioGAC reactor described two enrichment cultures (KR1 and YZ1) that both contained *Hydrogenophaga* strains (Reinauer et al. 2008).

Identification of organisms in self-inoculated BioGAC reactors is important as it can provide insights into the range of naturally occurring organisms that can degrade MTBE and TBA. Identification of these organisms can also potentially provide approaches to predict whether treatment of contaminated ground water sources could rely on self-inoculated BioGAC systems or whether bioaugmentation would be required to overcome a lack of native organisms with appropriate metabolic capabilities. Last but not least, identification of organisms in self-inoculated reactors can potentially also provide supporting evidence for the possibility of ongoing MTBE and TBA biodegradation within the aquifers from which contaminated ground water is extracted.

In this study, we have used ^{13}C-DNA-SIP to characterize TBA-utilizing organisms in self-inoculated BioGAC reactors used to treat TBA-contaminated ground water at sites in New York and California. The BioGAC sample obtained from California was obtained from the same reactors previously studied by Reinauer et al. (2008). In addition to identifying the TBA-metabolizing organisms, we were also interested to investigate whether these organisms possessed genes that have been implicated in TBA-oxidizing activity in pure cultures of TBA-metabolizing strains. The results of this study demonstrate ^{13}C-DNA SIP enabled the identification of several novel TBA-utilizing organisms from self-inoculated BioGAC reactors. Several genes that have been associated with TBA biodegradation in pure

cultures of TBA-oxidizing organisms were also detected in the ^{13}C-labeled metagenomic DNA obtained in our study. These findings have potential implications for decisions regarding bioaugmentation and self-inoculation of reactors intended to treat TBA-impacted groundwater.

Materials and methods

Materials

Carbon samples were obtained from self-inoculated BioGAC reactors at sites in Hampton Bays, Long Island (NY) and an undisclosed site in Southern California (CA). The samples were stored refrigerated (5°C) in sealed Mason jars until used for experiments. *Methylibium petroleiphilum* PM1 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM strain LMG 22953) (Gent, Belgium). $^{13}C_4$-TBA (99 at.%^{13}C) (98% purity) was obtained from Isotec (Sigma-Aldrich, Isotec, Miamisburg, OH). CsCl was obtained from J.T. Baker Ultrapure Bioreagents. Denaturing gradient gels were made with OmniPur® Acrylamide/Bis (37.5:1) and OmniPur® formamide obtained from EMD Chemicals Inc. (Gibbstown, NJ), and molecular biology grade urea obtained from Eastman Kodak Company (Rochester, NY). PCR primers were obtained from Eurofins MWG Operon (Huntsville, AL). Compressed gases (H_2, N_2, air) used for gas chromatography were obtained from local industrial vendors.

Microcosms

Microcosms were assembled with BioGAC (5 g wet weight) added to sterile glass serum vials (160 ml) using flame-sterilized spatulas. Mineral salts medium (25 ml) (Yeager et al. 1999) was added and the vials were sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton, Millville, NJ). Sterile control microcosms were constructed in the same manner except the vials were autoclaved (25 min at 121°C) three times on three separate days. When required, TBA (^{12}C or ^{13}C) (130 μmol) was added to microcosms as a neat compound using heat-treated (350°C for 30 s) glass microsyringes. When required, TBA was replenished in the microcosms by opening the microcosms in a sterile laminar flow hood. After allowing re-aeration of the gas phase for 5 min, the vials were resealed and TBA added as described previously. All microcosms were incubated in the dark at 25°C on a rotary shaker (150 rpm).

Cell cultures

Cultures of *M. petroleiphilum* PM1 and *Escherichia coli* BW545 were grown in glass vials (160 ml) sealed with butyl rubber stoppers and aluminum crimp seals and incubated on a rotary shaker (150 rpm) at either 30°C (PM1) or 37°C (*E. coli*). Strain PM1 was grown in mineral salts (25 ml) (Yeager et al. 1999) with TBA (120 μmol) as the sole carbon source. *E. coli* was grown in M9 minimal media (Sambrook and Russell 2001) (20 ml) containing D-^{12}C-glucose. Cells were harvested by centrifugation (10,000×*g* for 10 min) and DNA was isolated from the pellet using the Ultraclean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA).

Analytical methods

Consumption of TBA in all microcosms was monitored by gas chromatography (GC). Aqueous phase samples (2 μl) were taken directly from each microcosm using a heat-treated (350°C for 30 s) microsyringe and injected into a gas chromatograph (Shimadzu model GC-8A) (Kyoto, Japan) fitted with a flame ionization detector. The chromatograph was fitted with stainless steel column (0.3 by 183 cm) filled with Porapak Q (60/80 mesh) (Waters Associates, Framingham, Mass.). The column was used at a temperature of 160°C while the injection port and detector temperatures were 200 and 220°C respectively. Nitrogen was used as carrier gas and the chromatograph was interfaced with a Hewlett-Packard (Palo-Alto, CA) HP3395 integrator for data collection. The aqueous concentration of TBA was determined from a 10 point calibration plot ($r^2 \geq 0.997$) developed by adding known amounts of TBA to sterile water in a sealed glass serum vial (160 ml).

DNA isolation

The contents of each microcosm were stored at −20°C in sterile 50 ml conical tubes until processing. Samples were thawed at 50°C and then centrifuged (10,000×*g* for 10 min). DNA was isolated from each

BioGAC pellet with the PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions.

CsCl density gradient ultracentrifugation and DNA extraction

^{12}C-DNA was separated from ^{13}C-DNA by CsCl density gradient ultracentrifugation. CsCl gradients were assembled in polyallomer quick-seal ultracentrifugation tubes (11.5 ml) (Sorvall, Kendro Laboratory Products, Newtown, CT). Each tube contained TE buffer pH 8, the sample DNA solution (~30 µg total DNA), 200 µl of ethidium bromide (1%), and CsCl (1 g ml^{-1}). The tubes were centrifuged in a Beckman L8-55 ultracentrifuge using a Sorvall T-1270 rotor (140,000×*g* for 69 h at 20°C). Separated DNA fractions were visualized with UV light and carefully removed from each tube using a sterile 20-gauge needle and a 1 ml syringe (Neufeld et al. 2007). DNA was isolated from each fraction by *n*-butanol extraction (5 times) and ethanol precipitation. The DNA pellet was resuspended in 0.5× TE buffer pH 8 and visualized on a 1% agarose gel stained with ethidium bromide (1%). ^{12}C-DNA (100 ng) from *E. coli* was also added to each gradient as a control. ^{12}C- and ^{13}C-DNA fractions were analyzed for the presence of *E. coli* DNA as previously described (Sabat et al. 2000, Singleton et al. 2005) in PCR reactions using both 25-cycle and 40-cycle DNA amplifications. In 25-cycle reactions, *E. coli* was not detected in any fractions, but in 40-cycle PCR reactions, *E. coli* could be strongly detected in all ^{12}C fractions and in some ^{13}C fractions. To avoid amplification of traces of ^{12}C-DNA in ^{13}C-DNA fractions, subsequent molecular analyses were conducted with 25-cycle reactions.

Molecular analyses

Purified ^{12}C- and ^{13}C-DNA samples from CsCl gradients were used as templates in PCR reactions. For denaturing gradient gel electrophoresis (DGGE), PCR was performed using purified DNA (3 ng to 5 ng), primers 341GC forward (Muyzer et al. 1993) and 907 reverse (Lane et al. 1985) (0.5 µM each), molecular grade PCR water, and an Illustra PuReTaq Ready-To-Go™ PCR Bead (GE Healthcare Life Sciences) in a final volume of 25 µl. The hot start PCR consisted of a touchdown protocol as follows: denature at 94°C for 5 min; 10 cycles of denature at 94°C for 45 s, anneal at 65°C (decreasing by 1°C/cycle after the initial cycle to 55°C) for 45 s, and extend at 72°C for 90 s; 25 cycles of denature at 94°C, anneal at 55°C, and extend at 72°C for 90 s; and a final extension at 72°C for 7 min. Separation of PCR products was performed by DGGE using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). DGGE gels were made according to manufacturer's instructions for 6% polyacrylamide and a 30–55% denaturing gradient where 100% denaturant consisted of 7 M urea and 40% deionized formamide. Gels were electrophoresed for 14 h at 70 V and 60°C in TAE running buffer. Gels were post-stained for 25 min on a rotary shaker (30 rpm) in TAE running buffer containing ethidium bromide (1%). Gels were destained for 20 min under static conditions in TAE running buffer. DGGE gel bands were visualized with a FOTO/Prep™ UV transilluminator (Fotodyne Inc., Hartland, WI) and excised using sterile blades and forceps. Excised bands were transferred to sterile 1.5 ml tubes and washed for 15 min with 300 µl of 0.5× TE buffer pH 8. Wash buffer was removed and replaced with 50 µl fresh 0.5× TE buffer pH 8. Tubes were stored overnight (4°C) after which the gel slices were discarded. Eluted DNA was stored at −20°C. PCR was performed as previously described using extracted DNA (2 µl) as template. PCR products were purified using the QIAquick nucleotide purification kit (Qiagen, Germantown, MD), and sent to Eurofins MWG Operon (Huntsville, AL) for sequencing. All partial 16S rRNA sequences were deposited in GenBank (accession numbers HQ291074 through HQ291082).

Phylogenetic analyses

PCR sequences of partial 16S rRNA genes were analyzed and a phylogenetic tree was constructed with the Tree Builder application from the Ribosomal Database Project (RDP) Release 10 (Cole et al. 2007; Wang et al. 2007).

Gene level analysis

Primers were designed using Primer3 (Rozen and Skaletsky 2000) and GenBank sequences for *M. petroleiphilum* PM1 plasmid RPME01 (CP000556.1)

Table 1 Primer sequences for PCR amplification and DGGE analysis of *M. petroleiphilum* genes in ^{13}C-metagenomic DNA

Gene	PCR primer sequences forward (top) and reverse (bottom)
Mpe_B0532	5′-GACAACCTGCGCATCATCTA-3′
	5′-GTGCCTCTTCCTCGTAGTCG-3′
Mpe_B0541	5′-ACGCGAAGATCATGAAGGAG-3′
	5′-GTTGTCGTACGGGTGGATCT-3′
Mpe_B0555	5′-CGAGCGACACATGTACACAC-3′
	5′-ACGAAAACCAGATCGACCAC-3′
Mpe_B0561	5′-CAGCAGGTTGATGTCGTTGT-3′
	5′-TTCGATGACCTTCTGGAACC-3′
GC clamp sequence	5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC-3′

genes Mpe_B0532 (481668..482915), Mpe_B0541 (490848..492536), Mpe_B0555 (502407..503819), and Mpe_B0561 (509373..510266). Primer sequences, including the GC clamp sequence that was added to the 5' end of all forward primers, are shown in Table 1. As previously described, purified ^{13}C-DNA from BioGAC microcosms and total DNA from PM1 cultures were used as templates in PCR reactions. PCR products were separated on a DGGE gel (6% polyacrylamide with a 40% to 70% denaturing gradient where 100% denaturant consisted of 7 M urea and 40% deionized formamide), electrophoresed for 11.5 h in TAE running buffer. Bands were extracted from the DGGE gel and sequenced as previously described. Sample sequences were aligned with the corresponding GenBank gene sequence from *M. petroleiphilum* PM1 using Clustal version 2.0.8 (Larkin et al. 2007) and translated to amino acid sequences based on the *M. petroleiphilum* PM1 reading frame.

Results

Biodegradation of TBA in microcosms

When TBA (130 µmol) was added to sterilized microcosms containing BioGAC material there was an immediate and dramatic (>80%) decrease in the aqueous concentration of TBA (Fig. 1). However, after this initial decrease, the concentration of TBA remained close to constant throughout the remainder of the incubation (18 days). When TBA was added to sterile microcosms that contained mineral salts medium but no BioGAC material, this immediate decrease in TBA concentration was not observed. However, the aqueous concentration of TBA also remained close to constant throughout the remainder of the incubation. Based on these observations, the immediate decrease in TBA concentration that occurred in the presence of BioGAC was attributed to sorption of TBA by the GAC materials. In active microcosms, the same initial drop in TBA concentration was also observed. However, unlike the sterile control microcosms, all of the initial TBA was subsequently consumed over the following 12 days in the microcosm containing NY BioGAC and within 9 days in the microcosm containing CA BioGAC. In the case of NY BioGAC, a separate microcosm showed that subsequent cycles of re-aeration and addition of $^{13}C_4$-TBA resulted in more rapid rates of TBA consumption. In each case newly added TBA was consumed within 24–48 h.

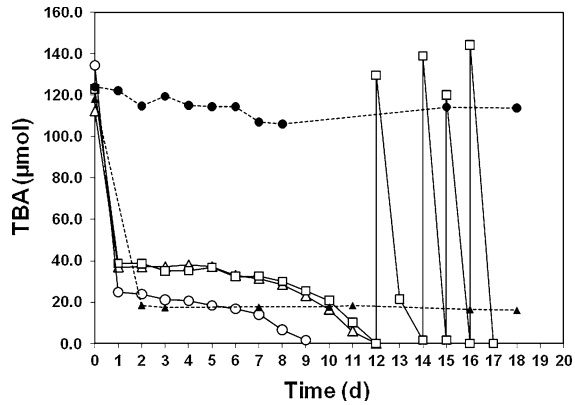

Fig. 1 Biodegradation of TBA in BioGAC microcosms. The Figure shows the time course for TBA consumption (130 µmol) in sterile (*closed symbols*) and active (*open symbols*) microcosms constructed using BioGAC samples obtained from either New York (NY) or California (CA). The microcosms were constructed as described in the "Methods" section. The symbols correspond to the following treatments; (*closed circle*) mineral salts alone + $^{13}C_4$ TBA, (*closed triangle*) mineral salts + autoclaved NY BioGAC + $^{13}C_4$ TBA, (*open triangle*) mineral salts + active NY BioGAC + a single $^{13}C_4$-TBA addition, (*open square*) mineral salts + active NY BioGAC + five sequential additions of $^{13}C_4$-TBA and (*open circle*) mineral salts + active CA BioGAC + a single $^{13}C_4$-TBA addition

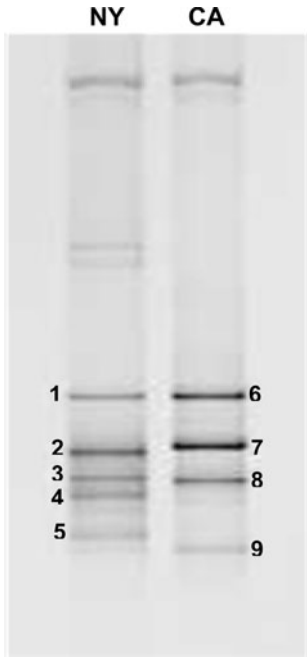

Fig. 2 DGGE analysis of partial 16S rRNA genes PCR-amplified from ^{13}C-DNA fractions The Figure shows a DGGE analysis of partial 16S rRNA genes amplified from ^{13}C-DNA obtained from the microcosms of BioGAC samples from New York (NY) and California (CA) exposed to a single addition of $^{13}C_4$-TBA (See Fig. 1). Numbered bands were extracted, sequenced, and compared to type strain 16S rRNA sequences available in the Ribosomal Database Project. Type strain isolates with closest matching partial 16S rRNA sequences are listed in Table 2

Sequence and phylogenetic analyses

After the single-pulse $^{13}C_4$-TBA incubations described in Fig. 1 were completed, total DNA was extracted and ^{12}C- and ^{13}C-DNA were separated using CsCl density gradient centrifugation. The 16S rRNA genes present in the recovered ^{13}C-DNA were PCR amplified and analyzed by DGGE (Fig. 2). Individual DGGE gel bands were excised, PCR-amplified, and sequenced as described in the Methods section. Searches in the Ribosomal Database Project Release 10 showed that multiple DGGE-resolved 16S rRNA sequences derived from ^{13}C-DNA fractions were related to *β-proteobacteria* (Table 2). Comparison of ^{13}C sequences from the NY and CA microcosms showed both similarities and differences in the microbial diversity of the TBA-oxidizing populations. Sequences related to organisms belonging to the genera *Cupriavidus* and *Methylibium* were obtained from both the NY and CA BioGAC samples. Differences in the two populations were highlighted by sequences related to *Polaromonas aquatica, Polaromonas jejuensis,* and *Rhodoferax ferrireducens* (NY only) and *Hydrogenophaga taeniospiralis* and *Paucibacter toxinivorans* (CA only). The *Hydrogenophaga* sequence for CA band 7 (Fig. 2 and Table 2) was closely related to the nearest BLAST sequence match (Genbank accession no.

Table 2 List of GenBank accession numbers and related Type Strain organisms for DNA sequences recovered following DGGE analysis of partial 16S rRNA genes amplified from ^{13}C-metagenomic DNA

DGGE Band	GenBank accession number	Related Type Strain isolates in RDP 10 (% sequence similarity)
NY Band 1	HQ291075	*Cupriavidus necator* (98.8%)
		Cupriavidus basilensis (98.8%)
NY Band 2	HQ291076	*Polaromonas aquatica* (100%)
		Polaromonas jejuensis (100%)
NY Band 3	HQ291077	*Rhodoferax ferrireducens* (99.1%)
NY Band 4	HQ291078	*Methylibium fulvum* (99.7%),
		Roseateles aquatilis (99.7%)
		Mitsuaria chitosanitabida (99.7%)
NY band 5	HQ291079	*Methylibium petroleiphilum* PM1 (99.7%)
		Methylibium aquaticum (99.7%)
CA Band 6	HQ291074	*Cupriavidus oxalaticus* (99.7%)
CA Band 7	HQ291080	*Hydrogenophaga taeniospiralis* (98.5%)
CA Band 8	HQ291081	*Paucibacter toxinivorans* (99.0%)
CA Band 9	HQ291082	*Methylibium aquaticum* (99.4%)

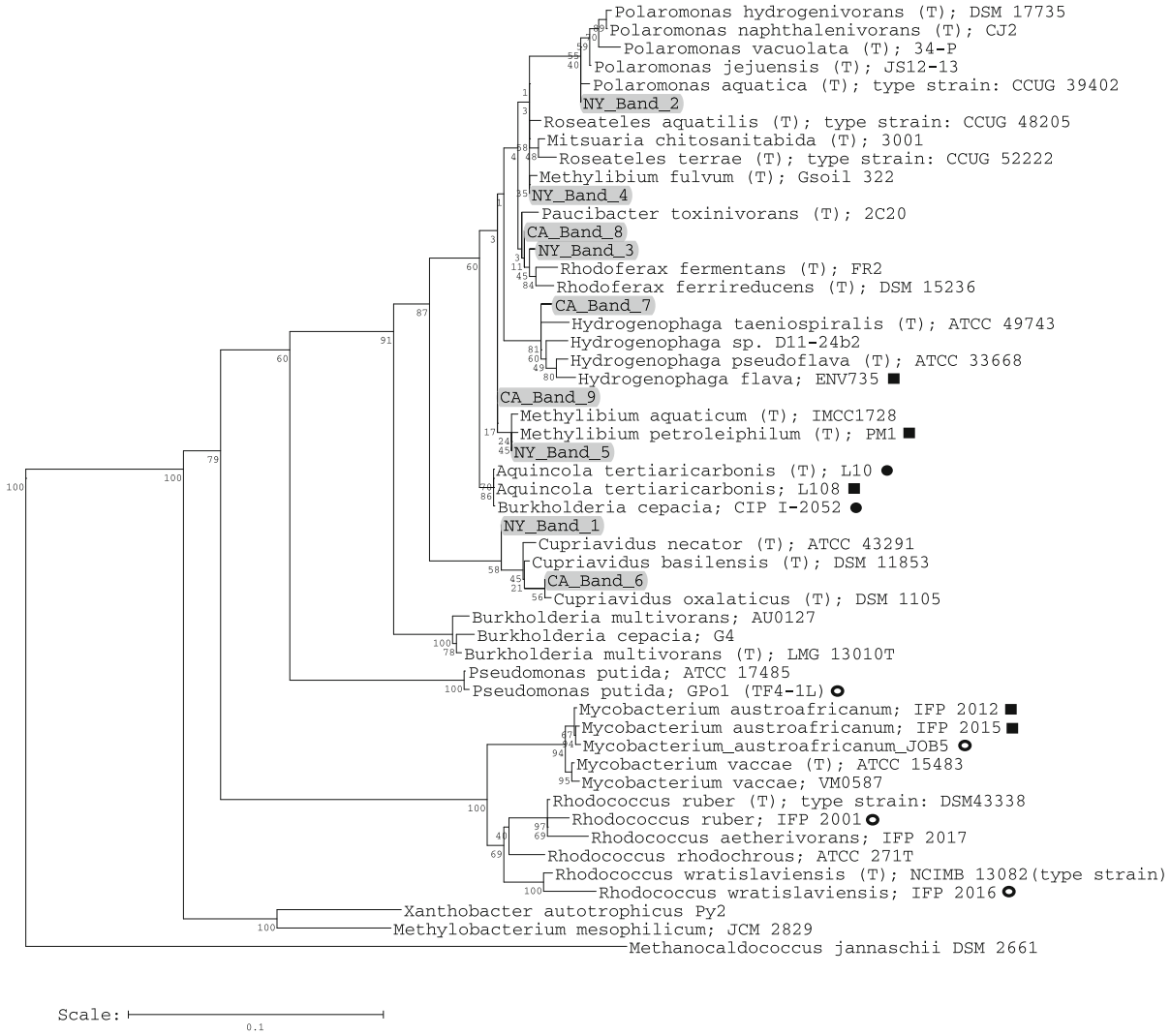

Fig. 3 Phylogenetic tree of DNA sequences recovered from DGGE analysis of partial 16S rRNA genes amplified from ^{13}C-metagenomic DNA. Symbols indicate aerobic organisms capable of (*closed circle*) growth on TBA, (*closed square*) growth on MTBE and TBA, and (*open circle*) MTBE cometabolism

AM403226) for a microbial isolate (Reinauer et al. 2008) derived from the same CA bioreactor used for the SIP experiments described in the present study. A phylogenetic analysis (Fig. 3) of the recovered DNA sequences from ^{13}C-enriched DNA indicates these sequences cluster tightly within the *Burkholderiales* order of the *β-proteobacteria,* along with other well known TBA-metabolizing organisms such as *Methylibium petroleiphilum* PM1 (Hanson et al. 1999), *Hydrogenophaga flava* ENV735 (Steffan et al. 1997), and *Aquincola tertiaricarbonis* L108 (Müller et al. 2008). In contrast, organisms associated with cometabolic MTBE biodegradation, such as *Mycobacterium austroafricanum* JOB5 (Smith et al. 2003) and *Pseudomonas putida* GPo1 (Smith and Hyman 2004) are more widely dispersed throughout the *Actinobacteria* and *γ-proteobacteria,* respectively.

Crossfeeding assessment

In stable isotope probing studies crossfeeding can occur when organisms that are not primarily

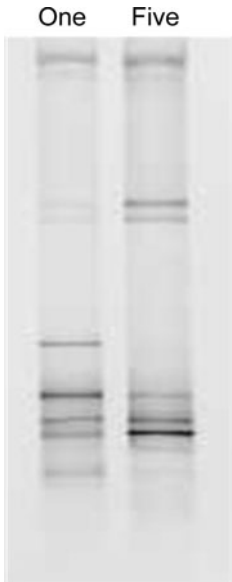

Fig. 4 Effect of TBA additions on microbial diversity in NY BioGAC samples. The Figure shows a DGGE analysis of partial 16S rRNA genes PCR-amplified from ^{13}C-DNA fractions obtained from microcosms constructed with BioGAC materials from New York (NY) treated with either one or five sequential additions of ^{13}C-TBA (See Fig. 1)

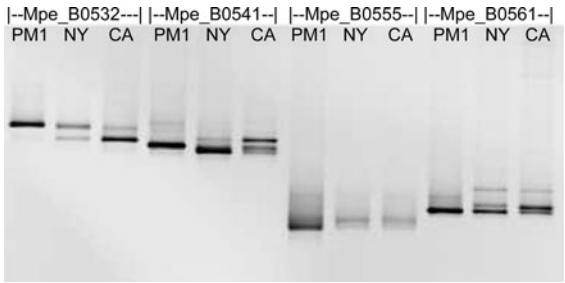

Fig. 5 Analysis of gene diversity in ^{13}C-metagenomic DNA. The Figure shows a DGGE analysis of partial genes PCR-amplified from either *M. petroleiphilum* PM1 or ^{13}C-metagenomic DNA obtained from microcosms constructed with BioGAC obtained from New York (NY) or California (CA) exposed to a single addition of $^{13}C_4$-TBA

responsible for metabolism of the isotopically-labeled test substrate assimilate and grow on labeled metabolites excreted by primary degraders. To address the possibility of this crossfeeding effect in the present study, we compared DGGE profiles of the partial 16S rRNA sequences derived from purified ^{13}C-DNA extracted from NY BioGAC samples receiving either one or multiple additions of $^{13}C_4$-TBA (See experiment described in Fig.1). Five distinct DNA bands were detected for the microcosm that received only a single $^{13}C_4$-TBA addition while only three bands were detected for the microcosm that received five sequential $^{13}C_4$-TBA additions (Fig. 4). While higher amounts of TBA might have been expected to increase the likelihood of crossfeeding, our results suggest that multiple additions of $^{13}C_4$-TBA decreased the diversity of organisms detected. One possible explanation of this effect is that repeated additions of TBA effectively made these microcosms an enrichment culture and selected for the faster growing strains of TBA-utilizing organisms.

Gene level analysis

Genomic DNA from pure cultures of *M. petroleiphilum* PM1 and ^{13}C-DNA obtained from both NY and CA BioGAC that received a single $^{13}C_4$-TBA addition were further analyzed for the presence of specific genes that have been associated with TBA biodegradation in *M. petroleiphilum* PM1 (Hristova et al. 2007). PCR reactions targeting four PM1 genes (Mpe_B0532, Mpe_B0541, Mpe_B0555, and Mpe_B0561) were conducted on all three DNA samples, as described in the Methods section. The products of these amplification reactions were further analyzed by DGGE (Fig. 5). All visible bands were extracted from the DGGE gel, purified, sequenced, and aligned, as described in the Methods section. In all cases, the DGGE analysis resulted in a single clear band for each PM1 gene and all DGGE-recovered PM1 sequences were identical to the partial PM1 gene sequences available in GenBank and as described in Methods and Materials. The differences in band migration for amplification products generated from ^{13}C-DNA from our microcosm studies and strain PM1 suggested that the DNA sequences of these products were different than the sequences for the products obtained from strain PM1. However, in most cases, the deduced amino acid sequences encoded by these differently migrating DNA fragments were highly similar (Table 3). For example, for gene Mpe_B0555 there were 14 nucleotide differences between sequences amplified from ^{13}C-DNA obtained from our microcosms as compared to the PM1 sequence. However, only two of these DNA sequence changes encode amino acid substitutions. In contrast, for gene Mpe_B0541, 52 nucleotide differences were detected in DNA sequences from ^{13}C-DNA obtained from our microcosms compared to the corresponding gene sequence for strain PM1. In this instance 13 of the 52 differences encode for amino acid substitutions.

Table 3 Heterogeneity analysis for genes found in DGGE-separated DNA sequences from NY and CA BioGAC samples

Gene ID	PM1 gene length (bp)	Sample sequence overlap of PM1 sequence (bp)	% gene coverage	Number of nucleotide differences in overlapping region (% similarity to PM1 gene sequence)	Number of nucleotide differences coding for amino acid substitutions	Amino acid substitutions
Mpe_B0532	1248	417	33	10 (97.6)	3	Val → Leu
						Pro → Ser
						Iso → Met
Mpe_B0541	1689	462	26	52 (88.7)	13	
Mpe_B0555	1413	493	35	14 (97.1)	2	Try → Ser
						Val → Leu
Mpe_B0561	894	423	47	19 (95.5)	4	Pro → Ala
						Lys → Glu
						Iso → Val
						Val → Iso

Discussion

The results of this study demonstrate that ^{13}C-DNA SIP using $^{13}C_4$-TBA enabled us to identify several novel TBA-utilizing organisms from self-inoculated BioGAC reactors. The 16S rRNA gene sequences recovered from NY BioGAC microcosms incubated with a single pulse of ^{13}C-TBA indicated that at least five different sequences representing five potentially different microorganisms were recovered. The DNA sequences of partial 16S rRNA genes showed these organisms all belonged to class *Betaproteobacteria*, order *Burkholderiales* and included representatives from the genera *Polaromonas*, *Cupriavidus, Rhodoferax, and Methylibium*. *Polaromonas* strains have previously been shown to be important members of surface-attached microbial communities in GAC-based water purification systems (Magic-Knezev et al. 2009). *Cupriavidus* strains are well known for their metabolic versatility (Lykidis et al. 2010; Pérez-Pantoja et al. 2008). However, as far as we are aware, no members of three of the four genera identified from our studies of NY BioGAC have previously been shown to grow on either TBA or MTBE.

For the CA BioGAC, all four of the 16S rRNA sequences obtained from the DGGE analysis were also related to organisms belonging to the *Betaproteobacteria.* However, the organisms represented by these sequences were different from those obtained from the NY BioGAC and included representatives of the genera *Methylibium*, *Hydrogenophaga, Paucibacter,* and *Cupriavidus*. Among these genera, only two out of four are related to organisms that have previously been shown to metabolize TBA. The best-characterized aerobic MTBE- and TBA-utilizing organism is *M. petroleiphilum* PM1. This organism was originally isolated from a peat moss biofilter in California and similar strains have been found at several sites in that state (Kane et al. 2001). The *Hydrogenophaga* sequence we obtained was closely related (98.5% sequence similarity) to the closest BLAST sequence match (GenBank accession no. AM403226) previously reported for a microbial isolate obtained from the only other previous study of aerobic TBA-metabolizing bacteria in BioGAC systems (Reinauer et al. 2008). This *Hydrogenophaga* strain was the dominant member of a mixed TBA-degrading culture, derived from samples of the

same BioGAC reactor used in our present SIP-based study. This result illustrates that SIP was successful in identifying at least one TBA-oxidizer belonging to the same genus as an isolate recovered from the same BioGAC. However, our results also illustrate that SIP can identify a wider diversity of metabolically active microorganisms than those identified using enrichment culture techniques. Even so, SIP may also under represent the diversity of active metabolizers in a community, as evidenced by the decrease in the number of different 16S rRNA sequences recovered when the number of ^{13}C-TBA pulses was increased from one to five (Fig. 4).

A concern with all SIP-based approaches is that labeled substrates are often used at concentrations that may not be environmentally relevant (Madsen 2006) and that this can cause shifts in the microbial population so that detected organisms are no longer representative of the targeted natural community. The BioGAC samples used in our analyses came from NY- and CA-based reactors treating groundwater with TBA levels as high as 10 and 350 ppm, respectively. As we have also shown in this study, the sorptive properties of the GAC can cause substantial decreases in aqueous phase TBA concentrations and presumably correspondingly large increases in the concentration of organics experienced by surface-attached bacteria. We also focused on results obtained when BioGAC samples were exposed to a single addition of $^{13}C_4$-TBA and also demonstrated that for NY BioGAC, multiple TBA exposures decreased rather than increased the microbial diversity detected in our samples. We therefore conclude that the aqueous TBA concentration in our microcosms ($\sim$300 ppm) is comparable, albeit at the high end, to concentrations of TBA these reactor samples may have experienced during field operation. Additionally, we can conclude that the microorganisms identified by the recovered 16S rRNA sequences are representative of the primary TBA-metabolizers in the BioGAC microcosms rather than organisms that have assimilated ^{13}C label through crossfeeding processes. However, it should be recognized that operational BioGAC reactors often contain several thousand kilograms of original GAC and the spatial and temporal distribution of surface-attached TBA-utilizing organisms within these reactors is unknown. As this present study made use of small samples (<100 g wet weight) of GAC material taken from a single location within a reactor we do not expect that our SIP analysis has fully captured the diversity of TBA-utilizing organisms within each reactor. As this initial study did not examine the reproducibility of our SIP analysis between subsamples of BioGAC we also do not know whether small-scale heterogeneities exist within these materials. We are currently completing a more detailed SIP analysis of the colonization and distribution of TBA-utilizing organisms within an operational BioGAC reactor. This study directly assesses these issues of local and reactor-scale heterogeneity and relates microbial distribution to reactor performance through a concurrent analysis of reactor pore water.

Our functional gene analysis targeted four genes that have been associated with TBA biodegradation by pure cultures of *M. petroleiphilum* PM1. Genes Mpe_B0532, Mpe_B0541, Mpe_B0555, Mpe_B0561 were selected for our study based on the results of a comparative transcriptome analysis which indicated they were differentially expressed (3- to 12-fold upregulated) when PM1 was grown on MTBE versus ethanol (Hristova et al. 2007). Similar genes were all detected in our NY and CA BioGAC ^{13}C-DNA samples, suggesting they may be frequently detected in TBA metabolizing organisms. Additionally, the deduced amino acid sequences for genes Mpe_B0555, Mpe_B0532, and Mpe_B0561 detected in our BioGAC samples were highly similar to the corresponding sequences for PM1, further suggesting that the predicted proteins are well conserved. Our findings are particularly relevant for Mpe_B0555 that codes for a phthalate dioxygenase-like enzyme, a protein recently detected during a proteomic analysis of Aquincola tertiaricarbonis L108 after growth on TBA (Schäfer et al. 2007). Detection of these genes, specifically Mpe_B0555, may indicate a site's potential for TBA-degrading activity and therefore could inform decisions regarding whether self-inoculation of BioGAC reactors intended to treat TBA-impacted ground water is likely to be an effective strategy.

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