Biodegradation (2009) 20:695–707
DOI 10.1007/s10532-009-9257-z

ORIGINAL PAPER

# Microbial community analyses of three distinct, liquid cultures that degrade methyl *tert*-butyl ether using anaerobic metabolism

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Received: 14 October 2008 / Accepted: 18 March 2009 / Published online: 2 April 2009


**Abstract** Methyl *tert*-butyl ether (MTBE) is a prevalent groundwater contaminant. In this study, three distinct MTBE-degrading, anaerobic cultures were derived from MTBE-contaminated aquifer material: cultures NW1, NW2 and NW3. The electron acceptors used are anthraquinone-2,6-disulfonate (AQDS; NW1), sulfate (NW2) and fumarate (NW3), respectively. About 1–2 mM MTBE is consistently degraded within 20–30 days in each culture. The 16S rDNA-based amplified ribosomal DNA restriction analysis (ARDRA) was used to analyze the microbial community in each culture. Results indicate novel microorganisms (i.e. no closely related known genera or species) catalyze anaerobic MTBE biodegradation, and microbial diversity varied with different electron acceptors. *Tert*-butyl alcohol (TBA) accumulated to nearly stoichiometric levels, and these cultures will be critical to understanding the factors that influence TBA accumulation versus degradation. The cultures presented here are the first stable anaerobic MTBE-degrading cultures that have been characterized with respect to taxonomy.

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**Keywords** Methyl *tert*-butyl ether (MTBE) · *Tert*-butyl alcohol (TBA) · Anaerobic biodegradation · Microbial community analysis

## Introduction

Fuel oxygenates were employed as gasoline additives to improve combustion efficiency and to reduce carbon monoxide emission to the atmosphere since the 1970s. About 85% of the oxygenate use in the United States was methyl *tert*-butyl ether (MTBE) because of its favorable characteristics with respect to refined fuel (Dewsbury et al. 2003). By 1996, 30% of gasoline consumed in the United States was amended with MTBE (Squillace et al. 1996), and by 1998 MTBE production was the fourth largest among domestic bulk chemical production (Song et al. 2006). It has been phased out nationwide and most often replaced by ethanol (Gold et al. 2002; McElliott 2002; Sim and McElligott 2002). MTBE contamination arose from gasoline spills and leaking underground fuel storage tanks, transportation systems and industrial wastewater. MTBE is very water soluble ($\sim$50,000 mg/l at 25°C) and does not significantly adsorb to subsurface solids due to its low octanol–water partition coefficient (log $K_{ow} = 1.2$) (Schmidt et al. 2004). Gas-phase partitioning is minimal because of its Henry's law constant ($5.9 \times 10^{-4}$ atm $m^3$ $mol^{-1}$). Plumes in groundwater can migrate from the source area and

threaten sensitive receptors such as drinking water intake wells. MTBE influences the taste and odor of water even at low concentration (<30 μg/l) (Fischer et al. 2005), and is regarded as a potential human carcinogen (Squillace et al. 1996).

Bioremediation has been considered a cost-effective strategy for several years (Schmidt et al. 2004). The majority of previous pure or mixed culture studies on MTBE biodegradation were performed under aerobic conditions. Microorganisms capable of degrading MTBE aerobically have been isolated (Deeb et al. 2000; Hanson et al. 1999; Mo et al. 1997; Munoz-Castellanos et al. 2006; Salanitro et al. 1994; Zhong et al. 2007) or studied as part of MTBE-degrading consortia (Raynal and Pruden 2008; Salanitro et al. 1994). These cultures can degrade MTBE with oxygen as terminal electron acceptor and require dissolved oxygen concentrations greater than 2 mg/l.

Biodegradation in anoxic environments has been well-documented (Finneran et al. 2001; Schmidt et al. 2004; Seagren and Becker 2002; Wilson et al. 2005), but there have been no reports to date on the specific microorganisms involved in Fe(III)- or sulfate-reducing MTBE biodegradation, which are considered prevalent processes for in situ, anaerobic MTBE biodegradation. In previous anaerobic investigations results with materials from different sites varied considerably, and conclusions of different investigations were often contradictory (Schmidt et al. 2004). What was missing from all anoxic studies were model cultures that could be manipulated in the same manner as the aerobic microorganisms so that single variables could be tested while other conditions remained constant in reasonable time frames (days or weeks rather than months or years). The only reported "culture" has been a sulfate-reducing culture that was maintained as a solids-containing aquifer enrichment rather than a liquid enrichment (Somsamak et al. 2001). The number of studies that can be performed with cultures in sediment is limited, and to date the dominant organisms have not been identified.

The primary limitation to understanding microbial physiology, biochemistry, and molecular ecology of anaerobic MTBE metabolism has been the lack of liquid cultures that maintain consistent activity with repeated transfers, in a reasonable timeframe for experimentation. Although anaerobic MTBE biodegradation has been reported in sediment incubations (microcosms) (Schmidt et al. 2004; Wilson et al. 2005), and one enrichment that was maintained in sediment (Somsamak et al. 2001), to date stable liquid cultures have not been characterized in terms of activity or microbial community composition. Three such cultures and the accompanying phylogenetic data are presented below.

We have enriched three distinct liquid, anaerobic cultures: NW1, NW2, and NW3, from MTBE-contaminated aquifer material. All cultures are stable in liquid medium (i.e. solids free) and activity is very consistent with each MTBE re-amendment and transfer to new medium. The cultures use different electron acceptors, and 16S rRNA gene analyses of the total communities indicate novel Bacteria involved in anaerobic MTBE biodegradation. *Tert*-butyl alcohol (TBA) has accumulated under the culture conditions, suggesting these cultures will be critical to understanding the environmental factors that influence TBA accumulation versus degradation.

## Materials and methods

### Chemicals

MTBE (ACS reagent >99%), anthraquinone-2,6-disulfonate (AQDS), sodium sulfate, and sodium nitrate were purchased from Sigma–Aldrich (Milwaukee, WI). HPLC-grade methanol, ethanol, benzene and toluene were purchased from Sigma Chemical Co. (St. Louis, MO). Poorly crystalline Fe(III) (hydr)oxide was synthesized as previously described (Lovley and Phillips 1986).

### Sample collection

Sediments were collected from a petroleum-contaminated aquifer of a gasoline station site in California. The material was originally sent to the University of Oklahoma for use in MTBE biodegradation microcosm studies and compound specific stable isotope analysis (CSIA). A sub-sample of the sediment was stored anoxically and shipped overnight in sealed containers to the University of Illinois laboratory, where they were stored at 4°C until use.

### Medium and enrichment incubation

The growth medium was a defined freshwater medium (Lovley et al. 1993), prepared as described below. The

medium contained (g/l unless otherwise specified): $NaHCO_3$, 2.5; $NH_4Cl$, 0.25; $NaH_2PO_4 \cdot H_2O$, 0.6; KCl, 0.1; modified Wolfe's vitamin and mineral mixtures (each 10 ml/l) and 1 ml/l of 1 mM $Na_2SeO_4$. The final concentrations of vitamins in the freshwater medium (1 l) were: 20 μg biotin, 20 μg folic acid, 100 μg pyridoxine HCl, 50 μg riboflavin, 50 μg thiamine, 50 μg nicotinic acid, 50 μg pantothenic, 1 μg B-12, 50 μg *p*-aminobenzoic acid, 50 μg thioctic acid; the final mineral concentrations were: 15 mg nitrilotriacetic acid (NTA), 30 mg $MgSO_4$, 5 mg $MnSO_4 \cdot H_2O$, 10 mg NaCl, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, 1 mg $CoCl_2 \cdot 6H_2O$, 1.3 mg $ZnCl_2$, 100 μg $CuSO_4 \cdot 5H_2O$, 100 μg $AlK(SO_4)_2 \cdot 12H_2O$, 100 μg $H_3BO_3$, 250 μg $Na_2MoO_4$, 240 μg $NiCl \cdot 6H_2O$, 250 μg $Na_2WO_4 \cdot 2H_2O$ and 189 μg $Na_2SeO_4$. The final pH of the medium was 6.8–7.0, buffered by 80:20 $HCO_3^-$:$CO_2$. 9 ml of medium was dispensed into each anoxic pressure tube, and then flushed with anoxic $N_2$/$CO_2$ (80:20) which had passed through a heated, reduced copper filled glass tube to remove trace oxygen. The medium tubes were sealed with thick butyl rubber stoppers and crimped with aluminum caps, and then sterilized by autoclave at 121°C for 1 h.

Enrichment incubations were set up using strict anoxic techniques as previously described (Finneran et al. 2002). Briefly, the sediments were homogenized in an anoxic glovebag filled with $N_2$, $CO_2$ and hydrogen, and then approximately 1 g of sediment was distributed into each anaerobic pressure tube containing defined freshwater media. The tubes were resealed and once taken out of the glovebag the headspace was flushed with $N_2/CO_2$ (80:20) to remove $H_2$ and to increase the $CO_2$ partial pressure. Sterile controls were prepared by autoclaving the sediment tubes at 121°C 1 h per day for three consecutive days. The electron acceptors used included anthraquinone-2,6-disulfonate (AQDS) (5 mM), fumarate (10 mM), poorly crystalline Fe(III) (hydr)oxide (50 mmol/l), sulfate (10 mM) and nitrate (10 mM). The amount of MTBE added ranged from 1 to 2 mM according to the types and concentrations of electron acceptors amended. *Un-amended controls* were set up by adding only MTBE without any electron acceptor. *Uninoculated controls* were set up by adding MTBE and the corresponding electron acceptors without culture inoculum. All reagents were added from sterile, anoxic stock solutions.

The enrichments were initially incubated at 18°C in dark without shaking. After MTBE degradation was consistent in these original incubations, transfers were made and were placed at 18, 30 or 37°C. MTBE degradation was tracked as described below. Activities under different temperatures were compared in order to optimize culturing conditions and to determine the influence of temperature as well. Positive MTBE degrading enrichments were re-amended with MTBE and the corresponding electron acceptors. Once MTBE degradation was consistent in the re-amended enrichments, 10% liquid was inoculated to new anoxic freshwater medium with the same concentration of MTBE and electron acceptor. Electron acceptor reduction was determined visually by observing color change (AQDS), precipitation (sulfate–sulfide production with Fe(II) added), or turbidity (fumarate).

## Analytical techniques

MTBE loss in the original enrichment incubations was initially tracked by sampling 0.1 ml of headspace with a gas lock syringe (Hamilton) and analyzed by gas chromatography connected to a flame ionization detector (FID) (Hewlett Packard Series 6890A), with an HP-1 capillary column (Hewlett Packard). Starting with the first transfer (to fresh medium) MTBE and potential degradation products were measured by gas chromatography equipped with mass spectrometry (GC-MS; Varian 4000, Varian Inc.) for greater sensitivity as described below. At each sampling point, 0.2 ml liquid was taken via an anaerobic syringe from each enrichment culture and injected into a specific vial for GC-MS auto-sampler. The vial was heated at 90°C for 10 min, and then 1 ml heated headspace sample was taken and injected into a capillary column (Varian, fused silica, VF-5MS 0.25 mm × 0.25 μm × 30 m) with the gas tight syringe. The oven temperature was held at 30°C for 3 min, and then increased by 25°C/min to 220°C. MTBE and TBA standards were measured each time together with samples and four-point calibration curves were made to quantify the concentrations of the compounds.

## DNA extraction

Genomic DNA from the active MTBE-degrading, sediment-free enrichment cultures was extracted by using the FastDNA® Spin Kit (MP Biomedicals). Briefly, cells were harvested by centrifugation (3 ml culture liquid; 5,000*g* for 6 min) and resuspended in

sterile water. Cells were lysed with a bead-beating device according to the manufacturer's instruction. DNA extraction was performed according to the protocol provided with the kit. The extracted DNA was confirmed on 2% agarose gel, and quantified by spectrophotometer.

## Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA) was applied after DNA extraction to analyze community compositions in the cultures. The main steps included: 16S rDNA PCR, cloning and PCR amplification, restriction enzyme digestion of amplified DNA, sequencing and phylogenetic analysis.

The 16S rDNA PCR was performed with Eubacterial universal primers 338F (5′-ACT CCT ACG GGA GGC AGC-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′). Temperature conditions for PCR were: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min 30 s, the final extension at 72°C for 8 min and then holding at 4°C. Positive amplification was determined by electrophoresis on 2% agarose gel stained with ethidium bromide (BioRAD) and visualized under UV light. Tris-Acetate-EDTA (TAE) buffer (0.5× made from 10× buffer by Sigma) and molecular grade agarose (BioRAD) were used.

Cloning and transformation were performed using the TOPO TA Cloning® Kit for Sequencing with One Shot® Mach1TM-T1® competent cells (Invitrogen). The cells were grown on LB agar plates containing 50 μg/ml kanamycin. Blue and white screening was performed to identify positive clones. Individual white colonies were picked randomly and added directly to PCR reactions. M13 primers (M13 for-21: 5′-GTA AAA CGA CGG CCA GT-3′; M13rev-24: 5′-AAC AGC TAT GAC CAT G-3′) targeting locations on the plasmid were used as recommended in the kit. The initial denaturation condition was 94°C for 10 min to lyse the cells prior to the PCR temperature program began. The PCR conditions were: 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min 30 s, the final extension at 72°C for 8 min and then holding at 4°C. The quality of PCR products were verified by gel electrophoresis. More than 50 clones were amplified for each sample. The M13-primer PCR products were digested with two restriction enzymes, HhaI and MspI (NE BioLabs), in the presence of bovine serum albumen (100 μg/ml) and NE Buffer 2 (NE BioLabs). The digested products were separated on a 3% Metaphor agarose gel (Cambrex Bio Science). The DNA band patterns were visualized and recorded using Molecular Imager Gel Doc XR System (Bio-RAD).

Clone representatives for unique ARDRA patterns were selected and sequenced using M13 for-21 primer. The selected PCR products for sequencing were purified using a QIAquick PCR purification kit (QIAGEN). The sequencing was performed by W. M. Keck Center for Comparative and Functional Genomics at University of Illinois, Urbana-Champaign.

## Microbial community analysis

To describe microbial population distributions of the MTBE-degrading cultures, the frequency of each clone in a library was calculated and clones that represented at least 10% of the total clones were operationally defined as "dominant". The sequences were compared with GenBank databases using the nucleotide BLAST (National Center for Biotechnology Information), and the relative with the highest identity value in the BLAST results was used to classify each distinct clone. Clones of which the closest known relatives were of less than 92% similarity were classified as "unidentified". A phylogenetic tree was constructed using the program Geneious 3.7.1 using maximum likelihood of PHYML with manual sequence alignment (Guindon and Gascuel 2003). The confidence of tree topologies was examined by bootstrap re-sampling for 1,000 replicates. Reference microorganisms were sulfate reducing bacteria, Fe(III)-reducing bacteria and previously reported MTBE- and/or TBA-degrading microorganisms.

## Alternate substrate utilization

The potential for these cultures to grow on other substrates was characterized. Substrate tested included: lactate (10 mM), acetate (10 mM), formate (10 mM), ethanol (10 mM), methanol (10 mM), benzene (2 mM) and toluene (2 mM). Cultures were inoculated to freshwater media (5% inoculum) via strict anoxic techniques as described before. Controls were set up with the same amount of inoculation and electron acceptors but no substrate amendment. Growth was determined spectrophotometrically at 600 nm for sulfate- and fumarate-amended cultures, and at 450 nm for

AQDS amended cultures to detect the concentration of anthrahydroquinone-2,6-disulfonate ($AH_2QDS$).

## Results

### Culture development

Enrichment incubations were set up to determine if MTBE- or TBA-degrading organisms in the native incubations were recoverable in viable liquid cultures. There were significant lag periods for each of the electron acceptors tested, ranging from 240 to 315 days prior to complete degradation (data not shown). MTBE was not degraded in the nitrate-amended enrichments (and to date activity is absent, data not shown.) AQDS amended, sulfate amended and fumarate amended enrichments completely degraded MTBE (not TBA) relative to uninoculated controls, and were further characterized.

MTBE was re-added to each bottle that had originally degraded MTBE, and the original enrichment (for each electron acceptor) was re-spiked with MTBE until the continuous degradation timeframe was approximately 30 days (AQDS and sulfate amended) or 20 days (fumarate amended) (data not shown). Once activity was continuous in the original (sediment bearing) enrichment, the material was transferred to fresh medium (10% vol/vol transfer) with MTBE plus the original electron acceptor. Each transfer was re-spiked at least three times to maintain activity, and each culture was transferred a minimum of five times such that approximately 0.001% of the possible sediment remained in each liquid culture (a negligible mass). At this point the cultures were given names and designated stable, liquid cultures. The names given were: NW1 (AQDS reducing), NW2 (sulfate reducing), and NW3 (fumarate reducing).

Culture NW1 completely degrades 1 mM MTBE with nearly stoichiometric production and accumulation of TBA (Fig. 1). Degradation is complete within 20–30 days (at 30°C) depending on when the MTBE is re-spiked or when the culture is transferred. Degradation is fastest at 30°C; however, culture NW1 can also degrade MTBE at 18 and 37°C (data not shown). $AH_2QDS$ is re-oxidized using poorly crystalline Fe(III) oxide each time MTBE is re-spiked. TBA increased only slightly after the fourth MTBE re-amendment, when compared to the increases during the first three additions which were stoichiometric. The capacity for the culture to degrade TBA as the sole substrate was tested by regenerating the electron acceptor AQDS by adding Fe(III) (which re-oxidizes the hydroquinone to the quinone), without adding MTBE. However, no significant TBA degradation was quantified (data not shown). TBA was also not degraded when it was added as the sole carbon and energy source for NW1 (data not shown). The uninoculated control did not degrade MTBE within a similar time frame to the active culture NW1 (embedded plot, Fig. 1). Identical control results were obtained with cultures NW2 (embedded plot, Fig. 2) and NW3 (embedded plot, Fig. 3).

Culture NW2 completely degrades 2 mM MTBE with stoichiometric TBA accumulation in approximately 20 days (Fig. 2). TBA has not been completely degraded by culture NW2 and it does not degrade TBA when it is added as the sole substrate (data not shown). Sulfate reduction was evident by the immediate formation of a black precipitate when anoxic $FeCl_2$ was added to active tubes. In addition, the characteristic odor was present when spent tubes were opened for DNA analyses.

Culture NW3 completely degrades 2 mM MTBE with near stoichiometric accumulation of TBA, and TBA does not further degrade (Fig. 3). The degradation timeframe is consistently 20–25 days depending on the time of re-spike or transfer (Fig. 3). Succinate was not analyzed, but $OD_{600}$ increases associated

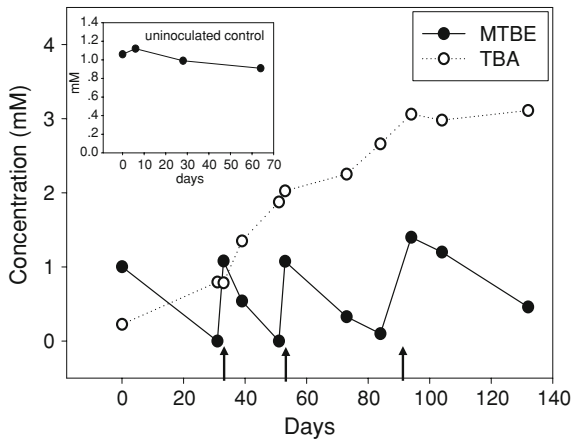


**Fig. 1** Anaerobic MTBE degradation in culture NW1 with 5 mM AQDS as terminal electron acceptor. MTBE was added at an initial concentration of 1 mM. Arrows indicate re-amendments of MTBE. Embedded plot: MTBE concentration change within 64 days in the uninoculated control

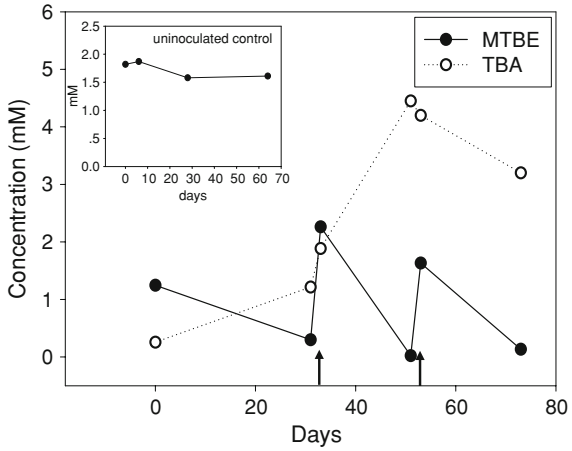


**Fig. 2** Anaerobic MTBE degradation in culture NW2 with 10 mM sulfate as electron acceptor. MTBE was added at an initial concentration of 1 mM. Arrows indicate re-amendments of MTBE. Embedded plot: MTBE concentration change within 64 days in the uninoculated control

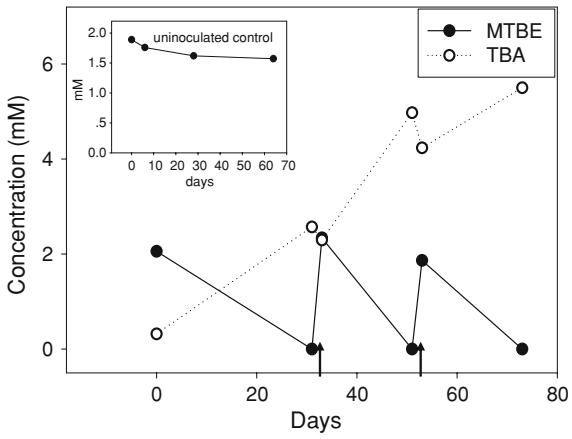


**Fig. 3** Anaerobic MTBE degradation in culture NW3 with 10 mM fumarate as electron acceptor. MTBE was added at an initial concentration of 2 mM. Arrows indicate re-amendments of MTBE. Embedded plot: MTBE concentration change within 64 days in the uninoculated control

with MTBE degradation in inoculated versus uninoculated controls or no-fumarate controls (both of which had no detectable increase in optical density at 600 nm) demonstrates biomass growth associated with fumarate reduction (data not shown).

## Microbial community analyses

Three clone libraries were constructed using amplified 16S ribosomal DNA restriction analysis (ARDRA) and distinct clones were assigned to different major phyla, as shown in Fig. 4. Clones that comprised 10% or more of the total clones were operationally defined as *dominant* (Fig. 4). Dominant clones were assigned alphanumeric designations (e.g. MA1). Culture NW1 had 13 different ARDRA patterns from 50 total clones. The highly dominant clone group MA1 represented 48% of the entire clone library. It is closely related to an uncultured bacterium clone anNCS10 (GenBank EF034939.1) derived from an Arctic permafrost soil from Spitsbergen, Northern Norway. The closest known (*cultured* = *known*) microorganism, *Desulfuromusa kysingii*, is only 84.7% related to MA1 (Table 1). The next most dominant clone MA2 comprised a much smaller fraction of the community than MA1 (10%), and was found to have the highest identity value to a clone TfC20H73 (GenBank DQ676393.1) from a suboxic freshwater pond. Neither of the dominant clones in NW1 was closely related to any known species. Other phylotypes were primarily affiliated with the phyla Firmicutes and Actinobacteria.

The sulfate-reducing culture NW2 was comparatively diverse. About 29.8% of the total clones classified as the phylum Actinobacteria; the two dominant representatives were MS3 and MS4, and the closest relationships were obtained with uncultured actinobacterium clone MVS-103 (GenBank DQ676393.1) derived from environmental sediment samples, with 98.1 and 98.3% similarities, respectively (Table 1). The most dominant single clone was MS1 with the highest frequency 19.3%; it was 100% related to an uncultured Spirochaetes bacterium (GenBank EU266876.1) detected in a tar oil contaminant plume where anaerobic toluene degradation occurred (Winderl et al. 2008). About 15.8% of 57 clones were classified into the class Deltaproteobacteria, predominated by MS2 which was related to *Desulfomicrobium* sp. STP10 (GenBank AJ006611.1) with 99.5% similarity. About 11 sequences were assigned to the order Clostridiales under the phylum Firmicutes, representing 19.3% of the total population.

Betaproteobacteria were the most abundant phyla in culture NW3 (Fig. 4). All of the sequences within this phylum were highly related to *Formivibrio citricus* (GenBank Y17602.1) with identity values between 97 and 98%, and clone MF3 as the majority phylotype. Another dominant clone was closely related to *Clostridium* sp. Kw12 (GenBank AB277862.1) (MF1 in Table 1), which is a member of the phylum Firmicutes. Two other dominant clones MF2 and MF4 were

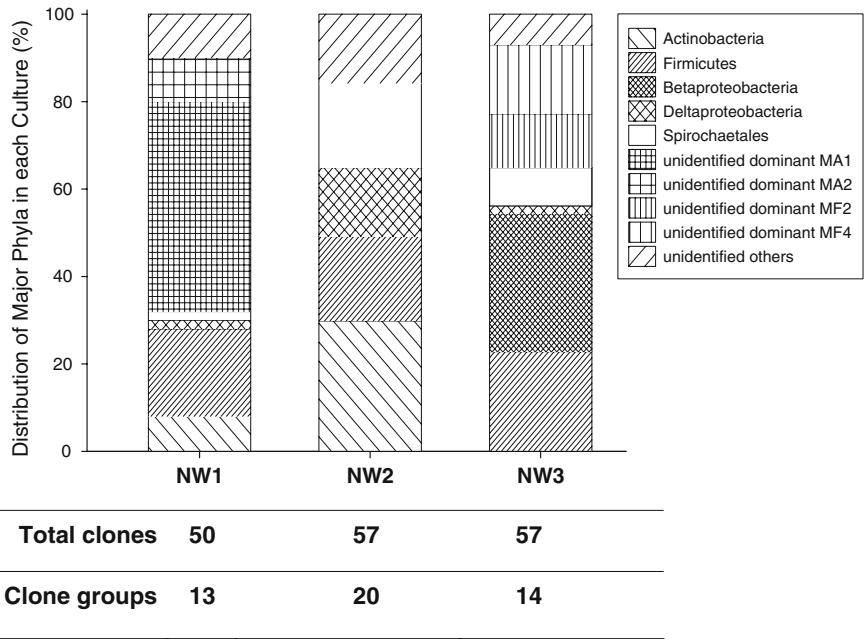


**Fig. 4** Microbial community composition in cultures NW1, NW2, and NW3 based on 16S rDNA analysis. The relative proportion of the clones within each culture is grouped by major phyla. Operationally-defined "dominant" clones were not relegated to a distinct phylum based on low similarity to known microorganisms

**Table 1** Dominant clones in all MTBE-degrading cultures compared with known (isolated and cultured) microorganisms and environmental clone sequences

| | Clone ID | Percentage | Phylogenetic group (NCBI BLAST) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Closest relative | Similarity (%) | Phylum/Class | Closest identified microorganism | Similarity (%) |
| NW1 | MA1 | 48.00 | Uncultured bacterium clone anNSC10 | 99.8 | Unidentified | *Desulfuromusa kysingii* | 84.7 |
| | MA2 | 10.00 | Uncultured bacterium clone TfC20H73 | 99.8 | Unidentified | *Rubrobacter taiwanensis* strain LS-293 | 88.6 |
| NW2 | MS1 | 19.30 | Uncultured spirochaetales bacterium | 100 | Spirochaetes | *Spirochaeta stenosstrepta* | 88.9 |
| | MS2 | 14.04 | *Desulfomicrobium* sp. STP10 | 99.5 | Deltaproteobacteria | | |
| | MS3 | 14.04 | Uncultured actinobacterium clone MVS-103 | 98.1 | Actinobacteria | *Rubrobacter taiwanensis* strain LS-293 | 91.4 |
| | MS4 | 10.50 | Uncultured actinobacterium clone MVS-103 | 98.3 | Actinobacteria | *Rubrobacter taiwanensis* strain LS-293 | 91.4 |
| NW3 | MF1 | 22.81 | *Clostridium* sp. Kw12 | 100 | Firmicutes | | |
| | MF2 | 15.79 | Uncultured bacterium clone G3DCM-82 | 98.8 | Unidentified | *Owenweeksia hongkongensis* | 86.1 |
| | MF3 | 14.04 | *Formivibrio citricus* | 98.5 | Betaproteobacteria | | |
| | MF4 | 12.28 | Uncultured bacterium clone C6 | 99 | Unidentified | *Acidaminococcus fermentans* | 93.2 |

associated with uncultured clones from environmental samples and had low similarities (86–93%) to currently known species.

A phylogenetic tree was constructed to illustrate the relationship of the dominant clones derived in our cultures to known sulfate reducers, Fe(III)/AQDS

reducers and aerobic MTBE and/or TBA degraders (Fig. 5). The dendrogram indicated that clones from the three different cultures correlated with each other and assembled into two main groups. MA2, MS3, MF4 were closely associated and are affiliated with *Rubrobacter taiwanensis*, as also indicated in Table 1. Additional related species included *Mycobacterium austroafricanum* and *Rhodococcus* sp. DEE5151, both of which are known aerobic MTBE degraders (Ferreira et al. 2006; Kim and Engesser 2004). The other distinct group included MA1, MS1, MS4, MF1 and MF3. A closely related species was *Shewanella oneidensis*, an organism that is capable of growing on a vast range of electron acceptors (Nealson et al. 2002). The cluster associated with this group consists primarily of microorganisms involved in MTBE/TBA degradation

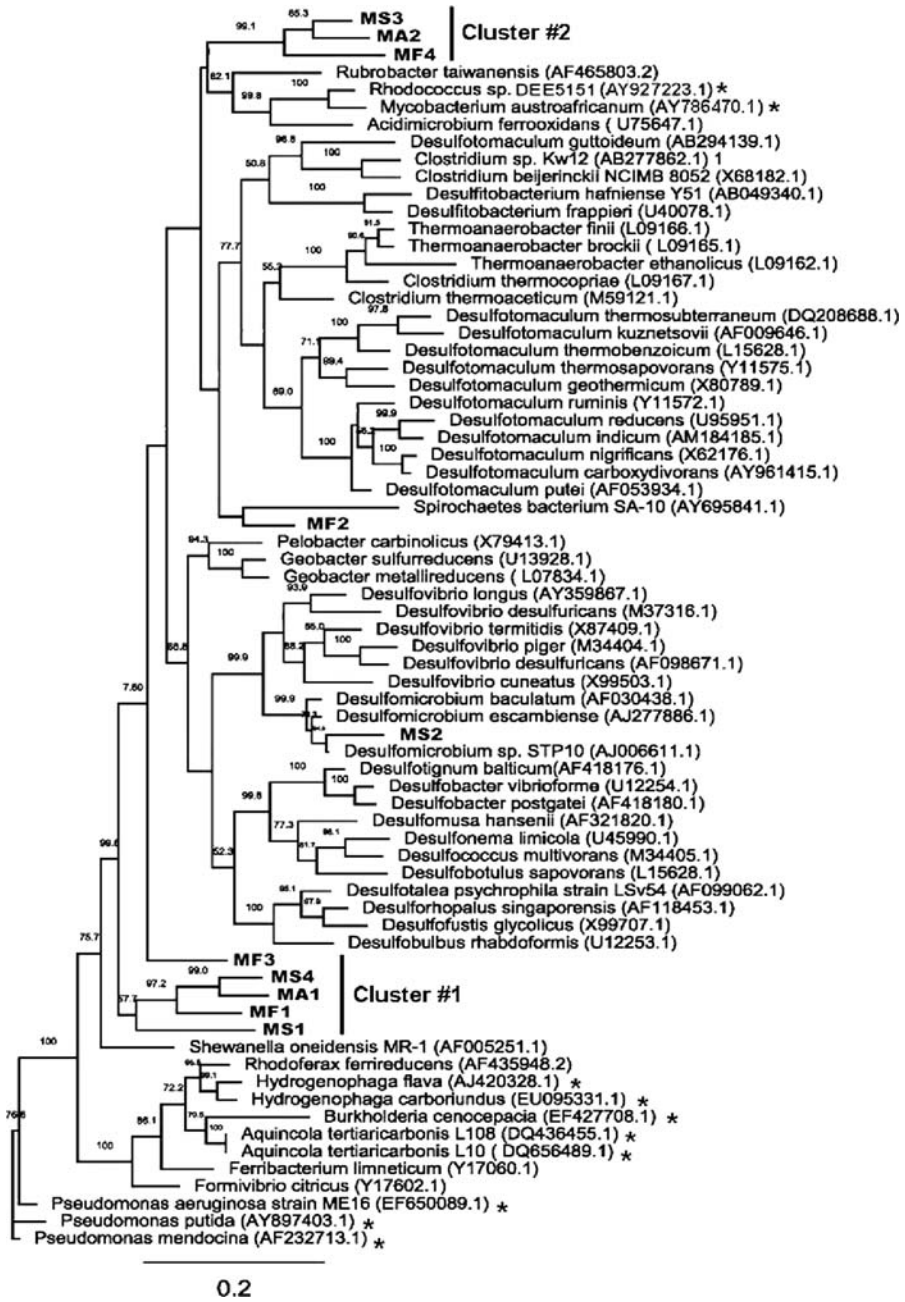


**Fig. 5** Phylogenetic positions of dominant clones based on maximum likelihood analysis of 16S rDNA gene sequences. ML bootstrap values above 50% (1,000 replicates) are presented. Numbers in brackets indicate the NCBI GenBank accession number of 16S rDNA sequences of reference cultures. The scale bar represents 0.2 base substitutions per site. Dominant clones retrieved in this study are highlighted in bold. Sequences marked with asterisk represent known MTBE (and/or TBA) degraders with oxygen as electron acceptor

**Table 2** Growth of all MTBE-degrading cultures on substrates other than MTBE

| | Benzene (2 mM) | Toluene (2 mM) | Ethanol (10 mM) | Methanol (10 mM) | Acetatec (10 mM) | Lactate (10 mM) | Formate (10 mM) | No donor control |
|---|---|---|---|---|---|---|---|---|
| AQDS (5 mM) | + | − | + | − | − | + | + | − |
| Sulfate (10 Mm) | − | − | − | − | − | + | + | − |
| Fumarate (10 Mm) | − | + | − | − | − | − | − | − |

Concentrations are listed in parentheses

*Note* +, represents growth; −, represents undetected growth within 20 days

under oxic conditions. MS2 was among the cluster of sulfate reducing bacteria and was assigned to the genus *Desulfomicrobium* because of the high identity value with the known species in *Desulfomicrobium*; MF1 was affiliated with *Spirochaetes* bacterium SA-10.

All clone sequences have been submitted to the GenBank database and accession numbers are pending.

### Initial characterization of substrate utilization

Test results (Table 2) demonstrate that the utilization of substrates (carbon and electron donor) other than MTBE varied in the three cultures. The AQDS-reducing culture NW1 used benzene, ethanol, lactate and formate as alternative substrates within 20 days. The sulfate reducing culture NW2 grew only on lactate and formate. The fumarate-amended culture NW3 grew on toluene, but did not utilize any of other tested substrates. Growth on methanol or acetate was not quantified in any of the three cultures.

## Discussion

Anaerobic mixed liquid cultures degrading MTBE coupled to the reduction of AQDS, sulfate or fumarate were enriched from MTBE-contaminated aquifer material. They are the first stable, sediment-free (liquid) anaerobic MTBE-degrading cultures. In addition, these are the first data reporting the microbial community involved in anaerobic MTBE biodegradation.

AQDS is an analog of humic substance quinone moieties, which can be used by many microorganisms as electron acceptors (Coates et al. 1998; Lovley et al. 1996, 2000). It is possible that humics are the relevant in situ electron acceptor; our past data demonstrate that humics were critical to MTBE degradation (Finneran and Lovley 2001). Many dissimilatory Fe(III)-reducing microorganisms also use AQDS as a terminal electron acceptor (Lovley 2000a), which suggests that MTBE degradation can be mediated by Fe(III) reduction in situ. This would be important because Fe(III) is the dominant terminal electron acceptor in many aquifer sediments (Thamdrup 2000) and effective in situ bioremediation strategies must encompass Fe(III)-reducing conditions in many cases.

One previous study by Somsamak et al (2001) reported complete loss of MTBE in sulfate enrichment incubations with fuel contaminated estuary sediment. In their study, 1.1 mM MTBE was transformed to a stoichiometric amount of TBA with the concomitant reduction of sulfate after 1,160 days of incubation. Further transfers for MTBE degradation from the original incubation were not reported in the initial study. Follow up reports with compound specific stable isotope analyses (CSIA) and the AK or CC enrichments (from Somsamak's previous study) indicated that secondary sediment-containing enrichments developed from the original incubation did degrade MTBE (Somsamak et al. 2006). However, these enrichments have not been reported in liquid culture and the microbial community has not been characterized.

Culture NW2 (presented here) demonstrated continuous, transferable MTBE-degrading activity under sulfate reducing conditions. Methane has not been detected at any point with this enrichment; therefore clone analysis with Archaeal primers was not performed. Sulfate has been widely reported as a significant electron acceptor for in situ, anaerobic MTBE and TBA transformation (Bradley et al. 2001a, b, 2002; Somsamak et al. 2001). Therefore, culture NW2 will be critical to understanding

oxygenate degradation on a mechanistic level as well as the microorganisms involved.

Fumarate is an organic acid oxyanion that can be reduced to succinate. Fumarate and succinate are key carbon compounds in central metabolism (citric acid cycle), but as an electron acceptor fumarate (and its reduction product succinate) remain as extracellular molecules. Many known Fe(III)-reducing microorganisms use fumarate as an alternate electron acceptor (Lovley et al. 2004). The model Fe(III) reducer *Geobacter sulfurreducens* is often grown in continuous culture using fumarate because of the high cell yield and ease of use (Esteve-Nunez et al. 2005). Fumarate may be a good amendment to stimulate in situ MTBE degradation, as it is relatively nontoxic and completely soluble in water. Utilization of this different electron acceptor for MTBE biodegradation suggested that respiratory processes in anaerobic environments under which MTBE can be degraded are diverse.

TBA is produced in nearly a stoichiometric manner by all cultures. TBA accumulates in NW1 and NW3; however, it began to decrease slightly, together with MTBE degradation in NW2 after 50 days incubation. TBA is the primary intermediate of MTBE degradation and its degradation is site specific (Bradley et al. 1999; Mormile et al. 1994; Yeh and Novak 1994). In a microcosm study with bed sediment of the Ohio River, stoichiometric transformation of MTBE to TBA was reported and TBA accumulated as a dead-end product under methanogenic conditions (Mormile et al. 1994). In sulfate-reducing enrichments reported by Somsamak et al. (2001), MTBE degradation also ended with TBA. In alternate batch studies, [$^{14}$C]-TBA was mineralized under nitrate- or sulfate-reducing conditions (Bradley et al. 2001b, 2002) or in the presence of Fe(III), sulfate, or under methanogenic conditions (Finneran and Lovley 2001), indicating that TBA biodegradation under in situ anaerobic conditions is possible. These results demonstrate that methyl group attack is the initial step in anaerobic MTBE degradation, as supported by previous reports. TBA decreased in NW2 after the third MTBE addition, and less TBA accumulated in NW1 after the fourth addition relative to the concentration of TBA that accumulated in the three prior MTBE amendments. One reasonable explanation may be that TBA degradation is catalyzed by similar enzymatic systems as MTBE, but MTBE is needed to induce the enzyme systems. This has been suggested for several of the aerobic organisms identified in Fig. 5. Now that three different anaerobic cultures are available, we can identify the physiological factors that control MTBE and TBA biodegradation. In addition, the lack of TBA degradation may have been an artifact of limitations in the growth medium. Cobalamin and/or cobalt have been identified as cofactors that are needed in excess of typical freshwater medium concentrations for TBA oxidation and subsequent carbon assimilation from TBA (François et al. 2002; Rohwerder et al. 2006).

Methylotrophic growth may be critical to all of the MTBE-degrading cultures identified here. Methylotrophy is an important metabolism for aerobic MTBE degradation (Piveteau et al. 2001; François et al. 2002). The rapid growth of NW1 and NW2 on formate also suggests potential dependence of the cultures on C1 compounds during anaerobic MTBE/TBA degradation. To date, TBA has not substantially degraded with the cultures presented in this study. More research is being carried out to determine whether TBA amended directly into these cultures can be degraded and to understand the influencing factors and limitations. Now that continuous cultures are available we can determine if the limitation is microbial diversity, or an environmental impact on microbial metabolism.

Microbial community analysis indicated that the dominant populations in the MTBE-degrading cultures are not closely related to known species, suggesting that anaerobic microorganisms growing on MTBE are in fact *novel*. This was especially noticeable in culture NW1, in which unidentified organisms were approximately half of the total population. This dominant group was unique to NW1 and was not detected in the other two cultures, and thus might be an organism characterized by respiratory activities specific to quinones or Fe(III). Microbial communities in the cultures using sulfate or fumarate were also dominated by novel clones, and the assemblage formed by these clones was actually close to known aerobic MTBE degraders, implying that organisms represented by this cluster might have homologous enzyme systems for MTBE despite their different respiratory metabolism.

Comparison of the phylum-level microbial community compositions revealed significant differences between the cultures and considerable microbial diversity within individual cultures. Betaproteobacteria were found exclusively in the fumarate-utilizing culture, where they dominated the clone library of

NW3 together with Firmicutes. The related, known microorganisms (*Formivibrio citricus* and *Clostridium* sp. Kw12) are well-known fermentative organisms, and fermentation is possible in fumarate-amended incubations. However, fermenters often have respiratory pathways as well, and further work will be done to determine if MTBE is transformed coupled to fumarate reduction or secondary fermentation reactions (though the latter is unlikely).

Deltaproteobacteria represented by *Desulfomicrobium* were abundant in NW2, but appeared at low frequencies in the other two libraries. Such high ratio of sulfate reducing population in NW2 was consistent with the main respiratory pathway of the culture and indicates that *Desulfomicrobium* may be the species actively using MTBE. Actinobacteria were also abundant in NW2, while they were missing in NW3 and limited in NW1. These microbial community distinctions between NW1/NW3 and NW2 may provide a better understanding of why TBA accumulates versus degrades, given that TBA loss has been at least identified in culture NW2.

Phylogenetic analyses using the most dominant clones identified two major clusters. The first (cluster #1) is a group related to several aerobic and facultative anaerobic bacteria, several of which are known MTBE-degrading microorganisms (Fig. 5). The second (cluster #2) is related to common soil bacteria, with only two known MTBE-degrading organisms being closely branching. While the cultures described here do not grow using oxygen as an electron acceptor, it is interesting that there is phylogenetic similarity between known aerobic MTBE degraders and the two major clusters developed with these enrichments.

The initial characterization of alternate substrate utilization suggests that the cultures are distinct with respect to their available metabolic pathways. While we have not isolated individual cultures and therefore cannot identify which phylotype within each community is responsible for utilizing the tested substrates, it is significant that each culture had distinct use patterns. Benzene and toluene are two additional significant compounds in fuel-contaminated sites and have been reported to degrade under diverse anaerobic conditions (Anderson et al. 1998; Anderson and Lovley 2000; Kazumi et al. 1997; Lovley et al. 1995; Lovley 1997, 2000b). Ethanol is used increasingly in gasoline as a substitute for MTBE and thus appears frequently in the groundwater system near fuel spills. Ethanol is amenable to degradation and may influence the degradation of coexisting fuel contaminants by consuming available electron acceptors (Powers et al. 2001). The utilization of benzene, toluene or ethanol by NW1 and NW3 will allow us to develop better understanding on how common co-contaminants may impact the fate and transformation of MTBE under anoxic environment. On the other hand, NW1 and NW2 can use lactate and formate and generate substantial amount of biomass, implying that growth with the easily used substrates might be a way to help accumulate biomass within relatively short time for investigations that require considerable amount of biomass—such as bioaugmentation studies. However, further work is needed to determine whether the growth stimulated by lactate or formate will promote MTBE degradation within the microbial communities.

These cultures provide model systems for mechanistic studies of MTBE biodegradation that could not have been previously examined. Using these cultures, we will develop a better understanding of anaerobic MTBE biodegradation at the cellular level, with respect to actual microbial physiology, biochemistry, and biogeochemistry. In addition, we will use these cultures and the phylogenetic data to understand the microbial community involved in anaerobic MTBE biodegradation and to develop molecular probes to determine whether the requisite organisms are present in the aquifer systems for natural attenuation or accelerated bioremediation. These cultures provide the scientific community investigating anaerobic MTBE transformation with the tools that have thus far been relegated to aerobic MTBE degradation, which in practice is far ahead of anaerobic bioremediation. They will be shared with those involved in this research such that the gap between anaerobic and aerobic knowledge can be closed.

**Acknowledgments** We thank Tomasz Kuder (University of Oklahoma) for the sediment samples. This work is funded by British Petroleum/Atlantic Richfield Company and the American Petroleum Institute.

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