CHARACTERIZATION OF ANAEROBIC METHYL *TERT*-BUTYL ETHER (MTBE)-DEGRADING COMMUNITIES

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And approved by

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ABSTRACT OF THE DISSERTATION

Characterization of anaerobic methyl tert-butyl ether (MTBE)-degrading communities

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The wide use of methyl *tert*-butyl ether (MTBE), a synthetic fuel oxygenate, have caused extensive contamination in groundwater in past two decades and resulted in taste, odor, and potential toxicity problems in drinking water. MTBE contaminated groundwater aquifer is often depleted of oxygen and microorganisms can anaerobically degraded MTBE under a verity of redox conditions. It has been demonstrated that MTBE can be degraded anaerobically which makes *in situ* bioremediation of contaminated aquifers a potential solution to address this problem. Assessment and enhancement of MTBE bioremediation requires knowledge of the microorganisms that responsible for biodegradation process. However, it is challenge to isolate anaerobic MTBE-degrading microorganisms or to characterize the microbial communities. The aim of this study was to identify the organisms that mediate anaerobic biodegradation of MTBE in methanogenic or sulfidogenic cultures enriched from estuarine sediments. Stable isotope probing (SIP) combined with terminal restriction fragment length polymorphism (T-RFLP) analysis showed that *Ruminococcaceae* species were active in methanogenic MTBE-degrading community. T-RFLP coupled with clone library analysis of bacterial 16S rRNA genes from sulfidogenic enrichment cultures showed *Deltaproterobacteria* were highly enriched than the other phylogenetically diverse populations. We also

investigated MTBE-degrading communities by single cell using Raman Spectroscopy and SIP. The results confirmed the MTBE degraders are not abundant in the communities. This study provides crucial information for understanding the mechanisms of anaerobic degradation of MTBE as well as for assessment of the *in situ* bioremediation at contaminated field sites as the microbial/molecular tools.

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BACKGROUND

MTBE in fuels

Methyl tert-butyl ether (MTBE) is a synthetic organic compound manufactured via the chemical reaction of methanol and isobutylene. With the molecular formula of $(CH_3)_3COCH_3$, MTBE has a tertiary carbon structure and a high dissociation energy of the ether bond (approximately 360 kJ/mol) (Kim & Engesser, 2004). It is a volatile, flammable, and colorless liquid with high water solubility. In addition, the human olfactory system is highly sensitive to the offensive minty taste and odor of MTBE (USEPA, 1997; Davis & Farland, 2001).

In the United States, fuel oxygenates have been introduced to replace tetraethyl lead since 1979 (Kirchstetter *et al*., 1999; von Krauss & Harremoës, 2001). Oxygenates can raise the octane number in conventional gasoline and therefore increase the combustion efficiency of motor vehicles and improve air quality (Deeb *et al*., 2000). The Clean Air Act Amendments of 1990 mandated the use of oxygenated gasoline to reduce carbon monoxide and ozone levels in urban regions that did not meet National Ambient Air Quality Standards (USEPA, 1998; Franklin *et al*., 2000). In 1995, at least 2% percent oxygen by weight was required to be added in the reformulated gasoline by Federal Reformulated Gasoline (RFG) program in most polluted metropolitan areas (USEPA, 1998).

Compared to the other fuel oxygenates, such as such as ethanol, alkylates, aromatic compounds and ether compounds, MTBE has the advantages of low cost and ease in

production, transportation, and blending into gasoline (Squillace *et al*., 1997). Therefore, MTBE was chosen over the other oxygenates by most of the US petroleum companies. 87% of reformulated gasoline contained MTBE and up to 15% MTBE by volume was added to gasoline in order to meet these legislation requirements. In response to the huge demand, the production of MTBE increased approximately one thousand fold from 1990 to 1999, from 83 thousand barrels to 78.9 million barrels per day (USEPA, 2008). During the 1990s, MTBE was the second in rank among all the synthetic organic chemicals produced in the U.S. (Häggblom *et al*., 2007). California, Texas, Northeastern and Mid-Atlantic states accounted for about three quarters of MTBE consumption in 2001 (Squillace & Moran, 2000; Moran *et al*., 2006).

In 1996, the drinking water supply of the city of Santa Monica was shut down due to MTBE contamination of wellfields (Rosell *et al*., 2006). This was the first incident that brought public awareness of MTBE contamination, which eventually also resulted in California being the first state to phase out MTBE. Since then, many states started limiting or banning the usage of MTBE (USEPA, 2008). In 2005, the Energy Policy Act formally repealed the 2% oxygen requirement in the reformulated gasoline, and Renewable Fuels Standard was announced at the same time (McCarthy & Tiemann, 2006). As a consequence, the refinery companies switched from MTBE to ethanol in gasoline.

MTBE has also been used as a gasoline oxygenate worldwide but at relative low concentration. For instance, gasoline contained an average of 2% MTBE in the European

Union (Rosell *et al*., 2006). After the phase-out of MTBE, ETBE was the most popular substitute in EU countries. MTBE is still used as the gasoline oxygenate in the other parts of the world (Häggblom *et al*., 2007; Mirabella & Den Hertog, 2008).

Health concern and Drinking water quality

The Environmental Protection Agency (EPA) considers MTBE as a possible human carcinogen at high exposure doses based on animal studies (Chun *et al*., 1992; Belpoggi *et al*., 1997; Bird *et al*., 1997; USEPA, 1997). However, there is insufficient evidence to support the same conclusion at low exposure levels (Moolenaar *et al*., 1994; White *et al*., 1995; Hakkola *et al*., 1996; Dodd *et al*., 2013). The chronic inhalation exposure data from rats suggests that MTBE exerts adverse effects on the kidney (Robinson *et al.*, 1990; Chun *et al.*, 1992). One study on MTBE genotoxic effects has shown that it could induce a variety of DNA damages of human lymphocytes (Chen *et al*., 2008). MTBE can enhance the toxicity of pesticides in aquatic systems (Hernando *et al*., 2003), as well as cause significant reproductive dysfunction in zebrafish at the concentration of 110 μg/L (Werner *et al*., 2001; Moreels *et al*., 2006).

Although long-term exposure to MTBE might cause the chronic health effects, the major concern for many people is that MTBE makes drinking water non-potable as it has a low taste and odor threshold. The threshold of MTBE is 15 μg/L in drinking water, which is far below the toxicity concentration determined for laboratory animals (van Wezel *et al*., 2009). Therefore, USEPA established a drinking water advisory level at a range of 20-40 μg/L or lower, mainly based on organoleptic effects and consumer acceptability reasons

than on health effects (USEPA, 1997). By 2003, 46 states in US had adopted EPA standards to establish their primary or secondary drinking water standards, ranging from 5 μg/L to 400 μg/L (USEPA, 2008).

Occurrence of MTBE in environment

The huge consumption of MTBE as a fuel additive in the 1990s resulted in widespread MTBE contamination of the environment across the United States (Squillace *et al*., 1999). The major environmental sources of MTBE are mainly related to the storage, distribution, and the usage of MTBE-blended fuels, such as underground and aboveground storage tanks, pipelines, refueling spills, accidental spills during transport, damaging the fuel tank, emissions from older marine engines (Reuter *et al*., 1998; Deeb *et al*., 2003; Rosell *et al*., 2007a). In particular, high concentrations of MTBE were frequently detected in the proximity of leaking underground storage tanks (LUSTs), which was associated with large volumes of oxygenated gasoline leaking into soil and groundwater (Landmeyer *et al*., 1998; Odencrantz, 1998; Shih *et al*., 2004; Kuder *et al*., 2005; Zogorski *et al*., 2006). Nonpoint sources include exhaust and evaporative emission from vehicles, atmospheric deposition, stormwater runoff, discharge from contaminated groundwater, effluents from refineries and treated municipal wastewater (Reuter *et al*., 1998; Deeb *et al*., 2003; USEPA, 2008).

MTBE can enter drinking water sources such as wells, lakes and reservoirs from leaking or spill incidents (Reuter *et al*., 1998). The EPA National Water-Quality Assessment Program (NAWQA) study between 1992 and 2001 detected MTBE in ~18% of samples

and at ~10% of sites in the United States. This study has shown that MTBE was detected more frequently in surface water, but at higher concentrations (up to 23,000 μg/L) in groundwater (USEPA, 2008). In addition, MTBE was the third most frequently detected volatile organic compound (VOC) of groundwater and drinking water supply wells nationwide between 1985 and 2002. The high population density areas where MTBE reformulated gasoline was used and the areas with high rates of groundwater recharge have the greatest detection frequency (Moran *et al*., 2005; Moran *et al*., 2006). MTBE was also detected in VOC mixtures with toluene and chlorinated compounds at high frequency in public water supply wells (Squillace & Moran, 2007). More importantly, MTBE has been found in finished drinking water. EPA had monitored MTBE in drinking water systems between 2001 and 2003 and found that approximately 226 million people in US served by the public water systems that was contaminated with MTBE. According to this survey, Unregulated Contaminant Monitoring Regulation (UCMR 1) of MTBE used a Minimum Reporting Level of 5 μg/L for drinking water (USEPA, 2008).

Fate

As a typical VOC, MTBE volatilizes from surface waters and reacts with nitrate radicals and photochemically produced hydroxyl radicals in the air (Squillace *et al*., 1996; Squillace *et al*., 1997). Nevertheless, MTBE is a very persistent and mobile contaminant once released to groundwater because of its physical and chemical properties. The high water solubility and low Henry's law constant indicates that MTBE tends to dissolve in groundwater rather than volatilize to the air (Squillace *et al*., 1997). MTBE also has a low soil adsorption coefficient (K_{oc}), which means it has a tendency not to adhere to soil

solids and likely to stay in the aqueous phase (Stocking *et al*., 2000). As a consequence of these physical properties, MTBE migrates faster and farther in groundwater than the other components of gasoline, such as benzene, toluene, ethylbenzene and xylene. Additionally, MTBE is resistant to chemical and natural biological degradation, which is attributed to its tertiary carbon structure and the stable ether bond (Kim & Engesser, 2004). Therefore, MTBE is relatively persistent with little degradation over several years in subsurface and it is challenge to clean up MTBE from contaminated sites (Wilson *et al*., 2005a).

Bioremediation

Although many states in US have banned or restricted MTBE usage in fuel since 2000, MTBE is still persistent in groundwater that requires remediation (USEPA, 2006). Cleaning up MTBE-contaminated water supplies for public water systems may require up to $85 billion (AWWA, 2005; McCarthy & Tiemann, 2006; Carter *et al*., 2006; Sweet *et al*., 2010). Physical and chemical remediation techniques such as adsorption by granular activated carbon, air striping and advanced oxidation process (AOP) have been applied to remove MTBE contamination (Levchuk *et al*., 2014). However, MTBE has a weak tendency to volatilize from the aqueous phase, making it difficult to remove from groundwater by air sparing. Meanwhile, the low soil adsorption coefficient of MTBE makes it less efficient for removal by adsorption techniques. Advanced oxidation processes generate undesirable by-products and consumes relatively expensive reactants. Biological processes have been used to treat MTBE contamination both *in situ* and *ex situ* (Deeb *et al*., 2000; Stocking *et al*., 2000; Waul *et al*., 2009; North *et al*., 2012).

Monitored natural attenuation, an *in situ* bioremediation technique, overcame the drawbacks of physical and chemical remediation and therefore showed a great attraction to address MTBE contamination problems in groundwater within reasonable budgets and time frames (Bombach *et al*., 2010; ITRC, 2005; USEPA, 2004; USEPA, 2007). Efficient monitoring and quantifying of biodegradation processes and identification of the single species or microbial communities that responsible for MTBE degradation are the two important aspects for a successful assessment of monitored natural attenuation.

Aerobic biodegradation

MTBE is a recalcitrant compound due to its stable chemical characteristics. It was initially thought to completely resist microbial attack until Salanitro *et al*. (1994) first demonstrated aerobic degradation. Since then, the aerobic MTBE-degrading cultures have been intensely studied and several bacteria that are capable to degrade MTBE have been isolated and characterized (Table 1.3). Under aerobic conditions, MTBE can be fully metabolized by some bacteria, which are able to utilize both MTBE and its key metabolic product TBA as sole carbon and energy sources. Other bacteria, such as alkane-metabolizing stains, can cometabolically oxidize MTBE but not further metabolize TBA. The third types of bacteria can rapid oxidize MTBE but poorly oxidize TBA or *vice versa*.

With these pure cultures, the pathways of aerobic MTBE degradation, the enzymes involved in different metabolic steps and the genes that encode these enzymes have also been studied. Oxidization MTBE to TBA is usually initiated by a monooxygenase,

including several types of non-heme iron-containing alkane hydroxylases and heme-containing cytochrome P450s (Hyman, 2013). In addition, some alkane hydrolases are also responsible for the further oxidation of TBA and other MTBE oxidization products. In *Methylibium petroliphilum* PM1 and *Methylibium* sp. strain R8 the plasmid located gene *mpdA* encodes for an alkane hydroxylase (Hristova *et al.*, 2007). Gene *alkB* encodes for other types of alkane hydroxylases, which can be found in *Pseudomonas* and *Mycobacterium* strains (Steffan *et al*., 1997; Smith *et al*., 2003; Smith & Hyman, 2004). Cytochrome P450s encoded by *ethB* were mostly found in strains that oxidize MTBE after growth on different substrates (Steffan *et al.*, 1997; Malandain *et al.*, 2010).

Anaerobic biodegradation

MTBE contamination is often linked to leakage from underground gasoline tanks. Intensive microbial oxidation of gasoline hydrocarbons leads to complete consumption of oxygen in the subsurface aquifer. The high solubility and mobility of MTBE in groundwater resulting in large gasoline plumes will further cause technical difficulties of introducing oxygen to stimulate aerobic degradation (Anderson & Lovley, 2000; McMahon & Chapelle, 2008). Over the past two decades, anaerobic MTBE-degrading enrichment cultures or microcosms have been successfully established from groundwater, sediment and wastewater treatment sludge. These studies have demonstrated the anaerobic biodegradation of MTBE under different redox conditions, including denitrification (Bradley *et al*., 2001; Fischer *et al*., 2005), manganese (IV)-reducing (Bradley *et al*., 2002), iron (III)- reduction (Finneran & Lovley, 2001; Pruden *et al*., 2005; Wei & Finneran, 2009), sulfidogenesis (Somsamak *et al*., 2001; Youngster *et al*.,

2008; Key *et al*., 2013), and methanogenesis (Mormile *et al*., 1994; Somsamak *et al*., 2005; Wilson *et al*., 2005a; Sun *et al*., 2012). The complete depletion of MTBE generally takes several months, indicating that anaerobic degradation of MTBE is a relatively rare process in the environment. The anaerobic MTBE degradation process terminates after transformation to TBA, which is not a desirable endpoint, however, it is important to understand this critical first step in the breakdown of MTBE.

Knowledge about anaerobic biodegradation of MTBE is quite limited, in contrast to aerobic degradation studies. Only a few microbial communities have been characterized and no responsible microorganisms have been conclusively identified to date (Table 1.4). A characterization of the microbial community structure of MTBE-degrading microcosms and enrichment cultures will help us better understand their potential catabolic functions with respect to MTBE degradation under different redox conditions. Previous study has been shown that bacterial mediated O-demethylation is the first step of anaerobic biodegradation of MTBE (Somsamak *et al*., 2005). Acetogens are capable of cleaving ether bonds and utilizing the methyl group of various aryl ethers, suggesting their candidacy for initiating MTBE degradation. Additionally, anaerobic MTBE degradation was suggested to be a corrinoid-dependent process and possibly facilitated by methanogens and sulfidogens through consumption of acetate produced from acetogenic metabolism (Youngster *et al*., 2008). In the study by Youngster *et al*. (2010a), members of the *Firmicutes*, *Chloroflexi* and *Deltaproteobacteria* were the dominant phylotypes of the MTBE-degrading community enriched from New Jersey costal sediment under methanogenic conditions. Sun *et al*. (2012) have shown by stable isotope

probing that the species associated with *Ruminococcaceae* and *Sphingopyxis* were active in a methanogenic MTBE-degrading consortium from sludge sample of Michigan. These studies have provided further support that acetogens may mediate anaerobic MTBE degradation since *Ruminococcaceae* belongs to the phylum *Firmicutes* which is known to contain a range of acetogens. However, much less is known of the microbial communities involved in anaerobic degradation of MTBE under sulfate or iron reducing conditions. To date, three sulfidogenic MTBE-degrading microbial communities have been characterized, and *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetales* are the most common phyla detected in these communities (Wei & Finneran, 2009; Raynal *et al*., 2010; Key *et al*., 2013). *Proteobacteria*, *Firmicutes* and *Actinobacteria* are abundant under iron reducing conditions (Wei & Finneran, 2009; Raynal *et al*., 2010).

Monitoring MTBE biodegradation by compound-specific stable isotope analysis

The challenges of assessing MTBE biodegradation in *situ* include the unreliable metabolite analysis and geochemical approaches, such as detecting the MTBE intermediate tert butyl alcohol (TBA) or the depletion of electron acceptors (Häggblom *et al*., 2007). In recent years, compound-specific stable isotope analysis (CSIA) has been increasingly used as an important assessment tool for monitoring MTBE biodegradation *in situ* (Hofstetter & Berg, 2011). Isotopically lighter (^{12}C, ^{1}H and ^{16}O) molecules are easier to degrade by microorganisms due to less consumption of energy, which result in enrichment of heavier isotope (^{13}C ^{2}H) in the residual contaminant fraction. The shift in the stable isotope ratio of specific elements (carbon and hydrogen) present in MTBE can be measured by sensitive gas chromatography-isotope ratio mass spectrometry (GC-

IRMS) which enables improved evaluation of *in situ* biodegradation of MTBE.

CSIA has several advantages compared to the other in *situ* assessment methods. First, CSIA can demonstrate that the reduction of MTBE concentration is due to biological activities and not abiotic processes, because the shift in isotope ratios only result from biodegradation. Second, CSIA can provide information for characterizing the initial reaction mechanisms, because the isotopic enrichment factors (ε) mostly depend on the first bond cleavage of the molecules. In MTBE degradation experiments, the values of carbon isotope enrichment factors (εC) observed during anaerobic degradation are generally much higher than for aerobic degradation, and terminal electron accepting conditions may not affect εC among the anaerobic enrichment cultures (Table 1.2). Moreover, the bacterial isotope fractionation patterns can be affected by the employment of different enzymes for catalyzing initial MTBE metabolism such as oxidation and hydrolysis in aerobic biodegradation (Table 1.3). For instance, *Methylibium* sp. strains PM1 and R8 have very similar slopes of $\Delta_2H/\Delta_{13}C$ that might result from their *mpdA* genes. *Pseudomonas putida* GPo1 also has an alkane hydroxylase but shows a different slope than PM1 and R8, indicating that the alkane hydroxylase encoded by *alkB* is using a different mechanism during aerobic degradation. In combination with hydrogen isotope enrichment factors (εH), a two-dimensional isotope fractionation approach can provide a better understanding of the primary respiration processes driving biodegradation (Rosell *et al*., 2007b). Finally, CSIA can potentially quantify the extent of in situ biodegradation based on the appropriately measured compound specific isotope enrichment factors from lab or field studies. With the enrichment factors, MTBE concentrations at the

contaminated sites can be estimated using the Rayleigh equation (Elsner *et al*., 2007).

PURPOSE OF THIS STUDY

Even though MTBE is no longer used as fuel oxygenate in the U.S., MTBE contamination of groundwater is still an ongoing problem. The aim of this study was to shed light on the microorganisms responsible for anaerobic degradation of MTBE as well as to provide information about anaerobic MTBE degradation mechanisms. This knowledge is critical for assessment of biodegradation processes during monitored natural attenuation and for developing methods for enhancing MTBE degradation rates. For this purpose, we analyzed 16S rRNA genes of MTBE-degrading enrichment cultures by multiple molecular biological approaches, including stable isotope probing (SIP) and terminal restriction fragment length polymorphism (T-RFLP), pyrosequencing and clone library analyses. In addition, I attempted the identification of the MTBE degrading organism by Raman microspectroscopy coupled with SIP in order to provide a new strategy for linking the particular microbes to their ecological functions in the environment.

The specific objectives of this study were:

1. To analyze the community structure of enrichment cultures utilizing MTBE as the sole carbon and energy source;

2. To identify the microorganisms that are responsible for anaerobic degradation of MTBE using stable isotope probing techniques;

3. To identify the microorganisms that are responsible for anaerobic degradation of MTBE using Raman microspectroscopy coupled with Stable Isotope Probing techniques at the single cell level.

TABLE 1.1 Properties of MTBE and aromatic hydrocarbon components of gasoline

Compounds	Water solubility[1] (mg/L)	Vapor pressure[2] (mm Hg)	Henry's Law Constant[3]	Log Koc[4]
MTBE	50,000	251	0.055	1.1
Benzene	1780	86	0.22	1.9
Toluene	535	28	0.24	1.9
Ethylbenzene	161	10	0.35	2.7
m-xylene	146	8.3	0.31	2.3
o-xylene	175	6.6	0.21	1.8
p-xylene	156	8.7	0.31	2.4

Table modified from (Moyer, 2003)

1 indicates tendency to dissolve in water

2 indicates tendency to volatilize from the non-aqueous phase

3 indicates tendency to volatilize from aqueous phase

4 indicates tendency to adhere to soil

TABLE 1.2. Studies demonstrating stable-isotope fractionation during MTBE in microcosms and enrichment cultures.

Source	Redox conditions	εC (‰) (95%CI)	εH (‰) (95%CI)	Λ(≈εH/εC) (95%CI)	References
Vandenberg Air Force Base, CA microcosms 1	Oxic	-1.5±0.1	NA	NA	(Gray *et al*., 2002)
Vandenberg Air Force Base, CA microcosms 2	Oxic	-1.4±0.1	-66±3	45±2	(Gray *et al*., 2002)
Vandenberg Air Force Base, CA microcosms 3	Oxic	-1.8±0.1	-29±4	12±2	(Gray *et al*., 2002)
Borden enrichment Culture, Alliston, ON	Oxic	-1.74±0.25	NA	NA	(Hunkeler *et al*., 2001)
Port Hueneme microcosm CA	Oxic	-1.4 (CI NA)	NA	NA	(Lesser *et al*., 2008)
UK Chalk aquifer High O_2 microcosm	Oxic	-1.53±0.4	NA	NA	(Thornton *et al*., 2011)
UK Chalk aquifer field O_2 microcosm	Low O_2	-0.2±0.01	NA	NA	(Thornton *et al*., 2011)
South American industrial landfill PM 04	Anoxic	-26.44±0.15	-71.4±7.6	3	(Zwank *et al*., 2005)
South American industrial landfill	Anoxic	NA	-3.3±0.4	NA	(Zwank *et al*., 2005)
Parsippany NJ gas station microcosms	Methanogenic	-9.16±5.0	NA	NA	(Kolhatkar *et al*., 2002)
Parsippany NJ gas station Field groundwater samples	Methanogenic	-8.1±0.9	NA	NA	(Kolhatkar *et al*., 2002)
Parsippany, NJ gas station culture enriched from groundwater microcosm	Anoxic	-13.0±1.1	–16±5	1	(Kuder *et al*., 2005)
New York Harbor culture enriched from sediment plus syringic acid	Methanogenic	–7.0±0.2	–41±1	6	(Youngster *et al*., 2010b)
New York Harbor culture enriched from sediment	Methanogenic	–6.7±0.2	Insignificant	NA	(Youngster *et al*., 2010b)
Arthur Kill, NJ culture enriched from sediment	Sulfidogenic	–7.14±0.86	Insignificant	NA	(Youngster *et al*., 2010b)
Arthur Kill, NJ culture enriched from sediment	Methanogenic	-15.6±4.1	NA	NA	(Somsamak *et al*., 2005)
Coronado Cay, CA culture enriched from sediment	Methanogenic	-14.4±1.5	NA	NA	(Somsamak *et al*., 2005)
Coronado Cay, CA culture enriched from sediment	Sulfidogenic	-14.4±3.6	NA	NA	(Somsamak *et al*., 2005)

Carbon and hydrogen enrichment factors (ε) and Λ values (slope of the linear regression of $\Delta\delta 2$ H/$\Delta\delta 13$ C or approximated εH/εC)

TABLE 1.3. Summary of key enzymes, genes and stable-isotope fractionation during aerobic MTBE biodegradation by representative strains.

	Strain	Degradation of TBA	Enzyme	Encode Gene	Growth compounds	εC (‰) (95%CI)	εH[‰] (95%CI)	Λ
Metabolism	*Methylibium petroliphilum* PM1	Yes	alkane hydroxylase	*mpdA*	MTBE and TBA	-2.2±0.1 (n = 4)	-35±2 (n = 4)	18±3
	Methylibium sp. strain R8	Yes	alkane hydroxylase	*mpdA*	MTBE and TBA	-2.4±0.1 (n = 2)	-42±4 (n = 2)	15
	Hydrogenophaga flava ENV735	Yes	Constitutive monooxygenase	Unknown	MTBE and TBA	ND	ND	ND
	Aquincola tertiaricarbonis L108	Yes	monooxygenase	*ethB*	MTBE and TBA	-0.5±0.1 (n=1)	Insignificant	NA
	Variovorax paradoxus CL-8	Yes	Unknown	Unknown	MTBE and TBA	–1.1±0.2 (n=3)	–15±2 (n=3)	14
Cometabolism	*Pseudomonas putida* CAM	No	cytochromes P450	Unknown	CAM	ND	ND	ND
	Pseudomonas putida Gpo1	No	alkane hydroxylase	*alkB*	Octane	–1.4±0.1 (n=3)	–11±2 (n=3)	8 ± 1
	Pseudomonas mendocina KR1	No	alkane hydroxylase	*alkB*	n-alkanes	ND	ND	ND
	Rhodococcus ruber IFP 2001	No	cytochromes P450	*ethB*	ETBE	-0.3±0.1	Insignificant	NA
	Mycobacterium sp. IFP 2009	No	cytochromes P450	*ethB*	ETBE	ND	ND	ND
	Rhodococcus zopfii IFP 2005	No	cytochromes P450	*ethB*	ETBE	ND	ND	ND
	Rhodococcus aetherivorans	No	cytochromes P450	*ethB*	ETBE	ND	ND	ND
	Pseudonocardia tetrahydrofuranoxydans K1	No	tetrahydrofuran monoooxygenase	Unknown	THF	-2.3±0.2 (n=2)	-100±10 (n=2)	48±5
Oxidation	*Mycobacterium vaccae* JOB5	Yes slow	alkane monooxygenase	Unknown	Propane	–2.50±0.04 (n=3)	–4.2±0.9 (n=3)	1.7 ± 0.3
	Mycobacterium austroafricanum IFP 2012	Yes fast	alkane monooxygenase	*alkB*	MTBE and TBA	–2.64±0.08 (n=5)	Insignificant	NA

References of the organisms:
Methylibium petroliphilum PM1 (Deeb *et al*., 2001; Gray *et al*., 2002; Nakatsu *et al*., 2006; Hristova *et al*., 2007)
Methylibium sp. strain R8 (Hristova *et al*., 2007; Rosell *et al*., 2007b; Bastida *et al*., 2010; Rosell *et al*., 2012)
Hydrogenophaga flava ENV735 (Hatzinger *et al*., 2001)

Aquincola tertiaricarbonis L108 (Piveteau *et al*., 2001; Rohwerder *et al*., 2006; Rosell *et al*., 2007b)
Variovorax paradoxus CL-8 (Zaitsev *et al*., 2007; Youngster *et al*., 2010b)
Pseudomonas putida CAM (Steffan *et al*., 1997)
Pseudomonas putida Gpo1 (Smith & Hyman, 2004; Rosell *et al*., 2012)
Pseudomonas mendocina KR1 (Steffan *et al*., 1997; Hyman, 2013)
Rhodococcus ruber IFP 2001 (Chauvaux *et al*., 2001; Hernandez-Perez *et al*., 2001; Rosell *et al*., 2007b)
Mycobacterium sp. IFP 2009 (Béguin *et al*., 2003)
Rhodococcus zopfii IFP 2005 (Béguin *et al*., 2003)
Rhodococcus aetherivorans (Goodfellow *et al*., 2004; Auffret *et al*., 2009)
Pseudonocardia tetrahydrofuranoxydans K1 (Thiemer *et al*., 2003; McKelvie *et al*., 2009)
Mycobacterium vaccae JOB5 (Smith *et al*., 2003; Johnson *et al*., 2004; Rosell *et al*., 2012)
Mycobacterium austroafricanum IFP 2012 (François *et al*., 2002; Lopes Ferreira *et al*., 2006; Rosell *et al*., 2012)

TABLE 1.4. Summary of anaerobic MTBE-degrading microbial communities enriched under different redox conditions.

Source	Redox condition	Dominant phylotypes	Reference
Arthur Kill, NJ culture enriched from sediment	Methanogenic	*Firmicutes, Chloroflexi and Deltaproteobacteria*	(Youngster *et al*., 2010a)
East Lansing, MI microcosms from activated sludge	Methanogenic	*Firmicutes* and *Alphaproteobacteria*	(Sun *et al*., 2012)
Vandenberg Air Force Base, CA in situ microcosm	Sulfate-reducing	*Betaproteobacteria* and *Gammaproteobacteria*	(Key *et al*., 2013)
California gas station culture enriched from sediment	Sulfate-reducing	*Firmicutes, Spirochaetales*, Deltaproteobacteria and *Actinobacteria*	(Wei & Finneran, 2009)
Denver, CO culture enriched from air-stripping system of petroleum refinery	Sulfate-reducing	*Firmicutes*	(Raynal *et al*., 2010)
California gas station culture enriched from sediment	Anthraquinone-2,6-disulfonate and Fe(III)	*Deltaproteobacteria*, *Actinobacteteria*, *Firmicutes*	(Wei & Finneran, 2009)
California gas station culture enriched from sediment	Fumarate and Fe(III)	*Firmicutes*, *Betaproteobacteria*, *Actinobacteria*	(Wei & Finneran, 2009)
Port Hueneme, CA culture enriched from Porous-Pot reactor sample	Iron-reducing	*Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*	(Raynal *et al*., 2010)

Chapter 2

Identification of active methyl *tert*-butyl degrading bacteria in a methanogenic consortium by stable isotope probing

ABSTRACT

The widespread use of methyl *tert*-butyl ether (MTBE) has caused major contamination of groundwater and is of concern due to its taste and odor problems, as well as toxicity. MTBE can be degraded anaerobically which makes bioremediation of contaminated aquifers a potential solution to address this problem. Nevertheless, the organisms and mechanisms that are responsible for anaerobic MTBE degradation are still unknown. The aim of our research is to shed light on the organisms actively degrading MTBE. For this purpose we collected sediment samples from the New Jersey Arthur Kill intertidal straight and enriched them with MTBE as the sole carbon source under methanogenic conditions. The cultures were analyzed using stable isotope probing (SIP) combined with terminal restriction fragment length polymorphism (T-RFLP) and clone library analysis of bacterial 16S rRNA genes. In particular, phylotypes belonging to *Firmicutes* were predominant in the methanogenic cultures. Stable isotope probing (SIP) experiments showed the sequential incorporation of the ^{13}C labeled MTBE by the bacterial community and a bacterium most closely related to *Saccharofermentans acetigenes* was identified as the bacterium active in O-demethylation of MTBE.

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is one of a group of oxygenates which was initially introduced as a fuel additive to replace tetra-ethyl lead in the late 1970s (Deeb *et al*., 2000). MTBE enhances the octane level of gasoline and therefore improves the combustion efficiency and reduces hazardous tailpipe emissions (carbon monoxide) to the atmosphere (Franklin *et al*., 2000; Häggblom *et al*., 2007). In 1990, the United States Clean Air Act amendments set the requirement for fuel oxygenates, with MTBE chosen over other possible oxygenates due to its blending characteristics and reasonable price (Squillace & Moran, 2007). As a result, up to 15% MTBE by volume was amended to fuel, with more than 10 billion liters of MTBE produced annually between 1995-2002 to fulfill this requirement (Franklin *et al*., 2000; AWWA, 2005; Carter *et al*., 2006; Sweet *et al*., 2010).

The wide use of MTBE has caused extensive groundwater contamination (Zogorski *et al*., 2006). Drinking water that is contaminated by MTBE has unacceptable taste and odor. MTBE is also a potential carcinogen to humans and can cause reproductive mutations in aquatic wildlife (Shamsipur *et al*., 2012; Bermudez *et al*., 2012). In addition to the taste, odor and health concerns, an additional issue of MTBE is its persistence and mobility in groundwater (Stocking *et al*., 2000; Deeb *et al*., 2003; Ayotte *et al*., 2005; van Wezel *et al*., 2009). MTBE can be introduced into the environment from leaking underground storage tanks and pipelines, industrial plants and refueling facilities and accidental spills during transport (Deeb *et al*., 2003; Carter *et al*., 2006). After several decades of heavy use, MTBE is widely spread in US and became one of the most frequently detected

volatile organic compounds in groundwater aquifers and domestic and public wells (Juhler & Felding, 2003; Squillace *et al*., 2004). Therefore, twenty-five states had signed legislation to ban or restrict the use of MTBE in gasoline by 2005. Efforts are being undertaken to reduce the contamination of MTBE in groundwater, as well.

Monitored natural attenuation is an attractive solution to clean up MTBE from contaminated groundwater in recent years, as it is the most financially realistic treatment for most contaminated groundwater cases (Keller *et al*., 1998; Van Deuren *et al*., 2002). Among different natural processes, biodegradation is the most effective method to reduce the mass of contaminants in a sustainable way (Deeb *et al*., 2003; USEPA, 2005; Sarkar *et al*., 2005). Therefore, when a MTBE biodegradation approach is undertaken, the efficiency assessment of the *in situ* remediation, and the identification of the microorganisms that are capable and responsible for degrading MTBE are critical.

Stable isotope probing (SIP) is a powerful tool to assess the biodegradation potential during natural attenuation processes. SIP is a technique based on the incorporation of a ^{13}C-labeled substrate to cellular nucleic acid, separation of ^{13}C-labeled from ^{12}C-unlabeled nucleic acid by density gradient centrifugation, and identification of active populations carrying labeled substrates (Radajewski *et al*., 2000). SIP offers a way to link specific environmental functions with the responsible organisms, which can be employed to identify the key microorganisms in metabolism of certain contaminants (Wellington *et al*., 2003; Dumont & Murrell, 2005; Madsen, 2006; Kerkhof & Häggblom, 2008). For instance, SIP has identified degraders of benzene, pyrene, naphthalene and benzoate in

the environments (Yu & Chu, 2005; Gallagher *et al*., 2005; Singleton *et al*., 2006; Kunapuli *et al*., 2007).

Anaerobic MTBE degradation coupled to methanogenesis was demonstrated in enrichment cultures established with sediment from the Arthur Kill intertidal straight between New Jersey and the New York harbor (Somsamak *et al*., 2005.) The methanogenic cultures were subsequently enriched with MTBE as the sole carbon source and maintained under strict anaerobic conditions for more than a decade, and through successive transfers reached a the total dilution of 10^{-7}–10^{-9} of the original cultures. Previous studies have shown that these microorganisms can O-demethylate MTBE leading to accumulation of *tert*-butyl alcohol (TBA) in the cultures (Somsamak *et al*., 2001; Somsamak *et al*., 2005). Bromoethane sulfonic acid, a specific inhibitor of methanogenesis, did not inhibit initial MTBE degradation, indicating that biodegradation is mediated by bacteria (Youngster *et al*., 2008). CH_4, accumulation in the headspace suggests that methanogens might utilize the degradation products of MTBE (Somsamak *et al*., 2006). However, knowledge of the responsible microorganisms is still limited. In this study, we analyzed the bacterial community structure of Arthur Kill methanogenic enrichment cultures and applied DNA-SIP to identify the bacteria active in anaerobic biodegradation of MTBE.

MATERIALS AND METHODS

Experiment setup and Analytical Methods.

Anaerobic enrichment cultures used in this study were originally established with 10% sediment (v/v) from the Arthur Kill Inlet (AK) between New Jersey and the New York Harbor (Somsamak *et al*., 2001; Somsamak *et al*., 2005; Youngster *et al*., 2008). The enrichment cultures have been maintained for over a decade using strict anaerobic technique under methanogenic conditions at 28 ˚C. The cultures were repeatedly transferred at 3–6-month intervals and usually at a 1:10 dilution, into fresh medium and enriched with MTBE (Aldrich, Milwaukee, WI) as the sole carbon source (Somsamak *et al*., 2001; Somsamak *et al*., 2005; Youngster *et al*., 2008). MTBE concentration was regularly monitored using gas chromatography with flame ionization detection (Somsamak *et al*., 2001).

MTBE SIP cultures

In this study, six replicate enrichment cultures were set up for SIP experiments. These cultures were transferred to 60 mL glass serum vials, capped with Teflon-coated stoppers and aluminum crimp seals and incubated at 28 °C. Three experimental cultures were spiked with 100 μM MTBE with ^{13}C labeled O-methyl carbon (kindly provided by H. Richnow, UFZ, Leipzig). Three control cultures were spiked with 100 μM ^{12}C-MTBE. Samples were taken for analysis at three time points after approximately 0%, 20% and 50% MTBE removal.

Acetic acid SIP cultures

Based on previous findings (Youngster *et al*., 2008; Youngster *et al*., 2010a), we hypothesized that acetogenic bacteria are able to cleave the ether bond of MTBE and convert the methyl group to acetate, which eventually can serve as the carbon source for other members of the microbial community. To test this hypothesis, 150 μM ^{12}C or ^{13}C acetic acid (both carbons labeled), respectively, were added to ^{12}C MTBE amended cultures after 50% degradation had occurred. The species that can utilize acetate in the MTBE-degrading communities are likely not the primary MTBE degraders, but utilize the product of acetogenic metabolism. The community structures of ^{12}C and ^{13}C acetic acid cultures were analyzed after incubation for four days and one month at 28 °C.

DNA extraction and SIP Gradients

For each SIP sample, 100 ng ^{12}C *Halobacterium salinarium* DNA and 100 ng ^{13}C *H. salinarium* DNA was added. To obtain the carrier DNA, the archaeon *H. salinarium*, was grown in marine broth 2216 (Difco, Detroit, Michigan) and ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg, OH), diluted 1:10 with sterile 4.5M NaCl. Cultures were incubated at 37°C with shaking until they reached an appropriate density for harvesting and DNA extraction (Gallagher *et al*., 2005). After carrier addition, genomic DNA was extracted by a modified phenol-chloroform extraction procedure (Kerkhof & Ward, 1993; Sakano & Kerkhof, 1998). The extracted DNA (approximately 300 ng including the MTBE community and *H. salinarium* DNA) was then dissolved in 500 μl CsCl density gradient (~1 g/mL) containing ethidium bromide. ^{13}C-labeled DNA was separated from ^{12}C DNA by 36 hours of centrifugation at 225,000 g on a Beckman

Optima ultracentrifuge (Palo Alto, CA) using a TLA 120 rotor (Gallagher *et al*., 2005). This approach yields a top band containing ^{12}C DNA from the resident bacteria and a bottom band containing ^{13}C DNA from newly synthesized bacteria that utilized ^{13}C-labeled MTBE or acetic acid. 40 µl of ^{12}C top DNA and 20 µl of ^{13}C bottom bands were collected by pipette from the CsCl gradient under UV light visualization and samples were dialyzed (in 10 mM Tris) for 45 minutes (Gallagher *et al*., 2005; Kerkhof *et al*., 2011).

DAPI staining

One Arthur Kill methanogenic ^{12}C MTBE amended culture was selected for cell counting. One ml of cell culture was sonicated for 30 s and centrifuged at 2000 g for 3 min to remove FeS precipitates in the medium. The cells were then stained with the dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 12.5 μg/ml, Vector Laboratories Inc., CA) in the dark for 5 min. Before mounting onto a slide with a drop of SlowFade® Gold antifade reagent (Life technologies, CA), the stained cells were filtered onto a 0.2 µm black polycarbonate filter paper and flushed with PBS to wash the sample. The cells were counted manually through epifluorescence images (ImageXpressTM, AXON, USA) at λ ex = 358 nm, λ em = 461 nm.

16S rRNA gene amplification and T-RFLP

The 16S rRNA genes of genomic DNA were amplified with 20 pmol universal bacterial primers: 50-end 6-FAM-labeled 27 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1100 reverse (5'-AGGGTTGCGCTCGTTG-3') to avoid amplification of *H*.

salinarium. PCR amplification parameters were as follows: 94 °C for 5 min, and then 94 °C for 30 s, 57 °C for 30 s and 25 cycles with a final extension at 72 °C for 10 min (Foster City, CA, USA). Two μl of the first round PCR product, used as the template, was amplified with identical PCR parameters again for 30 cycles (Tuorto *et al*., 2013) Approximately 15-20 ng of the PCR products were digested with restriction enzyme Mnl I (New England BioLabs, MA) at 37 °C for 6 hours. Digested samples were precipitated with sodium acetate and ethanol, resuspended in formamide with a ROX standard and denatured at 94°C (Gallagher *et al*., 2005). Samples were analyzed on an ABI 310 automated sequencer (Applied Biosystems Instruments, Foster City, CA), which generated a fingerprint of each community by Terminal Restriction Fragment Length Polymorphism (TRFLP).

Cloning, sequencing and phylogenic analysis

One ^{12}C MTBE amended microcosm after 50% degradation was selected for clone analysis. The 16S rRNA genes from this culture were amplified by PCR with 27F and 1100R primers and a clone library was generated using the Promega pGEM®-T Vector System I cloning kit (Promega, Madison, WI). To identify the bacteria associated with corresponding T-RFs in the AK methanogenic enrichment cultures, each individual clone was screened by TRFLP to match predominant T-RFs in the community profile before sequencing. Plasmid DNA of selected clones was extracted using Zyppy Plasmid Miniprep Kits (Zymo, Irvine, CA) and sequenced by Genewiz Inc (North Brunswick, NJ). The T-RFs that composed 5% or more relative abundance in the communities were

recovered from the clone library allowing us to identify the predominant bacterial phylotypes of the culture.

Pyrosequencing

When 50% of amended ^{12}C MTBE had been degraded, the triplicate cultures were sampled and 16S rRNA genes of the bacterial community were amplified with 27F and 519R primers and sequenced by Molecular Research LP using a Roche 454 Genome Sequencer. A total of 12040, 7230 and 13371 quality-filtered reads, for each biological replicate, were clustered into 56 operational taxonomic units (OTUs) with 81.3% to 100% similarity, enabling identification of OTUs to the family level.

Data analysis

The 16S rRNA gene sequences were compared with the Basic Local Alignment Search Tool (Blast) and Ribosome Database Project (RDP) Seqmatch tool for taxonomic identification. Phylogenic trees were constructed using the program Geneious 8.1. Clone sequences and selected sequences of the most closely related cultivated species determined from Blast searches were used to construct a Maximum likelihood tree (Guindon & Gascuel, 2003; Babcock *et al*., 2007; Drummond *et al*., 2011).

RESULTS

Studies of anaerobic MTBE biodegradation

In order to monitor the MTBE degradation process for the SIP experiment, six replicate enrichment cultures were transferred 1:2.5 into fresh medium to yield a total dilution of

10^{-11} of the original culture that had previously been established with 10% Arthur Kill sediment. The cultures were amended with either 100 μM ^{13}C-labeled MTBE or with ^{12}C MTBE as the control, each in triplicate. MTBE depletion occurred after 21 days in both the ^{13}C- and ^{12}C-MTBE amended cultures with similar degradation rates (Figure 2.1). The concentration of TBA, the daughter product of MTBE degradation, increased along with the decrease in MTBE concentration.

Identification of active bacteria during MTBE degradation

The stable isotope probing technique was applied to determine the bacterial species that actively utilized MTBE in the communities. Figure 2.2 shows SIP-TRFLP profiles of the AK methanogenic cultures in biological replicates. For each replicate culture the TRFLP fingerprints at different time points illustrate the predominant peaks in active community (^{13}C) compared to the resident community (^{12}C). The 207 bp terminal restriction fragment (T-RF) was predominant in the ^{13}C experimental profiles (^{13}C bottom band), suggesting that this species could initially utilize the methyl group of MTBE (Figure 2.2c red). A maximum likelihood phylogenic tree of the clones and their closest cultured bacterial species was constructed (Figure 2.3). The 207 bp clone, the predominant ^{13}C MTBE utilizing and assimilating phylotype, clustered in the *Firmicutes* with *Saccharofermentans acetigenes* as its closest cultivated relative (94% 16S rRNA gene similarity over 1090bp compared). The most similar uncultured clone of 207 bp TR-F was from a PCB degrading community (Ho and Liu 2010) (99% similarity of 1069 bp compared). The other clones spread across diverse phyla, including *Synergistetes*,

Actinobacteria, *Proteobacteria* and *Firmicutes*. Many of the clones were closely related to uncultured environmental clones, but only distantly related to cultivated isolates.

Microbial community analysis by TRFLP

The AK methanogenic cultures had a relatively complex bacterial community containing variety of phylotypes (Figure 2.4). In time zero profiles, 57.9± 4.4% of the total TRFLP peak area was from the 95 bp, 121 bp, 167 bp, 210 bp and 236 bp T-RFs. These T-RFs represented the initial resident community structure of the AK methanogenic culture. After 20% MTBE degradation occurred, the predominant T-RFs of the resident communities were nearly identical between ^{12}C-MTBE-control and ^{13}C-MTBE-experimental treatments (Figure 2.2b and 2.2c blue). The only exception was the 282 bp T-RF, which appeared in the ^{12}C top band. The relative abundance of 95 bp, 121 bp and 210 bp T-RFs increased from 29.2±5.7% to 44.7±5.1% among all the OTUs as MTBE was degraded. No T-RFs showed in ^{12}C-MTBE-control SIP profile (^{12}C bottom band) indicating there was no amplification of *Halobacterium* DNA in ^{12}C controls (Figure 2.2b red). Again, 95bp, 121 bp, 131 bp, 210 bp, 222 bp T-RFs represented 50.1±10.9% of predominant species in the resident community.

Microbial community analysis by pyrosequencing

When MTBE degradation reached 50%, 16S rRNA genes of ^{12}C MTBE amended bacterial communities were amplified and sequenced using a pyrotag sequencing approach. The pyrosequencing results showed that 60.0 ±20.3% of the OTUs were within the family of *Desulfobulbaceae*, *Synergistaceae*, *Acidimicrobiales* and *Peptococcaceae,*

which was in general agreement with the TRFLP-clone library results of 50.1±10.9%. The relative abundance of *Ruminococcaceae* represented 3.4±1.9% of the bacterial community which was also similar to the TRFLP results of 3.7±1.7% (Figure 2.5). Most importantly, the species identified from 454 pyrosequencing clustered with the same species identified from the clone library, which confirmed the accuracy of clone library identification (Appendix Fig.1).

Utilization of acetate

Figure 2.6 shows the acetic acid utilizing bacterial community of the AK enrichment culture, suggesting that they may not directly utilize MTBE. 282 bp T-RF had shown the incorporation of ^{13}C acetic acid (Figure 2.6d red). The closest isolated species of the likely ^{13}C acetic acid utilizer 282 bp T-RF clone was *Syntrophobacter fumaroxidans* belonging to the *Deltaproteobaacteria* (Harmsen *et al*., 1998; Plugge *et al*., 2012). Interestingly, the closest described uncultured clone of 282 bp T-RF was also from the same PCB degrading community as for the 207 bp T-RF (99% similarity of 1111 bp compared) (Ho & Liu, 2010). The TRFLP fingerprints indicated that the resident communities of ^{12}C and ^{13}C acetic acid amendment cultures tended to share a similar structure. After 4 days incubation, T-RFs 95 bp and 210 bp were the only predominant T-RFs in both ^{12}C and ^{13}C acetic acid amendment cultures. No T-RF was detected that specifically corresponded to ^{13}C acetic acid incorporation (Figure 2.6a and 2.6b). After one month incubation, up to 57% of the community consisted of the 95 bp, 121 bp, 131 bp, 210 bp and 222 bp T-RFs. However, the relative abundance of 131 bp T-RF

significantly increased (Figure 2.6c and 2.6d) in comparison to the 20% MTBE degradation profile (Figure 2.2b and 2.2c).

DISCUSSION

Anaerobic biodegradation of MTBE has been shown in a number of microcosms and field studies under different conditions. Nevertheless, the specific responsible organisms and mechanisms are still largely unknown. Our previous study has shown that anaerobic biodegradation of MTBE is an O-demethylation process likely mediated by acetogenic bacteria with *Deltaproteobacteria*, *Chloroflexi*, and *Firmicutes* as the three predominant phylotypes in the community (Youngster *et al*., 2008). Here we provide evidence that the bacterium mediating the initial degradation of MTBE in a methanogenic enrichment culture is a member of the *Ruminococcaceae* family. The 207 bp R-TF clone clustered with *Saccharofermentans acetigenes*, a newly discovered species within the *Ruminococcaceae* family, of the order *Clostridiales* in the *Firmicutes*. *S. acetigenes* is an obligately anaerobic bacterium isolated from brewery wastewater treatment sludge (Chen *et al*., 2010).

Of particular interest is that the bacterium initially incorporating the methyl carbon of MTBE is most closely related to an acetogenic bacterium. Many acetogenic bacteria are capable of utilizing methyl groups to synthesize acetate with formation of ATP via the reductive acetyl-coenzyme A pathway (Fuchs, 2011). In a typical reductive acetyl-CoA pathway, both carbon monoxide and methyl groups are reduced from carbon dioxide prior to generating acetate (Drake *et al*., 2006). However, the methyl group can also be

O-demethylated from a range of natural methyl donors, such as ethers and aromatic compounds (Emde & Schink, 1987; Wagener & Schink, 1988; Tanaka & Pfennig, 1988; Schramm & Schink, 1991). In addition, anaerobic MTBE biodegradation is a corrinoid dependent process (Youngster *et al*., 2008). The methyl group of MTBE might be transported to tetrahydrofuran (THF), a methyl-accepting corrinoid protein, and then be transferred to coenzyme A by a methyltransferase system, eventually yielding acetate (Drake *et al*., 2006; Ragsdale & Pierce, 2008; Fuchs, 2011). Furthermore, methanogens in the community might assist anaerobic MTBE degradation via the reversed Wood–Ljungdahl pathway as a result of oxidization of acetate to methane and releasing two electrons to drive the reductive processes of the acetyl-CoA pathway (Somsamak *et al*., 2005; Wilson *et al*., 2005b; Drake *et al*., 2006; Ragsdale & Pierce, 2008; Sun *et al*., 2012). Unfortunately, due to the addition of the archaeal carrier DNA and the use of bacterial specific 16S rRNA gene primers to amplify the MTBE-degrading communities, the exact methanogens involved were not identified.

The theoretical energy available for growth may not translate to acetogenic cell yield (Drake, 1994). When CO is unitized as the substrate, the acetate-to-biomass ratio of *Clostridium thermoaceticum* is 60, which suggests that only very limited labeled carbon is assimilated into biomass of acetogenic bacteria (Daniel *et al*., 1990; Daniel & Drake, 1993). In this study, the estimated cell concentration of initial AK methanogenic culture was 4×10^5 cells/ml from DAPI staining data and the relative abundance of the 207 bp T-RF was 2-5% of the bacterial community (Figures 2.4 and 2.5). From the rate of MTBE loss (Fig. 2.1) and bacterial community abundance, the estimated MTBE degradation rate

was 2.1~5.3×10^{-5} μg MTBE/*Ruminococcaceae* cell/day. This estimation was based on the assumptions that (1) the archaeal population accounted for 10% of the community, (2) the total cell density remained essentially constant of the incubation period, and (3) MTBE was only utilized by *Ruminococcaceae* (Appendix 2). Although theoretically 100 μM O-methyl carbon labeled ^{13}C MTBE would provide sufficient carbon and energy for the acetogenic bacterial population to double twice, the combination of the low initial biomass and limited labeled carbon availability resulted in the low abundance of active ^{13}C incorporation T-RFs in the communities.

Only a few previous studies have also applied the SIP technique to identify microorganisms involved in MTBE degradation under anaerobic conditions (Busch-Harris *et al*., 2008; Sun *et al*., 2012; Key *et al*., 2013). According to their studies, the relative abundance of *Ruminococcaceae* was also less than 10% of the overall microbial communities. For instance, "bio-traps" and PLFA-SIP was used to investigate the microbial communities in MTBE/TBA contaminated groundwater aquifer. The results suggested that ^{13}C labeled MTBE was incorporated into cell phospholipids (Busch-Harris *et al*., 2008). Sun *et al*. (2012) examined the active MTBE-degrading microcosms established from activated sludge under methanogenic conditions by DNA-SIP. The bacteria from *Ruminococcaceae* and *Alphaproteobacteria* were identified from ^{13}C labeled DNA as putative MTBE degraders. Interestingly, *Ruminococcaceae* had also been identified in another SIP study of the MTBE contamination groundwater in California (Key *et al*., 2013). O-demethylation is the likely initial step in cleaveage of the ether bond of MTBE (Youngster *et al*., 2008), the evidence from this study along with

other community analyses (Sun *et al*., 2012; Key *et al*., 2013) suggest that the acetogens belonging to *Ruminococcaceae* have the capability for MTBE O-demethylation and assimilation of the methyl carbon.

The analysis of the acetic acid utilizing bacterial community suggested that acetate might not be immediately converted to biomass and incorporated to DNA, since the acetic acid experimental T-RFs were only present in the later time point. O-demethylating acetogens might feed methanogens, but then slowly from biomass decay various fermenting organisms could utilize the MTBE carbon. Several T-RFs were identified to closely relate to sulfate-reducing bacteria in the MTBE-degrading community, which were possibly supported by fermentation or the oxidation of ferric sulfide to sulfate in the media.

When acetic acid was added to the ^{12}C MTBE amendment cultures, the relative abundance of the 131 bp T-RF significantly increased during the incubation (Figure 2.6). One of the closest species of 131bp T-RF is *Paenirhodobacter enshiensis* (97% similarity of 1053 bp compared), which is a facultative anaerobic species in the *Alphaproteobacteria*. *P. enshiensis* grows chemoheterotrophically and can use acetate as a sole carbon source (Wang *et al*., 2014). Alternately, the 167 bp T-RF clustered with *Dechlorosoma suillum* which can also oxidize acetate (Achenbach *et al*., 2001), and might be a competitor of the 131 bp T-RF *Alphaproteobacteria*. The species related to the 95 bp, 121 bp and 210 bp T-RFs might be able to further consume fermentation products of acetate. These T-RFs are distantly related to *Thermanaerovibrio velox* (89% similarity of 1082 bp compared), *Gaiella occulta* (86% similarity of 1100 bp compared)

and *Desulfobulbus propionicus* (91% similarity of 1124 bp compared), respectively. *T. velox* is a member of the *Synergistetes* phylum that are able to ferment variety of organic substrates but not acetate (Zavarzina *et al*., 2000). *Actinobacteria* are frequently detected in anaerobic MTBE degrading studies (Wei & Finneran, 2009; Raynal *et al*., 2010; Sun *et al*., 2012). However *G. occulta* is a strictly aerobic organism that cannot assimilate acetate (Albuquerque *et al*., 2011). The 210 bp T-RF clustered with *D. propionicus* in the *Deltaproteobacteria*, which can also ferment a variety of substrates to propionate if sulfate is absent and H_2 and CO_2 are present (Widdel & Pfennig, 1982; Tasaki *et al*., 1993; Pagani *et al*., 2011). If sulfate is limited, *D. propionicus* can connect through nanowires of cable for electrons transport that help them compete with the other sulfate reducers (Pfeffer *et al*., 2012). Finally, *Syntrophobacter fumaroxidans*, the closet described species of the 282 bp T-RF (92% similarity of 1127 bp compared), is a propionate-oxidizing bacterium syntrophically cocultured with methanogens that produces acetate and CO_2 (Harmsen *et al*., 1998; de Bok *et al*., 2002; Plugge *et al*., 2012). *Syntrophobacter* species are found to associate with syntrophic degradation of propionate to acetate in methanogenic ecosystems by cleavage acetyl-CoA (Stams & Plugge, 1994). The 282 bp T-RF represented the only species in ^{13}C-acetic acid experimental profiles might possible due to inefficient incorporation of isotope labeled carbon to DNA.

In summary, acetogenic bacteria in the *Ruminococcaceae* family are hypothesized to initally O-demethylate MTBE via the reductive CoA pathway and are facilitated by methanogens that consume the acetate. The other microoganisms in the in AK enrichment culture community likely metabolize the decayed biomass of acetogens and

methanogens for further fermentation. *Ruminococcaceae* species are also the key bacteria active in the other SIP studies of MTBE degradation (Sun *et al*., 2012). A characterization of the metabolic pathways and identification of the microorganisms responsible for the activity will help us better understand anaerobic MTBE degradation processes in the field and determine biomarkers to use for monitoring natural attenuation.

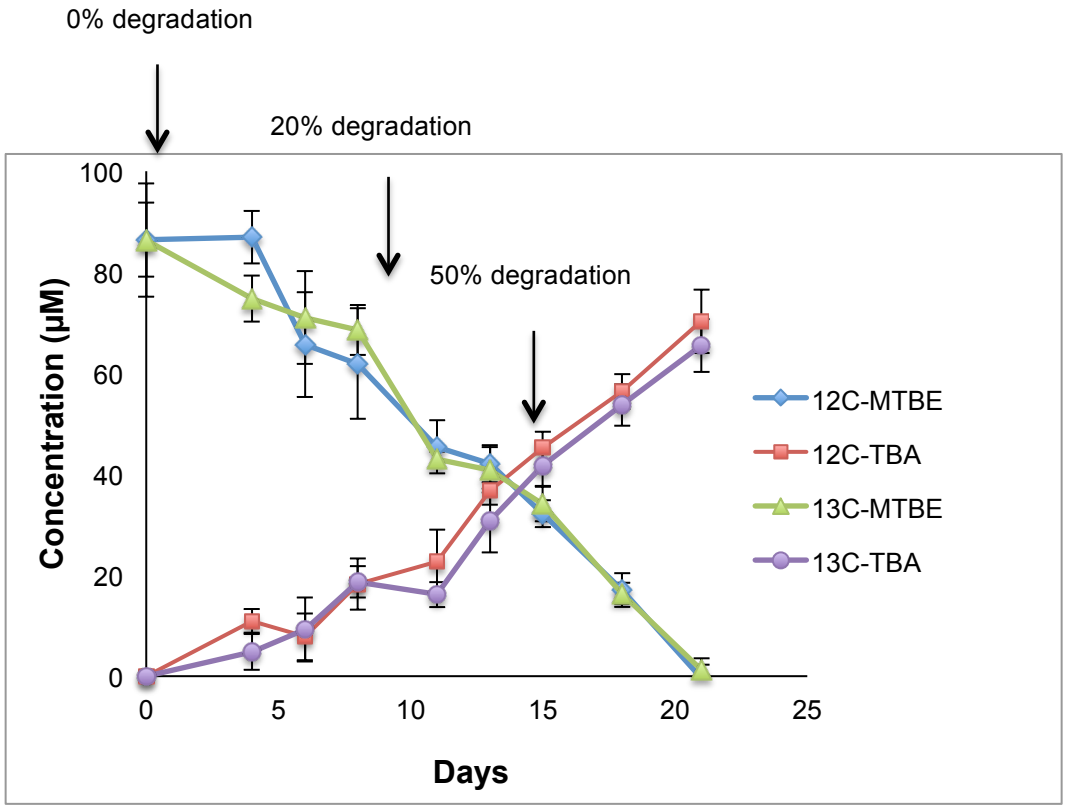


FIGURE 2.1. Biodegradation of ^{12}C MTBE and ^{13}C MTBE to ^{12}C TBA and ^{13}C TBA during anaerobic MTBE biodegradation by methanogenic Arthur Kill enrichment cultures. Error bars represent the mean and standard deviation of triplicate cultures. Arrows indicate sampling points for community analyses.

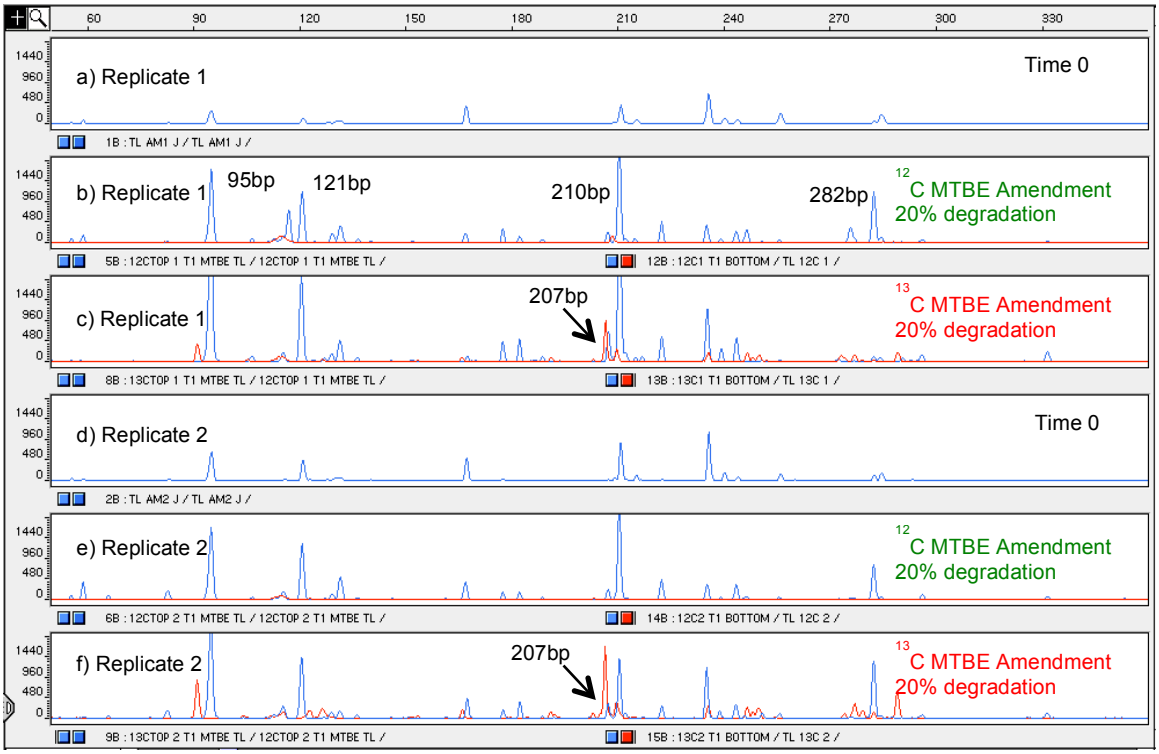


FIGURE 2.2. 16S rRNA gene T-RFLP fingerprints of AK methanogenic cultures of ^{12}C and ^{13}C DNA, demonstrating the resident (blue) and the active (red) communities, respectively. a) Time zero resident community; b) ^{12}C-MTBE-treatment control; c) ^{13}C-MTBE- experimental SIP profiles after 20% degradation of biological replicate 1. d) Time zero resident community; e) ^{12}C-MTBE-treatment control; f) ^{13}C-MTBE-experimental SIP profiles after 20% degradation of biological replicate 2.

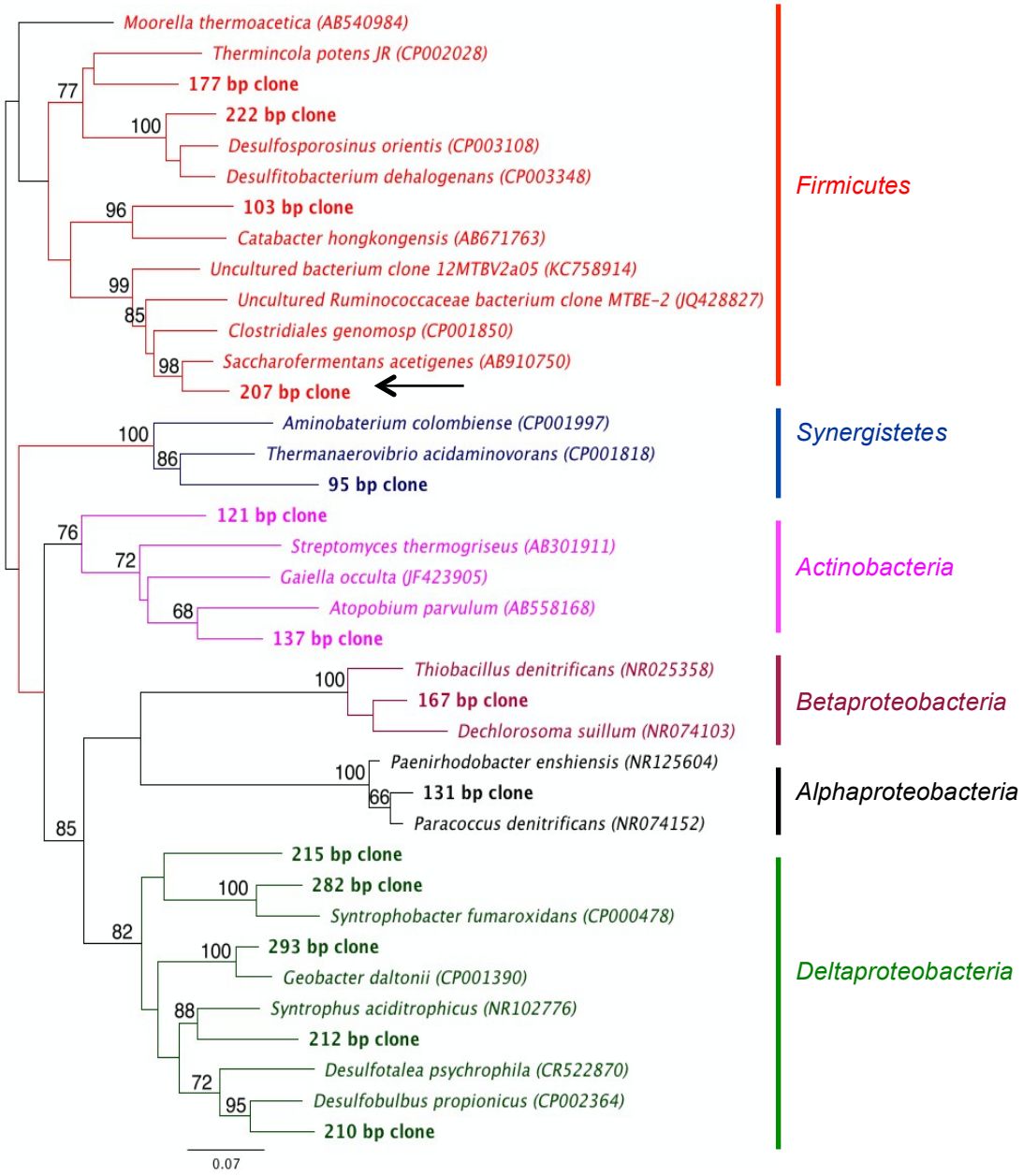


FIGURE 2.3. Maximum likelihood phylogenetic relationships of organisms detected in Arthur Kill MTBE-degrading methanogenic culture based on 16S rRNA gene sequences. 1073 bp unambiguously aligned nucleotide positions were used analysis. Numbers at nodes indicate bootstrap values from 100 replications. Accession numbers for reference sequences are indicated in parenthesis.

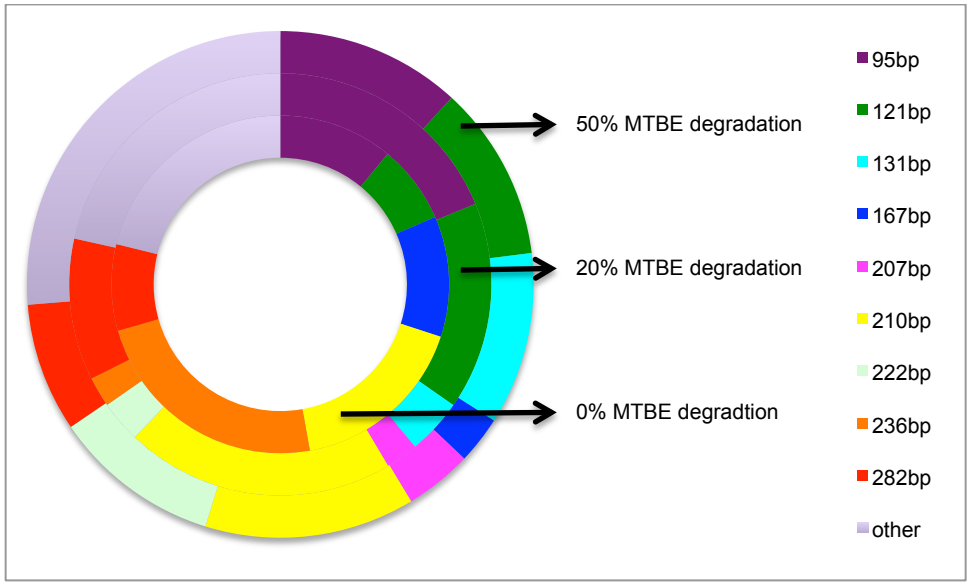


FIGURE 2.4. The relative percentages of bacterial species identified in the clone libraries as determined by Terminal Restriction Fragment Length Polymorphism (TRFLP).

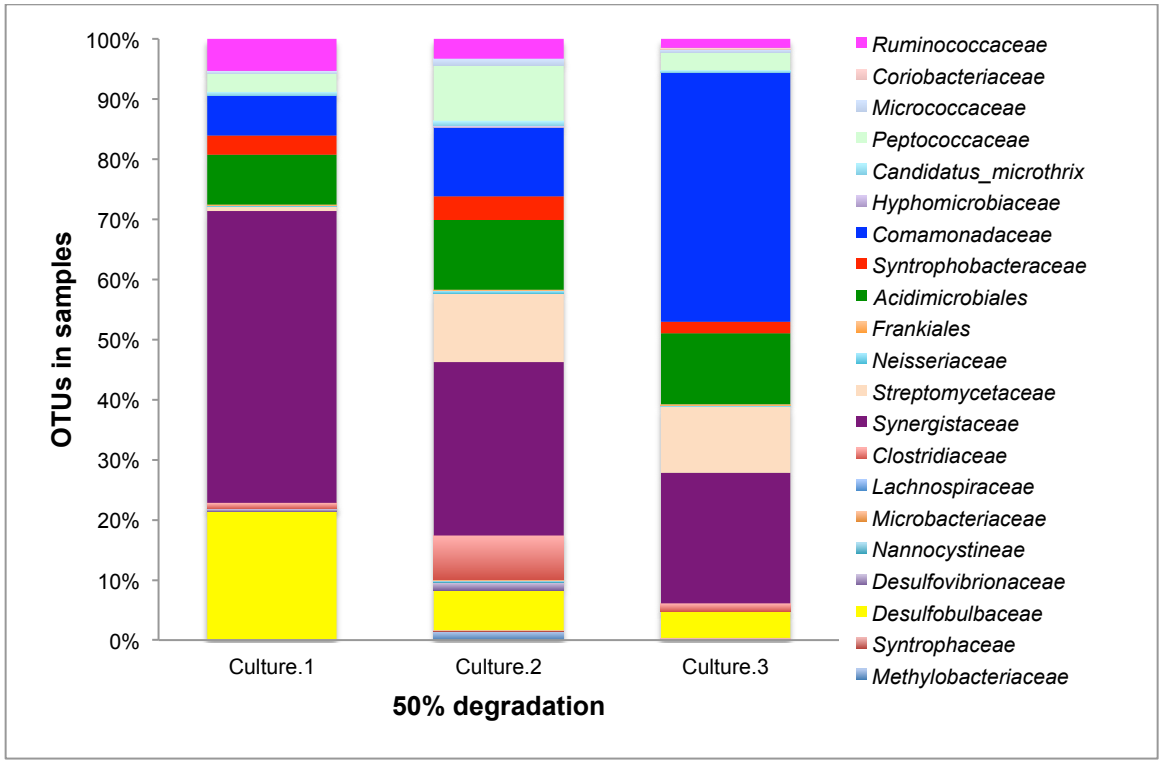


FIGURE 2.5. Relative abundance of bacterial species identified as determined by 16S rRNA gene pyrosequencing and clustered to the family level.

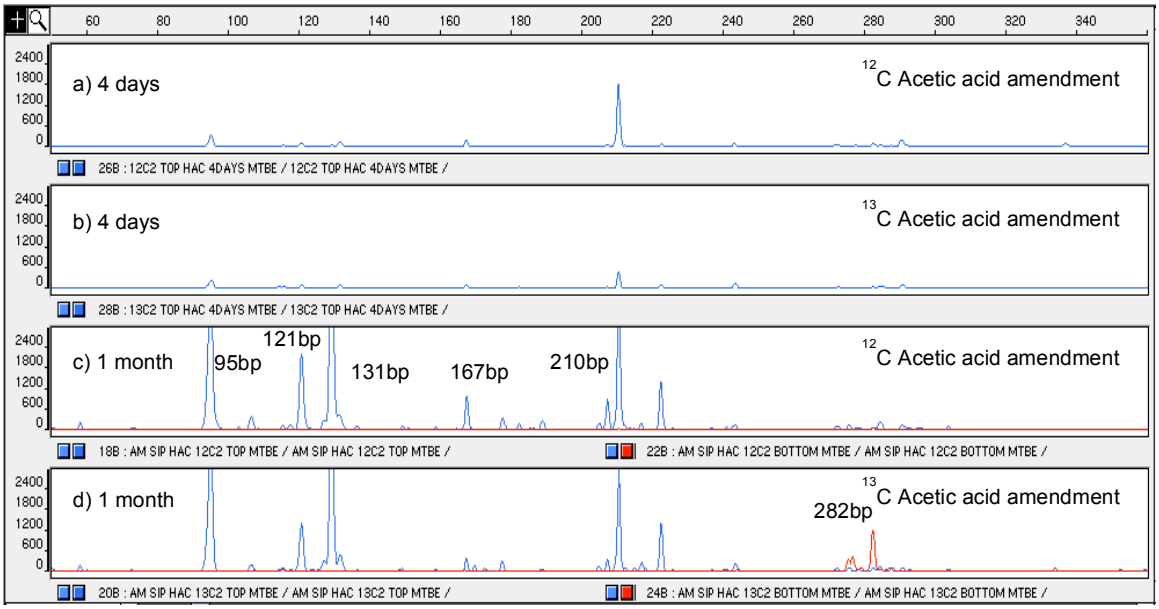


FIGURE 2.6. 16S rRNA gene TRFLP fingerprints of acetic acid amended cultures, demonstrating the resident (blue) and the active (red) communities, respectively. a) Four days incubation of ^{12}C-acetic acid- treatment control, b) four days incubation of ^{13}C-acetic acid experimental, c) one month incubation of ^{12}C-acetic acid treatment control, and d) one month incubation of ^{13}C-acetic acid experimental SIP profiles. Specific TRFs of interest are indicated in the SIP profiles.

Chapter 3

Characterization of anaerobic sulfate-reducing MTBE-degrading communities

ABSTRACT

MTBE was the dominant fuel oxygenate and used worldwide among several gasoline additives. The widespread use of MTBE has caused major contamination of groundwater and is of concern due to taste and odor problems, and potential toxicity. It is highly water soluble and resistant to microbial degradation. Nonetheless, anaerobic degradation of MTBE has been demonstrated, including in sulfidogenic sediment enrichment cultures. However, the responsible organisms and the degradation mechanism are unknown. The aim of this study was to identify the organisms that mediate anaerobic biodegradation of MTBE in sulfidogenic cultures enriched from estuarine sediments (New Jersey Arthur Kill and New York Harbor). Terminal restriction fragment length polymorphism (T-RFLP) coupled with clone library analysis of bacterial 16S rRNA genes showed that multiple phylotypes were likely involved in anaerobic MTBE degradation, including members of the *Synergistetes*, *Firmicutes, Actinobacteria* and *Deltaproteobacteria*. The members of *Deltaproteobacteria* were highly enriched during the degradation process.

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) was first introduced as an engine anti-knocking agent to replace lead in late 1970s (Squillace *et al*., 1996) as it enhances the octane number of gasoline and reduces hazardous combustion emissions. Since the Clean Air Act Amendments of 1990 mandated the use of reformulated oxygenated fuel, MTBE was chosen over the other oxygenates because it has a high octane level, low cost and is easily blended with gasoline (USEPA, 2008). However, MTBE has a very strong unpleasant taste and odor. Even relatively small amounts of MTBE may cause large reserves of groundwater to be nonpotable and therefore impact municipal water supplies (USEPA, 2008; van Wezel *et al*., 2009). MTBE is also associated with human and environmental health concerns, because it is a possible carcinogen of humans and cause of reproductive mutations of aquatic wildlife (Moreels *et al*., 2006; Shamsipur *et al*., 2012; Bermudez *et al*., 2012). Additionally, in contrast to benzene, ethylbenzene, toluene and xylenes (BTEX), MTBE tends to be highly mobile and persistent in groundwater as a result of its high water solubility (51 g/ liter), low Henry's law constant and low soil adsorption coefficient (Deeb *et al*., 2003; Fiorenza & Rifai, 2003; USEPA, 2008).

After several decades of heavy use, MTBE has become one of the most common contaminants in groundwater that affects the quality of drinking water (Moran *et al*., 2005). To date, most states in the U.S. have banned or limited MTBE use in fuel in order to protect drinking water supplies (USEPA, 2008). Nevertheless, due to its recalcitrant nature, MTBE contamination is a persistent problem that requires remediation. Typical MTBE contaminated sites are subsurface aquifers that lack oxygen for aerobic

biodegradation processes (McMahon & Chapelle, 2008). In addition, microbial degradation of gasoline hydrocarbon co-contaminants results in rapid consumption of oxygen in the aquifer, and it is technically difficult and expensive to introduce sufficient oxygen into these source zones to promote aerobic degradation (Kane *et al*., 2001; Häggblom *et al*., 2007).

Although the stable chemical structure makes MTBE resistant to microbial attack, it has been reported that microorganisms can anaerobically degrade MTBE under different redox conditions, including denitrification (Bradley *et al*., 2001), Fe^{3+} reduction (Finneran & Lovley, 2001; Pruden *et al*., 2005; Wei & Finneran, 2009), sulfidogenesis (Somsamak *et al*., 2001; Somsamak *et al*., 2006; Youngster *et al*., 2008; Youngster *et al*., 2010a), and methanogenesis (Mormile *et al*., 1994; Somsamak *et al*., 2005; Youngster *et al*., 2010b; Sun *et al*., 2012). Hence, anaerobic bioremediation could be a feasible method to clean up MTBE contamination *in situ*.

A challenge for MTBE bioremediation is to evaluate the potential of *in situ* anaerobic biodegradation. To date, none of the responsible organisms have been conclusively identified or isolated under anaerobic conditions and the knowledge of anaerobic MTBE-degrading communities is limited, as well. Therefore, phylogenic analysis of MTBE-utilizing enrichment cultures will provide the information for assessment of *in situ* bioremediation potential (Kane *et al*., 2001). Previous studies have shown that bacteria O-demethylated MTBE to TBA in both methanogenic and sulfidogenic enrichment cultures and that the methyl group can serve as a carbon source (Somsamak *et al*., 2001;

Somsamak *et al*., 2005). Terminal restriction fragment length polymorphism (T-RFLP) is a community gene fingerprinting technique, which can be applied to investigate complex microbial communities in diverse environments, based on variation in the 16S rRNA genes (Liu *et al*., 1997; Osborn *et al*., 2000; Kerkhof & Häggblom, 2008). In combination of clone library and 454 pyrotag sequencing analysis of 16S rRNA genes, TRFLP can elucidate the MTBE-degrading communities enriched in sulfidogenic cultures.

In this study, sulfidogenic cultures from estuarine sediments were enriched with MTBE as the sole carbon source for over one decade under strict anaerobic conditions. Characterization of the enrichment cultures through TRFLP analysis and pyrosequencing with identification the key microorganisms in the communities aimed to provide an initial understanding of microbes active in anaerobic MTBE biodegradation.

MATERIALS AND METHODS

Enrichment culture setup

Anaerobic enrichment cultures were originally established with 10% sediment (v/v) from the New York Harbor (NYH) and Arthur Kill (AK) inlet and maintained using strict anaerobic technique under sulfidogenic conditions for over one decade (Somsamak *et al*., 2001; Somsamak *et al*., 2005). The cultures were repeatedly transferred, at 6–12-month intervals and usually at a 1:10 dilution, into fresh medium and enriched with 50-150 μM MTBE (Aldrich, Milwaukee, WI) as the sole carbon source (Youngster *et al*., 2010a). Cultures were maintained in 50–150 mL glass serum vials capped with Teflon-coated

stoppers and aluminum crimps and incubated at 28 °C. The concentration of MTBE and its daughter product TBA were monitored regularly using gas chromatography with flame ionization detection as described previously (Somsamak *et al*., 2001). Selected enrichment cultures, representing up to 10^{-12} dilutions of the original sediment enrichments, were chosen for DNA extraction and microbial community analysis.

DNA extraction and purification with archaeal carrier

When 50% MTBE was depleted, a 1 ml liquid sample was amended with 100 ng *Halobacterium salinarum* DNA, and the community DNA then extracted using a modified phenol-chloroform extraction procedure (Kerkhof & Ward, 1993; Kerkhof *et al*., 2011). *H. salinarium* was grown in marine broth 2216 (Difco, Detroit, Michigan) at 25 °C for approximately 20 days. The cells of *H. salinarium* were harvested and extracted by the modified phenol-chloroform extraction procedure. The extracted DNA (approximately 300 ng including sample DNA and *H. salinarium* DNA) containing ethidium bromide was purified by Cesium Chloride density gradient centrifugation at 80,000 g for 16-24 hours. After centrifugation, the DNA band was visualized using UV light, directly collected by pipette tips from the gradient and dialyzed (in 10 mM Tris) for 45 minutes (Gallagher *et al*., 2005).

16S rRNA genes amplification and T-RFLP community analysis

The 16S rRNA genes of genomic DNA were amplified with 20 pmol universal bacterial specific primers: 50-end 6-FAM-labeled 27 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1100 reverse (5'-AGGGTTGCGCTCGTTG-

3') to avoid amplification of *H. salinarium* rRNA genes. PCR amplification parameters were as follows: 94 °C for 5 min, and then 94 °C for 30 s, 57 °C for 30 s for 31 cycles with a final extension at 72 °C for 10 min (Foster City, CA, USA). Approximately 15-20 ng of PCR products were digested with restriction enzyme MnlI at 37 °C for 6 h. Digested samples were precipitated and resuspended in formamide with a ROX standard and denatured at 94 °C (Gallagher *et al*., 2005). Samples were analyzed on an ABI 310 automated sequencer (Applied Biosystems Instruments, Foster City, CA), which generated a fingerprint of each community by terminal restriction fragment length polymorphism (TRFLP).

Cloning

One 10^{-11} dilution AK culture after the first feeding and one 10^{-9} dilution NYH culture were selected for clone analysis because T-RFLP results showed that these two cultures consisted of relatively few dominant T-RFs compared to the other enrichment cultures from the same sediment. The 16S rRNA genes from these two enrichment cultures were amplified with 27F and 1100R primers. The PCR products were cloned using the TOPO-TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Plasmids were transformed in DH5α™ -T1R chemically competent *Escherichia coli* (Invitrogen). Transformed cells were plated on Luria–Bertani (LB) agar plates containing ampicillin and X-gal. Colonies were grown overnight in LB media containing ampicillin and selected based on color. Plasmid DNA was individually screened by TRFLP analysis as described above to identify unique clones and extracted

using the Zyppy plasmid miniprep kit (Zymo research, Irvine, CA) according to the manufacturer's recommendations (Tuorto *et al*., 2013).

Sequencing and data analysis

The 16S rRNA genes of selected clones were Sanger sequenced by Genewiz Inc (North Brunswick, NJ). The clone library was used to identify the various terminal fragments (T-RFs) of the TRFLP profiles. The T-RFs that composed 5% or more relative abundance were operationally defined as "dominant" in the communities. The 16S rRNA gene sequences were compared with the Basic Local Alignment Search Tool (Blast) and Ribosome Database Project (RDP) Seqmatch tool for taxonomic identification. Phylogenic trees were constructed using the program Geneious 8.1. Clone sequences and those of the most closely related cultivated bacteria used to construct Maximum likelihood trees (Guindon & Gascuel, 2003; Babcock *et al*., 2007; Drummond *et al*., 2011)

Pyrosequencing

16S rRNA genes of the AK 10^{-11} dilution sulfidogenic culture, which had been respiked with MTBE for three times (Figure 3.2c), were amplified with 27F and 519R primers and sequenced at the Molecular Research LP using a Roche 454 Genome Sequencer. A total of 3,000 quality-filtered reads were clustered in OTUs with 77.2% to 99.8% similarity, enabling identification of OTUs down to the family level.

RESULTS

Anaerobic MTBE degradation in sulfidogenic enrichment cultures

The sulfidogenic Arthur Kill and New York Harbor enrichment cultures were transferred and fed with MTBE as the sole carbon source for over a decade (Somsamak *et al*., 2001; Youngster *et al*., 2008; Youngster *et al*., 2010a). Over the course of enrichment and sequential transfers, MTBE degradation with near stoichiometric accumulation of TBA was observed. In the most recent cultures, the MTBE degradation rate was faster in the AK than the NYH cultures, with depletion of approximately 100 μM MTBE within 10 days (Figure 3.1). In contrast, there was a 20 day lag period before degradation occurred in NYH cultures, and the degradation rate was slower, with complete depletion of approximately 100 μM MTBE within 40 days (Figure 3.1). In addition, the MTBE degradation rate of the cultures increased through repeated amendment with MTBE.

Analysis of AK sulfidogenic communities

MTBE as the sole carbon source, was generally spiked to the Arthur Kill sulfidogenic cultures for three times and followed by a transfer with 1:10 dilution into fresh media. After feeding with MTBE and approximately 50% MTBE depletion was observed, the cultures were sampled and DNA extracted for community structure analysis. Over the course of MTBE additions and degradation three community structure profiles of the AK sulfidogenic enrichment were analyzed (Figure 3.3). The TRFLP fingerprints of the AK sulfidogenic cultures over three consecutive transfers from the 10^{-10} dilution to the 10^{-12} dilution show that the number of T-RF peaks diminished over time, suggesting a reduction of community complexity. One exception was that more T-RF peaks appeared

after the third MTBE spike than with the first spike in the 10^{-11} dilution culture, suggesting that long term incubation with MTBE does not diminish the diversity of the community (Figure 3.2b and 3.2c). The 210 bp T-RF peak was the only dominant peak that persisted in the cultures over time, and its relative abundance accounted for 50% of the total T-RF area in the 10^{-12} dilution culture, suggesting that it might play a central role in MTBE biodegradation. The 95 bp T-RF peak represented 18% of the community profile in the 10^{-11} dilution culture after the first feeding and diminished to 5% in the 10^{-12} dilution culture after the first feeding (Figure 3.2b and 3.2d). Particularly notable is the increase in the relative abundance of the 121 bp, 222 bp and 235 bp T-RF peaks after the third feeding of the 10^{-11} dilution culture and which followed the significant decrease after transfer. These data suggest that these T-RFs might not be the main organisms involved in MTBE degradation, but are instead scavengers of metabolic products of MTBE degradation (Figure 3.2c).

The TRFLP profiles in Figure 3.3 demonstrate the dynamics of the bacterial communities of the AK cultures (10^{-12} dilution) during MTBE degradation. The triplicate cultures were transferred 1:10 from one 10^{-11} dilution culture (Figure 3.2c) and were shown to degrade approximately 130 μM MTBE within 35 days. The TRFLP fingerprint of the bacterial community at the 50% degradation time point had the fewest T-RF peaks compared to the 0% and 100% degradation profiles (Figure 3.3). There were 5.3±1.2 dominant T-RF peaks (>5% in the community) the initial time point (0% degradation) TRFLP profiles and 5.0±1.7 dominant T-RF peaks in the 100% degradation profiles, respectively. In contrast, 2.0±1.0 dominant T-RF peaks were observed in the 50% degradation profiles.

The 210 bp T-RF was the dominant peak in all the communities. Additional T-RF peaks, for instance, 95 bp, 131 bp, 177 bp and 284 bp T-RFs, appeared in the 100% degradation profile, suggesting that the species corresponding to these T-RFs might utilize the degradation products of MTBE.

Cloning and phylogenetic analysis

A 10^{-11} dilution AK culture after the first feeding and a 10^{-9} dilution NYH culture were selected for clone analysis. Each clone was screened by TRFLP and sequenced to identify the corresponding T-RF peaks in the profiles. All of the dominant T-RF peaks of the sulfidogenic enrichment cultures were identified by matching fingerprints of individual clones to the corresponding T-RFs in the community fingerprints, with the exception of the 236 bp T-RF peak that was not detected in the clone library. However, the 16S rRNA gene sequence corresponding to the 236 bp T-RF was identified from 454 pyrosequencing data. A phylogenic tree was constructed to illustrate the relationship of the identified clones and their closest cultured relatives using maximum likelihood methods (Figure 3.6). The dominant T-RFs were primarily assigned to *Synergistetes*, *Actinobacteria* and *Deltaproteobacteria,* representing deeply branching 16S rRNA gene phylotypes (Table 3.1).

The 16S rRNA genes of AK 10^{-11} enrichment culture were also amplified and sequenced using 454 pyrosequencing. A total of 41,452 quality-filtered reads enabled identification of OTUs at the family level (Figure 3.4). The dominant OTUs clustered within the *Micrococcaceae*, *Desulfobulbaceae*, *Acidimicrobiales*, *Peptococcaceae*,

Streptomycetaceae, and *Synergistaceae*, which agreed with the TRFLP and clone library identification. The relative abundance of most dominant OTUs was consistent between pyrosequencing and TRFLP coupled with clone library analysis, except that the relative abundance of *Micrococcaceae* species (235 bp T-RF clone) was 55% in pyrosequencing analysis but 24% in TRFLP analysis.

Comparison of sulfidogenic bacterial communities between AK and NYH enrichment cultures

The 10^{-11} dilution AK sulfidogenic culture and 10^{-9} dilution NYH sulfidogenic culture were compared to examine whether they had similar bacterial communities after long term enrichment with MTBE as the sole carbon source. The TRFLP profiles, with T-RF identification from clone libraries, showed that *Synergistetes*, *Firmicutes* and *Deltaproteobacteria* were shared between the two communities, revealing the similarity among the two enrichment cultures (Figure 3.5). The 210 bp T-RFs representing *Deltaproteobacteria* species was the main peak abundant in the two cultures. In addition, both AK and NYH sulfidogenic communities had *Synergistetes* species represented by 123 bp and 95 bp T-RF peaks, as well as *Nitrospirae* species represented by 240 bp T-RF peaks. This suggests that the compositions of the anaerobic MTBE-degrading communities were influenced by terminal electron accepting condition rather than the source of sediments.

DISCUSSION

Anaerobic MTBE degrading cultures established under various respiration conditions have been studied over the last decade (Mormile *et al*., 1994; Finneran & Lovley, 2001; Bradley *et al*., 2001; Bradley *et al*., 2002; Somsamak *et al*., 2005; Pruden *et al*., 2005; Wilson *et al*., 2005a; Somsamak *et al*., 2006; Busch-Harris *et al*., 2008; Wei & Finneran, 2009; Sun *et al*., 2012; Key *et al*., 2013). However, only a few of these primary cultures have been enriched on MTBE for longer time periods (Somsamak *et al*., 2005; Somsamak *et al*., 2006; Wei & Finneran, 2009). Hence, little is known about the responsible microorganisms, and to date no MTBE-degrading pure culture has been isolated under anaerobic conditions. Previous studies have found that MTBE degradation was mediated by bacteria in a corrinoid dependent process and the inhibition of methanogenesis or sulfidogenesis did not directly block MTBE utilization (Youngster et al. 2008). This suggests that acetogenic bacteria may likely mediate the degradation of MTBE by cleavage of the ether bond to generate TBA under both reductive conditions, and utilization of the methyl group to generate acetate and ATP (Youngster *et al*., 2008). Methanogens and sulfate reducers could oxidize acetate that accelerates the degradation process (Ragsdale & Pierce, 2008). Acetogenic bacteria with the potential of O-demethylation have been found in the *Firmicutes*, *Chloroflexi*, *Spirochaetes* and *Deltaproteobacteria*, and most known acetogenic bacteria are in the *Firmicutes* phylum (Drake *et al*., 2006; Pierce *et al*., 2008; Hug *et al*., 2013). A *Ruminococcaceae* species has been identified as MTBE degraders in AK methanogenic enrichment cultures (Chapter 2). Therefore, although none of the acetogenic bacterial isolates have been shown to utilize MTBE, the *Firmicutes* members observed in the MTBE-degrading

enrichment cultures were of particular interest in this study. The abundant phylotypes assigned to a variety of phyla suggests a considerable microbial diversity within individual cultures might be necessary for the overall carbon flow.

The most abundant phylotypes in the AK and NYH sulfate reducing enrichment cultures were *Deltaproteobacteria*, which contains several sulfate reducing and fermentative species. Although a few *Deltaproteobacteria* species are known to be acetogenic bacteria (Schink *et al*., 2002; Pierce *et al*., 2008), these acetogens are phylogenetically distant from the *Deltaproteobacteria* species identified in our cultures. Putative sulfate reducing bacteria clustered within the *Deltaproteobacteria* were likely playing a role in facilitating MTBE degradation by utilizing acetate, the metabolic product of acetogens, and providing electrons for the reductive acetyl-CoA pathway. The closet described species of the 210 bp T-RF clone is *Desulfobulbus propionicus*. This OTU was unlikely able to directly utilize MTBE, because similar phylotypes were not able to use ^{13}C MTBE in the AK methanogenic cultures (Chapter 2). In addition, a variety of substrates could be fermented or used as the electron donors by *Desulfobulbus propionicus* under sulfate reducing conditions (Widdel & Pfennig, 1981; Tasaki *et al*., 1993; Pagani *et al*., 2011), which implies that the bacterium corresponding to the 210 bp T-RF phylotype might be able to use MTBE degradation products. Furthermore, the microbial diversity of the community became more complex in the end point of degradation suggesting that the MTBE carbon had been incorporated through cross-feeding by the other members of the community (Figure 3.4). Possible syntrophic bacteria represented by the 284 bp T-RF OTUs were able to anaerobically respire electron donors such as propionate and fumarate

with sulfate or syntrophically use the acetate coupled with methanogens (Harmsen *et al*., 1998; de Bok *et al*., 2002; McInerney *et al*., 2008; Plugge *et al*., 2012).

Little is known about the physiology and metabolic functions of the *Synergistetes*, *Acinobacteria*, and *Nitrospirae* species growing in the MTBE utilizing sulfate reducing enrichment cultures. An *Actinobacteria* was the most abundant phylotype in one MTBE sulfidogenic enrichment culture originally from petroleum-contaminated sediment (Wei & Finneran, 2009). Two different phylotypes of *Synergistetes* were identified in the AK and NYH communities, respectively (Figure 3.6). The 240 bp T-RF clone is distantly related to *Candidatus Magnetobacterium* belonging to *Nitrospirae* that contains some sulfate reducers (Jogler *et al*., 2010). These three phyla were widely distributed in the environment that might be metabolically diverse groups in the degradation process.

As a consequence of MTBE degradation, TBA accumulated in our enrichment cultures and was not further degraded, which is not a desired end-point. Interestingly, TBA was shown to decrease or to not accumulate in the some other sulfidogenic MTBE-degrading cultures, suggesting that TBA might be degraded also under anaerobic conditions, (Bradley *et al*., 2002; Raynal *et al*., 2010; Wei & Finneran, 2011). However, no anaerobic enrichment cultures have been sustained with TBA as the sole carbon source.

MTBE is a synthetic chemical, which has not previously existed in the environment and, hence, its biodegradation is a challenge to microorganisms. The identified phylotypes in the cultures anaerobically grown on MTBE as the sole carbon source were members of

diverse phyla and not closely related to any cultivated species, suggesting that these novel microorganisms might be pre-adapted to an MTBE contaminated environment. However, the relative complex community suggests anaerobic degradation were conducted by multiple species in symbiotic relationships that can improve the energetic efficiency of microbial degradation reactions. The decayed biomass or the degradation product such as acetate might support the growth of the whole the community. In conclusion, the phylogenic data provided in this study is important for gaining and understanding of the microbial communities that are responsible for anaerobic MTBE degradation under sulfidogenic conditions, and therefore can potentially be used as molecular probes for assessment of *in situ* bioremediation.

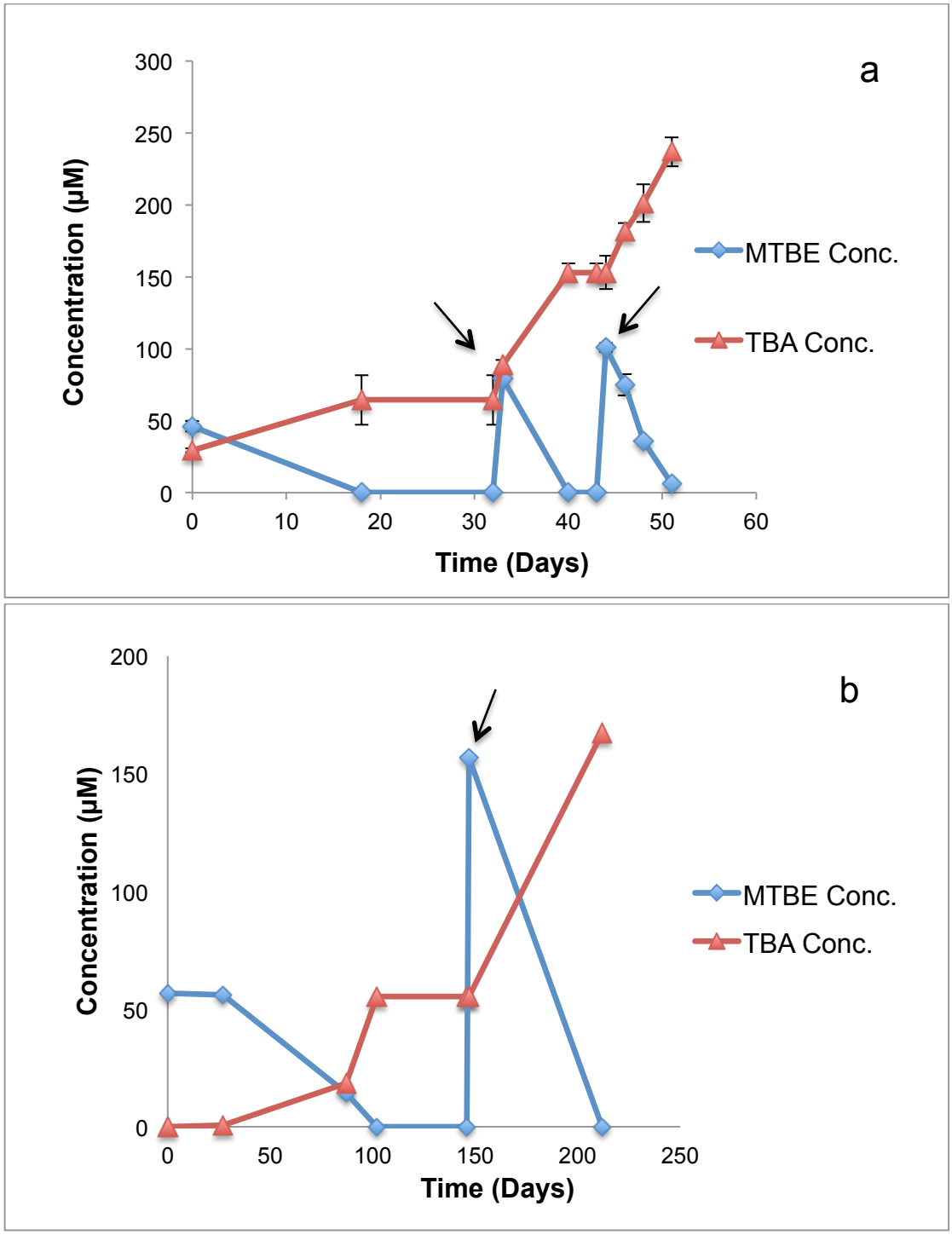


Figure 3.1. Anaerobic transformation of MTBE (solid diamonds) to TBA (solid triangles) in a) Arthur Kill 10^{-11} dilution and b) New York Harbor 10^{-9} dilution enrichment cultures under sulfidogenic conditions. Arrows indicate respiking of MTBE. Error bars represent the mean and standard deviation of triplicate cultures.

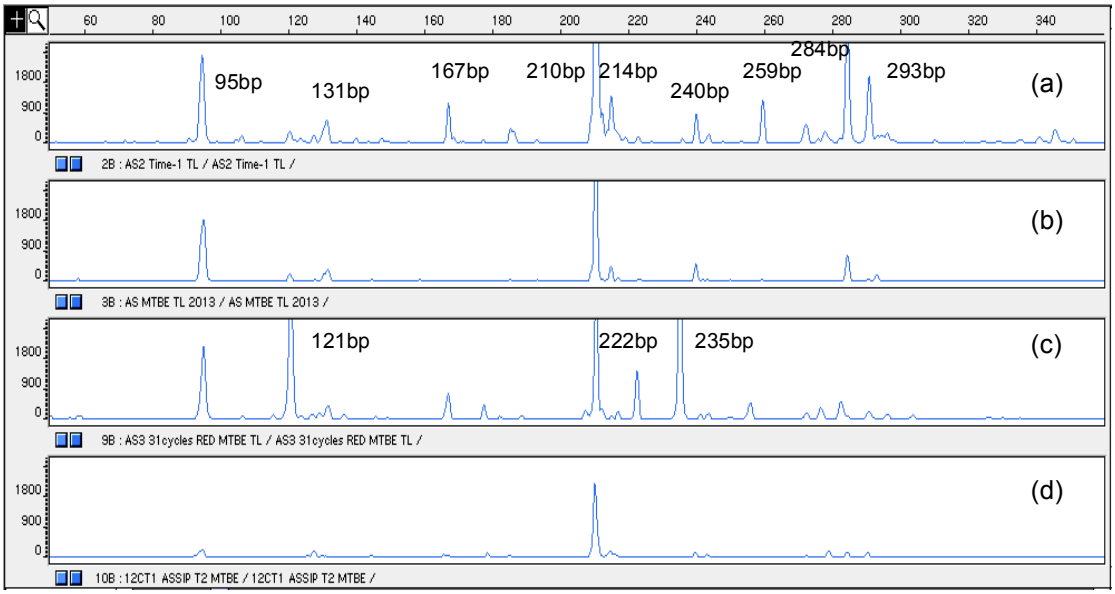


FIGURE 3.2. T-RFLP fingerprints of MTBE-degrading sulfidogenic communities from Arthur Kill sediment after sequential transfers and dilutions: (a) After 3rd MTBE feeding of 10^{-10}, (b) After 1st feeding of 10^{-11}, (c) after 3rd feeding of 10^{-11} and (d) after 1st feeding of 10^{-12} culture. Enrichments were fed three times of 50-100 μM MTBE before each transfer.

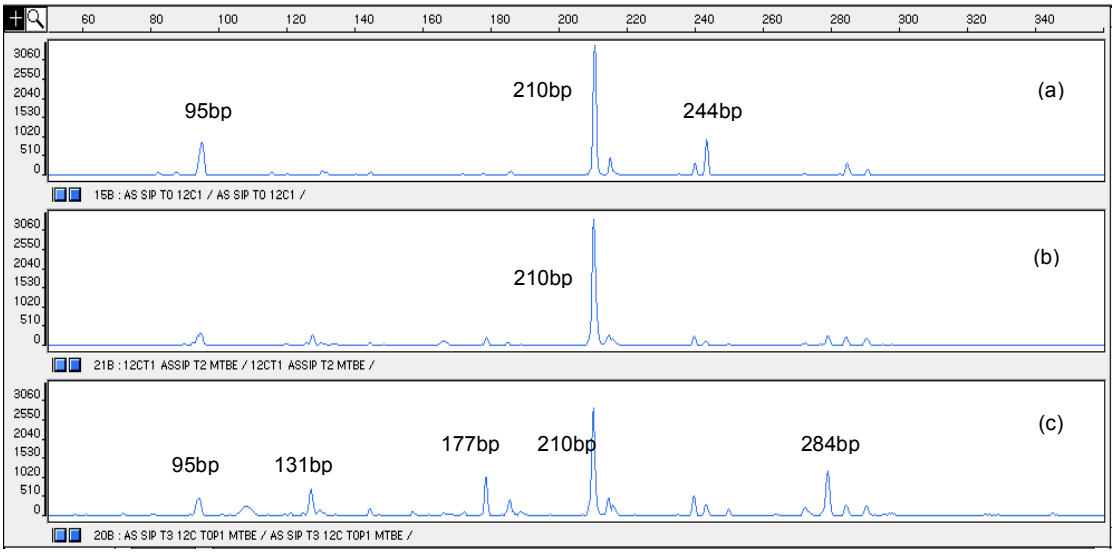


FIGURE 3.3. TRFLP fingerprints of the bacterial communities at different degradation time points: (a) 0%, (b) 50%, and (c) 100% of MTBE loss in a sulfidogenic Arthur Kill sediment enrichment culture diluted and transferred to 10^{-12} of the original culture.

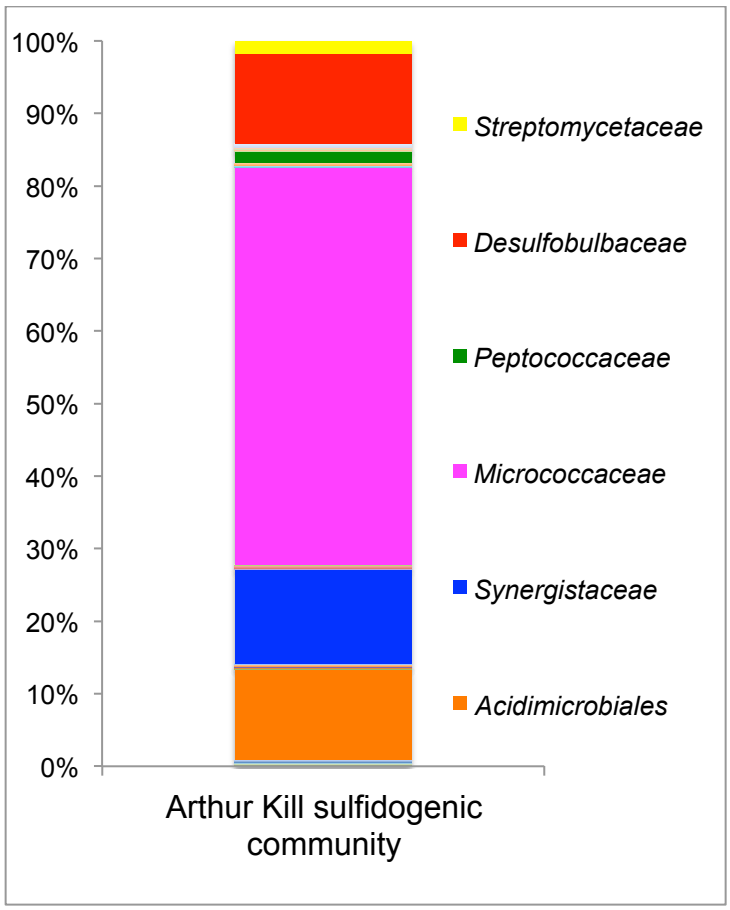


FIGURE 3.4. Relative abundance of different phylotypes from 3rd fed 10^{-11} dilutions AK sulfidogenic culture based on operational taxonomic units (OTUs) obtained by pyrosequencing of amplified 16S rRNA genes. Each bar represents the mean of duplicates PCR samples.

Table 3.1 Dominant clones in MTBE-degrading enrichment cultures compared with known (isolated and cultured) microorganisms and environmental clone sequences.

	Clone ID	Closest cultured Microorganisms	Similarity (%)	Closest Uncultured Microorganism	Similarity (%)	Phylum/Class
AK	95bp	*Thermanaerovibrio velox*	89%	Uncultured bacterium clone Er-MS-7	99%	Synergistetes
sulfide-genic	121bp	*Gaiella occulta*	86%	Uncultured bacterium clone LD_RB_37	98%	Actinobacteria
	210bp	*Desulfobulbus propionicus*	84%	Uncultured bacterium clone De133	90%	Deltaproteobacteria
	236bp	*Micrococcus luteus* *	99%	NA		Actinobacteria
NYH	123bp	*Thermovirga lienii*	91%	Uncultured bacterium clone BA128	99%	Synergistetes
sulfide-genic	210bp	*Desulfobulbus propionicus*	90%	Uncultured bacterium clone De133	96%	Deltaproteobacteria
	240bp	*Candidatus Magnetobacterium bavaricum*	88%	Uncultured bacterium clone HK_RBC7	98%	Nitrospirae
	284bp	*Syntrophobacter fumaroxidans*	92%	Uncultured bacterium clone Er-LAYS-31	97%	Deltaproteobacteria

* Identified from 16S rRNA genes pyrosequencing of Arthur Kill sulfidogenic culture

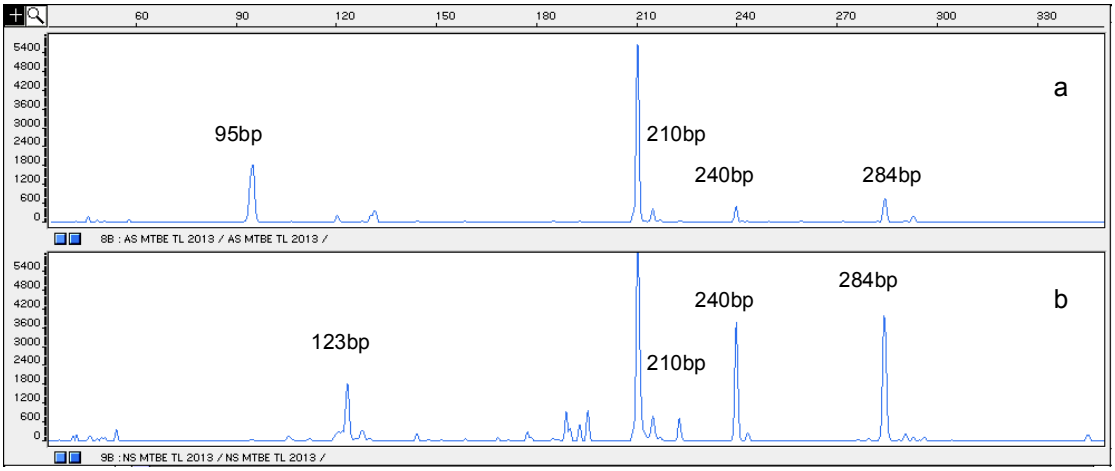


Figure 3.5. T-RFLP analysis of MTBE degrading enrichment cultures established from (a) Arthur Kill 1st fed 10^{-11} dilution sulfidogenic culture and (b) New York Harbor 10^{-9} dilution sulfidogenic culture.

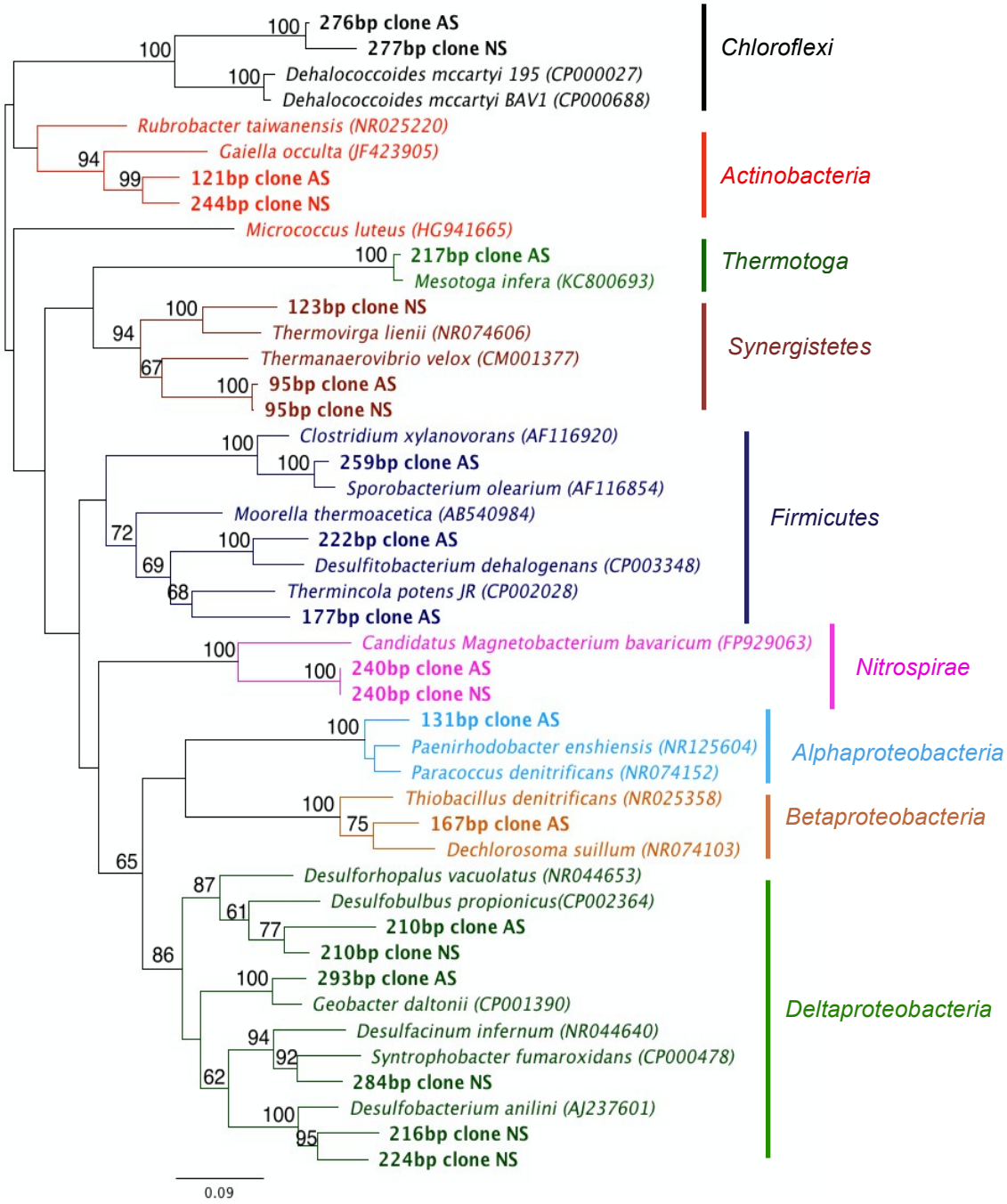


Figure 3.6. Maximum likelihood tree based on partial bacterial 16S rRNA gene sequences describing the relationship of bacteria that cloned from Arthur Kill sulfidogenic culture (AS) and New York Harbor sulfidogenic culture (NS). 1073 bp unambiguously aligned nucleotide positions were used for analysis. Numbers at nodes indicate bootstrap values from 100 replications. The scale represents 2% sequence divergence. Accession numbers for reference sequences are indicated in parenthesis.

Chapter 4

Investigation of MTBE-degrading communities by Single Cells Using Stable Isotope Probing and Raman Spectroscopy

ABSTRACT

In United States, the wide use of methyl *tert*-butyl ether (MTBE) makes it a significant contaminant in groundwater. The unpleasant taste, odor and the toxicity of MTBE in groundwater adversely affects drinking water quality. Currently, anaerobic bioremediation holds the greatest hope for cleaning up the contaminated aquifers. Previous work has characterized MTBE-degrading communities under different reductive conditions. However, knowledge of the responsible microorganisms that mediate the degradation process is still limited. Recently, Raman microspectroscopy in combination with stable isotope probing (SIP) has been applied for determination of the functional species at the single cell level in microbial communities. In this study, single cells from enrichment cultures amended with ^{12}C or ^{13}C MTBE were examined by Raman microspectroscopy. The Single Cell Raman Spectral (SCRR) analysis showed an intense cytochrome-like spectrum in some cells, indicating a strong electron transport capability. Meanwhile, the "redshift" phenomenon, from the change in the resonance spectra due to the incorporation of the stable isotope substrates into the macromolecules, was not observed in the ^{13}C MTBE fed enrichment cultures, suggesting that the MTBE degraders are not abundant in the communities.

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is one of the fuel oxygenates that was widely used in the United States to reduce hazardous emissions from motor vehicles and to meet the requirements of the Clean Air Action Act of 1990 (Deeb *et al*., 2000). Subsequently, MTBE became a significant contaminant in groundwater as it frequently leaked from underground storage tanks and pipelines (USEPA, 2008). Groundwater is an important source of drinking water. Drinking water contaminated by MTBE has a very unpleasant taste and odor, and therefore relatively small amounts of MTBE contamination can make drinking water non-potable (Deeb *et al*., 2000; Moran *et al*., 2002; Deeb *et al*., 2003).

Biodegradation can naturally remove MTBE from groundwater (Häggblom *et al*., 2007). MTBE contaminated groundwater is often depleted of oxygen due to biodegradation of gasoline hydrocarbon co-contaminants. Since oxygenation of groundwater is costly, anaerobic biodegradation may offer a feasible and economic way to clean up MTBE contamination *in situ*. Identification of the microorganisms that are responsible for MTBE degradation will allow us to evaluate the biodegradation potential of the contaminated groundwater aquifer. However, only a few anaerobic MTBE-degrading communities have been characterized (Busch-Harris *et al*., 2008; Wei & Finneran, 2009; Raynal *et al*., 2010; Youngster *et al*., 2010a; Sun *et al*., 2012; Key *et al*., 2013), but specific knowledge of the microorganisms that are responsible for anaerobic MTBE degradation is still limited.

Linking the ecological functions to the particular microbes in the environment is always a challenge for environmental microbiological studies. Traditional microbial cultivation and physiology studies are not suitable for most environmental samples since many microorganisms are difficult to grow and isolate (Amann *et al*., 1995). Therefore, molecular techniques based upon DNA extraction and PCR of 16S rRNA genes were developed to overcome the obstacles of the cultivation (Head *et al*., 1998). Currently, stable isotope probing (SIP) coupled with 16S rRNA gene sequence analysis has been used to identify the MTBE degraders that established a direct linkage between uncultured cells and its specific metabolic functions (Sun *et al*., 2012; Key *et al*., 2013). However, the identified species were only distantly related to cultured bacteria, which prevented the further comparison of functional genes and enzymes.

Single-cell Resonance Raman spectroscopy (SCRR, with a resolution down to 1 μm) is a non-invasive and non-fluorescence label technique allowing *in vivo* and multiple parameter analysis of individual living cells in microbial communities (Li *et al*., 2012). Raman spectroscopy rapidly detects the chemical bonding patterns associated with biological molecules through the inelastic scattering of incident laser light that provides an intrinsic fingerprint of the single cell (Huang *et al*., 2010). Single cell Raman spectra (SCRS) provides rich biological information of cells without special pretreatment or any external labeling (Huang *et al*., 2010). For instance, a variety of cell contents such as nucleic acids, proteins, polysaccharides, carbohydrate, and lipids, can be identified from Raman spectra in the range of 500-2000 cm^{-1} (Table 4.1). Such spectral information offers a signature of the molecular structures, cellular compositions, and physiological

states. In addition, metabolism of stable isotope labeled substrates can greatly shift some signature Raman bands in the spectrum. It offers an inherent and quantitative approach to identify active cells in the microbial communities for functional study (Figure 4.1) (Huang *et al*., 2004; Li *et al*., 2012).

In addition, if ^{13}C stable isotope substrates are incorporated into the macromolecules, the resonance spectra will significantly change and produce a "red shift" (Huang *et al*., 2009a) in comparison to the spectra with the incorporation of ^{12}C based substrates (Figure 4.1). Following mapping, activated cells can be distinguished from the inert cells and separated based on the ^{13}C-incorporation at the single cell level (Huang *et al*., 2004; Huang *et al*., 2010). The separated cells have the potential for further cultivation and genome amplification (Huang *et al*., 2009b). Therefore, SCRR microspectroscopy in combination with SIP may provide a direct method to screen and identify the cells actively incorporating MTBE in a microbial community, providing a means for mapping MTBE degraders and their ecological function in the natural environment.

MATERIALS AND METHODS

Sample collection

Anaerobic sediment enrichment cultures from the Arthur Kill Inlet (AK) between New York and New Jersey were previously established (Somsamak *et al*., 2001; Somsamak *et al*., 2005). The cultures were enriched under methanogenic or sulfidogenic conditions with MTBE (Aldrich, Milwakee, WI) as the sole carbon source using strict anaerobic technique as described in detail previously (Somsamak *et al*., 2001; Somsamak *et al*.,

2005). For the experiment 10^{-11} dilution methanogenic or sulfidogenic cultures were transferred into fresh media twice (1:10 dilution) without resazurin (as redox indicator, which would cause interference) and spiked with either 100 μM ^{13}C O-methyl carbon labeled MTBE (provided by Dr. H. Richnow, UFZ, Leipzig) or 100 μM ^{12}C MTBE as controls. MTBE concentration was regularly monitored using gas chromatography with flame ionization detection as previously described (Somsamak *et al*., 2001). The enrichment cultures that were selected for Raman micro-spectroscopy represented a dilution of 10^{-13} of the original sediment cultures. The culture samples were stored at -20 °C after approximately 50% MTBE depletion prior or analysis.

Detection of single cells in MTBE-degrading cultures by Raman micro-spectroscopy

Raman micro-spectroscopy was employed to examine ^{13}C- MTBE incorporation at the single cell level (Huang *et al*., 2004; Huang *et al*., 2009a). To remove the FeS precipitate in the medium 10 ml of cell culture was sonicated for 30s, followed by centrifugation 3 times at 2000 g for 3 min. The cells in liquid phase were then transferred to clean centrifuge tubes and washed with deionized water before analysis by Raman microscopy as described previously (Huang 2004). Briefly, each cellular suspension (1 μl) was spread on a calcium fluoride (CaF_2) slide and allowed to air dry before Raman analysis. The SCRR spectra were acquired using a confocal Raman microscope (LabRAM HR, HORIBA Scientific, London, UK) equipped with an integrated Olympus microscope (Model BX41). A 100× magnifying dry objective (NA. 0.90, Olympus, Essex, UK) was used to observe and acquire Raman signals from single cells. The laser beam was targeted on the cell using a charge-coupled device camera monitor and a motorized XY stage (0.1-μm step). The Raman scattering was excited with a 532-nm Nd:YAG laser

(Torus Laser, Laser Quantum, Manchester, UK). The laser power on a single cell was 3.5 mW. Each Raman spectrum was acquired between the range of 557 and 2172 cm^{-1}, with 1021 data points and a resolution of 1.5 cm^{-1}. LabSpec software (HORIBA Scientific) was used to operate the Raman system and acquire Raman spectra. Acquisition time was 5-30 s for measurement of each single cell.

RESULTS AND DISCUSSION

The samples prepared for Raman microspectroscopy were the AK methanogenic and sulfidogenic enrichment cultures, fed by either ^{12}C or ^{13}C MTBE as the sole carbon source. In each 1μl sample, an average of 100 putative cells were examined by Raman microspectroscopy and 10-20% of them were identified as real cells because they contained the signature bands of Single Cell Raman Spectra (SCRS). The rest of the putative cells were unknown particles from media or cell debris destroyed by the pretreatment. Figure 4.2 is a typical SCRS of the MTBE-degrading community. The phenylalanine band (1002 cm^{-1}) was used as the signature band to identify the cells (Huang *et al*., 2007; Huang *et al*., 2009b). In total, around 1200 cells were examined (200 cells from ^{12}C MTBE cultures, 1000 cells from ^{13}C MTBE cultures). None of the SCRS exhibited the "red shift" phenomenon expected in ^{13}C MTBE amendment samples, indicating that the MTBE degraders were not abundant in the communities. Our previous study has shown that *Ruminococcaceae* species were responsible for MTBE degradation and that the relative abundance of *Ruminococcaceae* species was approximately 2-5% of the total community based on DNA-SIP results (Chapter 2). Following this calculation, it is likely that one isotopically labeled cell should be identified from five hundred cells.

However, no ^{13}C MTBE incorporated cells were discovered by SCRS, of more than 1000 analyzed, which might have resulted from the loss during FeS precipitate removal or due to the lower relative abundance caused by transfer and dilution. Furthermore, Raman microspectroscopy requires at least 50% incorporation of isotope labeled substrates for distinctive spectra (Li *et al*., 2013). Although the methyl group of MTBE was assumed to be the only carbon source for MTBE degraders, the cells might not meet this criterion (Figure 4.1).

Besides regular cell contents, some specific molecules synthesized by a cell could generate specific Raman signals which allows the identification of these molecules (Huang *et al*., 2010). In our observation, a few SCRS profiles contained bands of 747 cm^{-1}, 1128 cm^{-1}, 1316 cm^{-1} and 1582 cm^{-1}, which are different from the spectrum of *Escherichia coli* (Figure 4.3a). These signature bands are associated with cytochrome-like components (Johannessen *et al*., 2007; Pätzold *et al*., 2008; Okada *et al*., 2012; Ichimura *et al*., 2014). Cytochromes are rich in the microorganisms which use anaerobic respiration to obtain energy. They are membrane-bond proteins that primarily generate ATP through electron transport. For instance, acetogens are putative MTBE degraders through their capability of O-demethylation (Chapter 2). In certain acetogens, cytochromes are one of the carriers in the membranous electron transport system that can generate proton gradients and synthesize ATP (Drake *et al*., 2006). Interestingly, bacteria possessing cytochrome a P450-type monooxygenase are responsible for aerobic degradation of MTBE and the other oxygenates (Deeb *et al*., 2001; Ferreira *et al*., 2006). However, it is unclear whether this type of enzyme can catalyze anaerobic

biodegradation.

Raman microspectroscopy can rapidly measure intrinsic molecular information of cells *in vivo* without cultivation and DNA extraction. This information provides the cellular phenotype and chemical fingerprints of the cell that could be used for identification purpose. In addition, Raman microspectroscopy armed with optical tweezers can further sort the cells of interest for single cell genomic analysis, which can provide the opportunity for studying the functional species in their natural microbial communities without external labeling and prior cultivation (Huang *et al*., 2009a; Huang *et al*., 2010; Li *et al*., 2013).

TABLE 4.1. Assignment of some bands frequently in Raman spectra respective of biological query and putative assignments shifted by ^{13}C substitution (Modified from Huang *et al*., 2004 and Huang *et al*., 2010).

Frequency at 100% ^{12}C (cm^{-1})	Frequency shift at 100% ^{13}C (cm^{-1})	Assignment
720	15.20	Adenine
781	15.03	Cytosine, Uracil (ring, str)
897	36.65	C-O-C str
1002		Phenylalanine
1032	16.05	Phenylalanine; C–N str
1098		Phosphate C-C skeletal and C-O-C str
1128	27.40	Carbohydrate (C-N and C-C stretching)
~1130		=C–C=(unsaturated fatty acids in lipids)
1170		Tyrosine phenylalanine
1246		Thymine, cytosine, tyrosine, tryptophan
1254	46.81	Protein Adenine, amide III
1339		Adenine, Guanine, Tyrosine, Tryptophan
1431–1481		Protein marker band 1451
1482–1487	26.13	Nucleic acids
1575–1578	44.85	Guanine, Adenine (ring str)
1582, 1593		Protein
1609		Phenylalanine
1663	46.94	Protein (Amide I)
2942		C-H
3064		C=C-H aromatic str

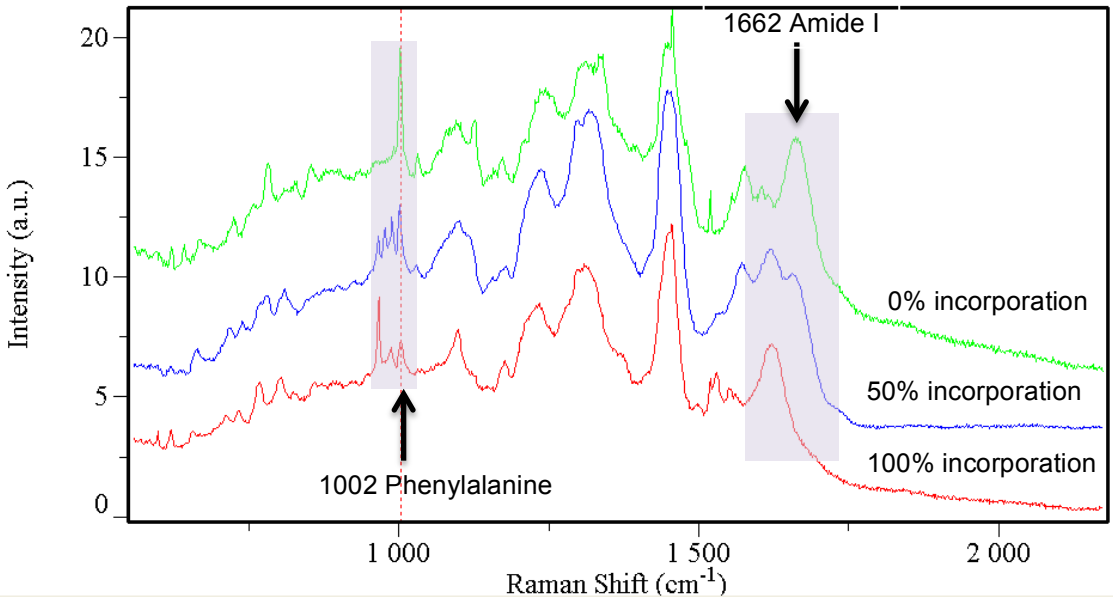


FIGURE 4.1. Examples of Single Cell Raman Spectra of ^{13}C-labeled *Escherichia coli* fed by glucose. Shaded areas are the red shifted phenylalanine and Amide bands (modified from Li *et al*., 2013).

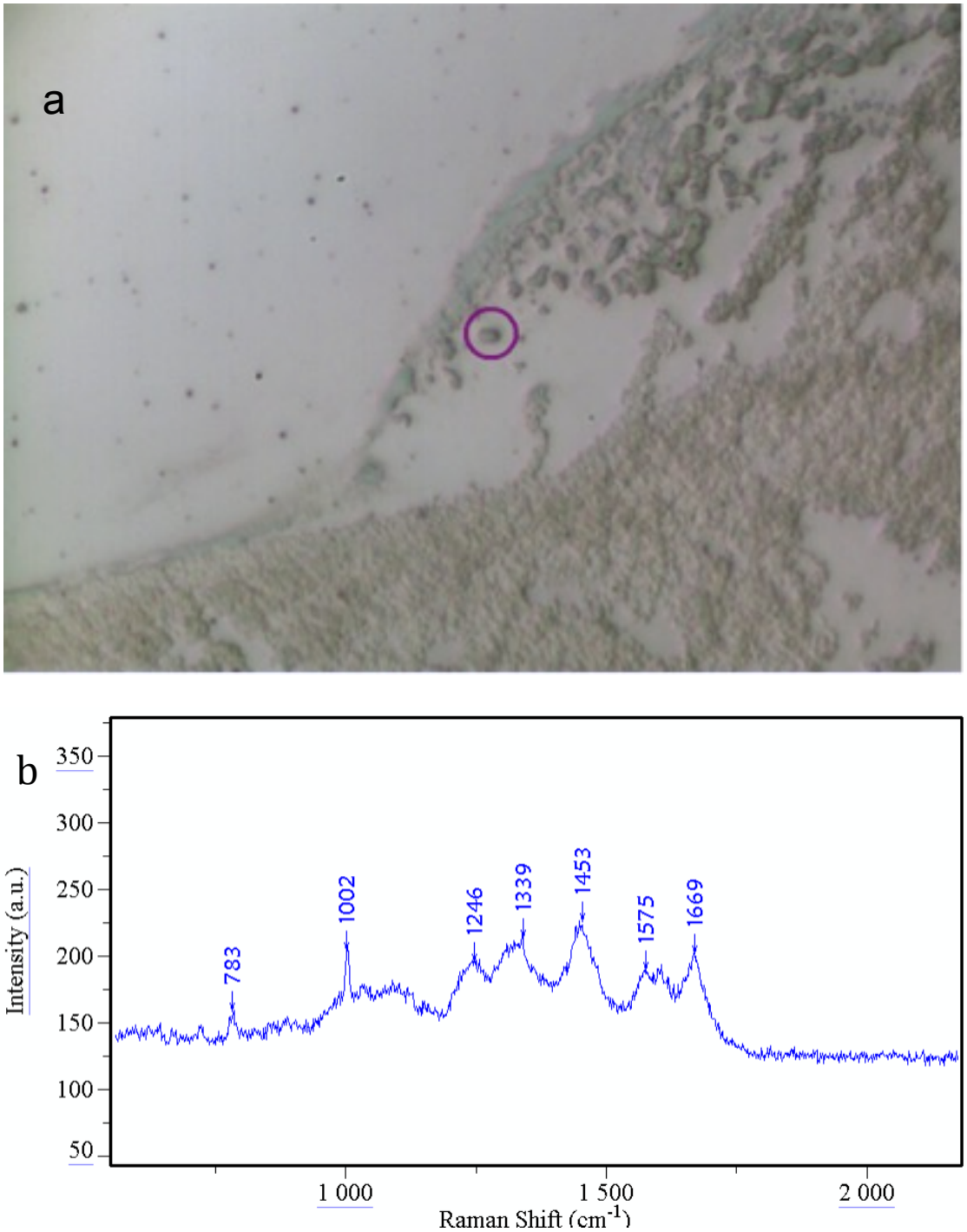


FIGURE 4.2. A typical cell in the ^{13}C MTBE-degrading community (a) and its Raman spectrum using a 532 nm laser (b). The laser on the single cell was 3.5 mW and acquisition time was 30s. Most Raman signals are at range of 600-2000 cm^{-1}.

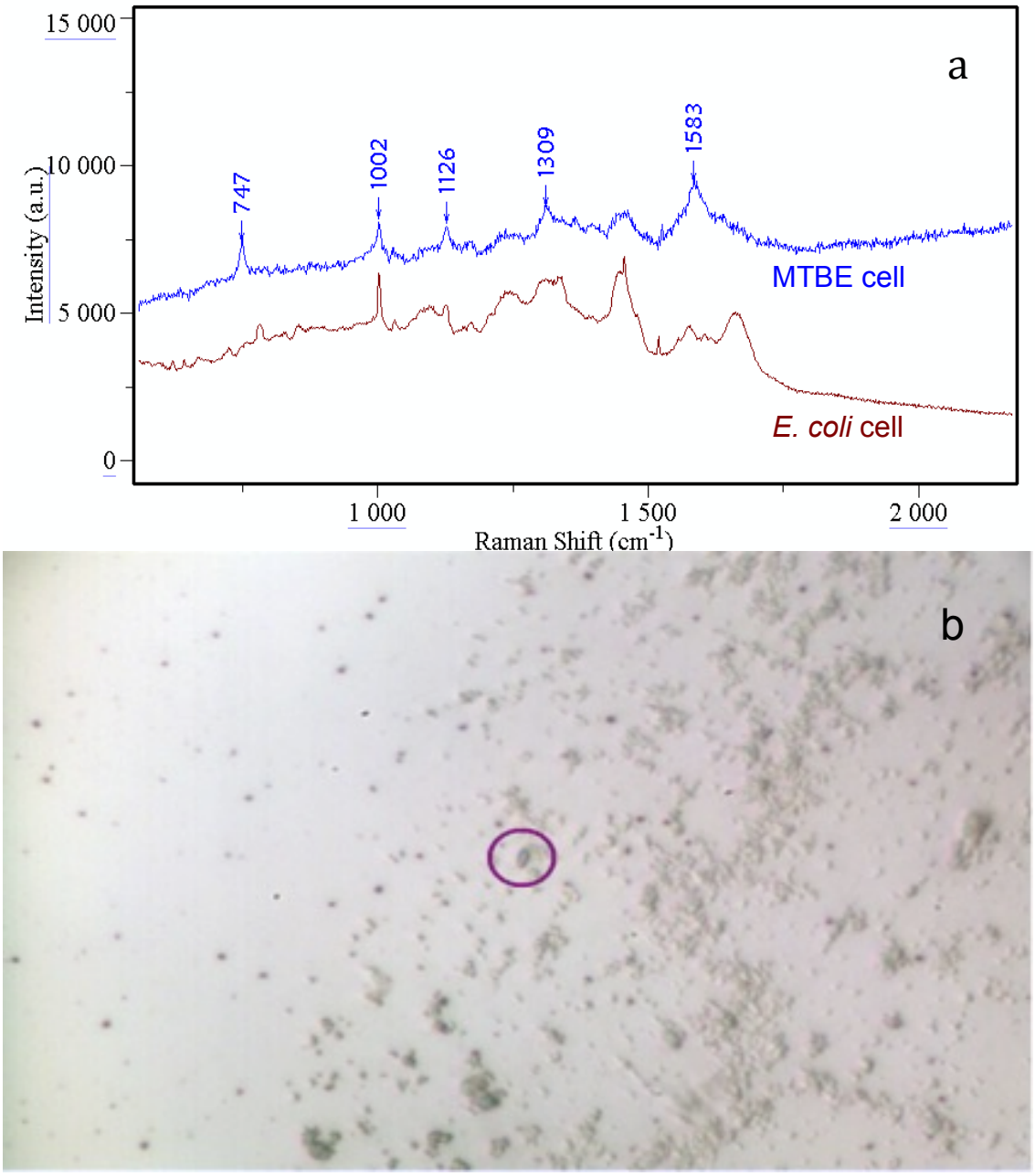


FIGURE 4.3. Comparison of the Raman spectra of a cell from the MTBE degrading community that contained signature cytochrome like bands with an *Escherichia coli* cell (a) and the morphology of the cell that contained cytochrome like components (b).

Chapter 5

Discussion

Methyl *tert*-butyl ether (MTBE) contamination is a persistent problem in groundwater that threatens drinking water quality. Bioremediation is the preferred solution of removal MTBE contamination from groundwater aquifer because it is more efficient and less costly than the other remediation techniques. MTBE contaminated field sites are often low in oxygen. Therefore, *in situ* anaerobic biodegradation is a particularly interesting application of bioremediation (Häggblom *et al*., 2007). To accurately assess the *in situ* biodegradation potential of contaminated sites, it is important to know the microorganisms responsible for anaerobic MTBE degradation. The microbial community structure and their metabolic functions can also provide information with respect to evaluation of degradation potential (Bombach *et al*., 2010). MTBE is a recalcitrant compound and resistant to microbial attack. As a consequence, the responsible microorganisms are not usually abundant in the communities, which increases the difficulty of characterization. To date, there are only a few anaerobic MTBE-degrading communities that have been characterized to greater detail since Mormlle *et al*. (1994) published the first study demonstrating anaerobic degradation of MTBE (Mormile *et al*., 1994; Wei & Finneran, 2009; Raynal *et al*., 2010; Sun *et al*., 2012; Key *et al*., 2013) and no responsible microorganisms have been isolated.

We have successfully established and maintained anaerobic enrichment cultures from sediments for more than one decade, which have been continuously amended with MTBE as the sole carbon source (Somsamak *et al*., 2001; Somsamak *et al*., 2005; Somsamak *et al*., 2006; Youngster *et al*., 2008; Youngster *et al*., 2010a; Youngster *et al*., 2010b; Chapters 1 and 2). These previous studies demonstrated that acetogenic bacteria are very

likely to mediate anaerobic biodegradation of MTBE rather than sulfidogenic or methanogenic organisms in the community (Youngster *et al*., 2008). These bacteria utilize the methyl group of MTBE (O-demethylation) with *tert*-butyl alcohol (TBA) accumulating as an end-product. Many acetogenic demethylation reactions are corrinoid dependent and O-demethylation of MTBE to TBA is also a corrinoid-dependent process that reinforced the possibility of acetogenic bacteria unitization of methyl groups (Youngster *et al*., 2008). The initial characterization of MTBE-degrading Arthur Kill methanogenic enrichment culture showed that *Firmicutes*, *Chloroflexi* and *Deltaproteobacteria* were the three dominant phylotypes of the microbial communities (Youngster *et al*., 2010a).

We identified the bacteria responsible for MTBE degradation by amending the enrichment cultures with ^{13}C labeled MTBE for DNA stable isotope probing (SIP) analysis. Terminal restriction fragments length polymorphism (TRFLP) coupled with clone library analysis of the ^{13}C labeled 16S rRNA genes were used to identify the bacteria responsible for anaerobic degradation of MTBE in the highly enriched Arthur Kill methanogenic community. Nearly full-length 16S rRNA genes (1100 bp) were sequenced in this study which increased the phylogenetic resolution for identification (Chapter 2). A 207 bp terminal restriction fragment (T-RF) was the dominant peak in the SIP profiles, indicating that the corresponding bacterial species was able to cleave the ether bond of MTBE and incorporate the labeled methyl group into its DNA (Figure 2.2). Phylogenic analysis of the 207 bp T-RF gene sequence assigned it to the *Ruminococcaceae* family, of the order *Clostridiales* in the *Firmicutes*. The most closely

described spices of the 207 bp T-RF clone (94% similarity) is *Saccharofermentans acetigenes*, a spore-forming anaerobic bacterium isolated from sludge of brewery wastewater treatment reactors (Chen *et al*., 2010). *S. acetigenes* is a newly isolated species that can ferment several hexoses, polysaccharides and alcohols. Glucose was fermented to acetate, lactate and fumarate, but *S. acetigenes* does not reduce sulfate. Of interest was that the 207 bp T-RF phylotype clustered with species identified from the other MTBE-degrading communities (Figure 2.3). This strong evidence indicates the acetogenic bacteria within the *Ruminococcaceae* family are responsible for anaerobic O-demethylation of MTBE.

We further investigated the carbon flow within a methanogenic MTBE utilizing community (Figure 5.2). Acetogenic bacteria are capable of utilizing methyl groups to synthesize acetate with formation of ATP via the reductive acetyl-coenzymeA pathway (Drake *et al*., 2006). Therefore, ^{13}C acetic acid was added to the MTBE degrading cultures to determine the phylotypes that can utilize this O-demethylation product. After one-month incubation, a 282 bp T-RF was the only peak in ^{13}C acetic acid SIP profiles, which corresponded to *Syntrophobacter fumaroxidans* (92% similarity) (Figure 2.6). *S. fumaroxidans* is known to grow syntrophically in co-culture with hydrogen- and formate-utilizing methanogens (Harmsen *et al*., 1998). Methanogens can convert acetate to methane and carbon dioxide that in turn facilitates the O-demethylation process (Ragsdale & Pierce, 2008). The other phylotypes in the community were classified as *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Actinobateria* and *Synergistetes* (Figures 2.4 and 2.5). They might survive through fermentation of decayed

acetogen and methanogen biomass. Follow-up SIP experiments will involve amendment of ^{13}C-labelled biomass (Isogro) to the enrichment cultures. This should allow us to identify the members of the enrichment community that utilize the biomass generated by the initial utilization of the methyl group of MTBE or from the acetate produced by acetogens.

Another two sets of anaerobic MTBE enrichment cultures originally established from Arthur Kill (AK) and New York Harbor (NYH) sediments were maintained under sulfate reducing conditions. TRFLP in combination with clone library analysis were used to characterize the microbial community structures of these two enrichment cultures (Chapter 3). In the AK sulfidogenic cultures, the complexity of the microbial community decreased during enrichment (Figure 3.2). The relative abundance of a 210 bp T-RF peak increased from 25% to 50% during transfers (from 10^{-9} to 10^{-11} dilution of the original culture), indicating that it might play an important role in the community. The 210 bp T-RF clone had 84% similarity to *Desulfobulbus propionicus*, a sulfate reducing *Desulfobulbaceae* species within the *Deltaproteobacteria* (Figure 3.3). *D. propionicus* and several other filamentous species within *Desulfobulbaceae* family are able to exchange the electrons by extracellular nanowires, which allow them compete with the other sulfate reducers in the culture (Pfeffer *et al*., 2012). Previous study suggests that MTBE degradation is not directly coupled to sulfidogenesis (Youngster *et al*., 2008), indicating that the species corresponding to the 210 bp T-RF clone might not mediate the degradation of MTBE, but possibly utilize the acetate generated. The 207 bp T-RF was also not found in AK sulfidogenic communities, suggesting that other acetogenic bacteria

with a similar mechanism might be responsible for the degradation (Chapter 2). During MTBE depletion, the dynamics of the AK sulfidogenic microbial diversity implies that other species in the community might be supported by fermentation of sulfidogenesis products (Figures 3.2 and 3.3). The phylotypes belonging to the *Synergistetes*, *Actinobacteria*, *Deltaproteobacteria* are abundant in AK sulfidogenic enrichment cultures.

Although *Synergistetes*, *Actinobacteria* and *Deltaproteobacteria* species are also abundant in the NYH sulfidogenic enrichment culture, it appears to have a slightly different community structure in comparison to the AK sulfidogenic culture (Figure 3.5), suggesting that different bacterial species with similar metabolic functions might be enriched during anaerobic MTBE degradation. This was also observed in earlier analysis of the MTBE-degrading enrichment cultures (Youngster et al. 2010a). Furthermore, more common T-RF peaks were shared between the AK sulfidogenic and methanogenic cultures than between the AK and NYH sulfidogenic enrichment cultures. This suggests that the electron-accepting condition did not dramatically impact the MTBE-degrading bacterial communities originally enriched from the same sediment even after one decade incubation (Figures 5.1). In addition, the microbial diversity of AK and NYH enrichment cultures were still complex during sequential transfers from 10^{-3} dilution to 10^{-9} dilution of the original enrichment cultures, but their community structures have greatly changed. This suggests that most phylotypes appearing in the communities did not directly utilize MTBE as carbon source (Youngster *et al*., 2010a). To date, only three other sulfidogenic MTBE-degrading communities have been characterized, but none of them shared the

same dominant phyla, indicating that the microbial diversity among these communities and more characterization should be done in the future (Wei & Finneran, 2009; Raynal *et al*., 2010; Key *et al*., 2013).

Single-cell Resonance Raman (SCRR) is culture-independent technique that can provide a fingerprint of a single cell through its unique spectrum. The Raman spectrum contains rich cell information which allows analyzing the individual living cells in microbial communities (Li *et al*., 2012). In addition, if ^{13}C stable isotope substrates are incorporated into cell macromolecules, a "red shift" spectrum will be produced (Huang *et al*., 2004) that can be used to identify MTBE degraders in a complex community. This method can overcome the cross feeding problems and the identified cells can potentially be analyzed in more detail through single cell genomic amplification (Huang *et al*., 2009a). We manually examined six hundred cells from methanogenic and sulfidogenic enrichment cultures amended with ^{13}C MTBE. Unfortunately, none of the Raman spectra showed a "red shift", which means that none of the examined cells had incorporated ^{13}C MTBE in sufficient amount for detection. This is in agreement with the SIP results, which suggested that MTBE degraders are not abundant in the enrichment cultures. Interestingly, some cells contained rich cytochrome-like components, suggesting that they are able to produce ATP and obtain energy from anaerobic respiration (Figure 4.3).

In this study, we have identified *Ruminococcaceae* species that are responsible for the anaerobic MTBE degradation in methanogenic cultures. The utilization of the methyl group of MTBE was demonstrated by SIP coupled with TRFLP methods. The other

species identified in the methanogenic cultures might support the degradation through consuming the degradation products generated by the MTBE-utilizing *Ruminococcacea*. Sulfate reducers belonging to *Deltaproteobacteria* were highly enriched in both AK and NYH sulfidogenic cultures, suggesting that they were very likely to utilize acetate that was produced by acetogens. The other phylotypes observed might be enriched from utilization of decayed biomass by a variety of fermentation processes in the sulfidogenic cultures. SCRR results confirmed that the MTBE degraders were not abundant in the communities. The spectra of cytochrome like components suggest that cytochromes are rich in the microorganisms in the MTBE-degrading communities.

TBA is a stable MTBE degradation product, which is also a contaminant widely distributed in the environment. In most cases, TBA is resistant to anaerobic biodegradation in laboratory studies, however, it has been suggested to be anaerobically biodegraded in field studies (USEPA, 2007). Therefore, the initial anaerobic biodegradation of MTBE to TBA is a key aspect for completele degradation of MTBE in groundwater. Currently, various methods have been developed for remediation of MTBE contaminated sites (Levchuk *et al*., 2014). Within these techniques, monitored natural attenuation holds the greatest promise for cleaning up MTBE contamination *in situ* because of its advantages of high efficiency and low cost (ITRC, 2005; USEPA, 2004; USEPA, 2005). Compound-specific isotope analysis is a useful tool for monitoring and quantifying active microbial transformation of MTBE during natural attenuation process. The microorganisms active in MTBE enrichment cultures can potentially be used as microbial/molecular tools to evaluate biodegradation capacity of MTBE contaminated

sites *in situ*. The combination of these tools will provide better assessment of monitored natural attenuation, which will guide future exploration of MTBE degradation processes in the field.

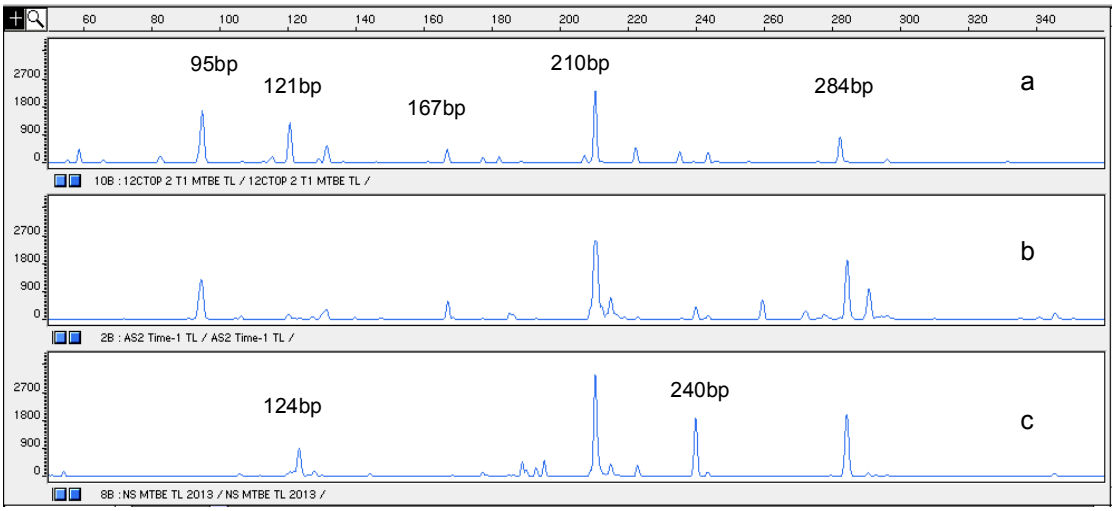


FIGURE 5.1. T-RFLP analysis of MTBE degrading enrichment cultures established from (a) AK 10^{-10} dilutions methanogenic culture, (b) AK 10^{-11} dilutions sulfidogenic culture, and (c) NYH 10^{-9} dilutions sulfidogenic culture.

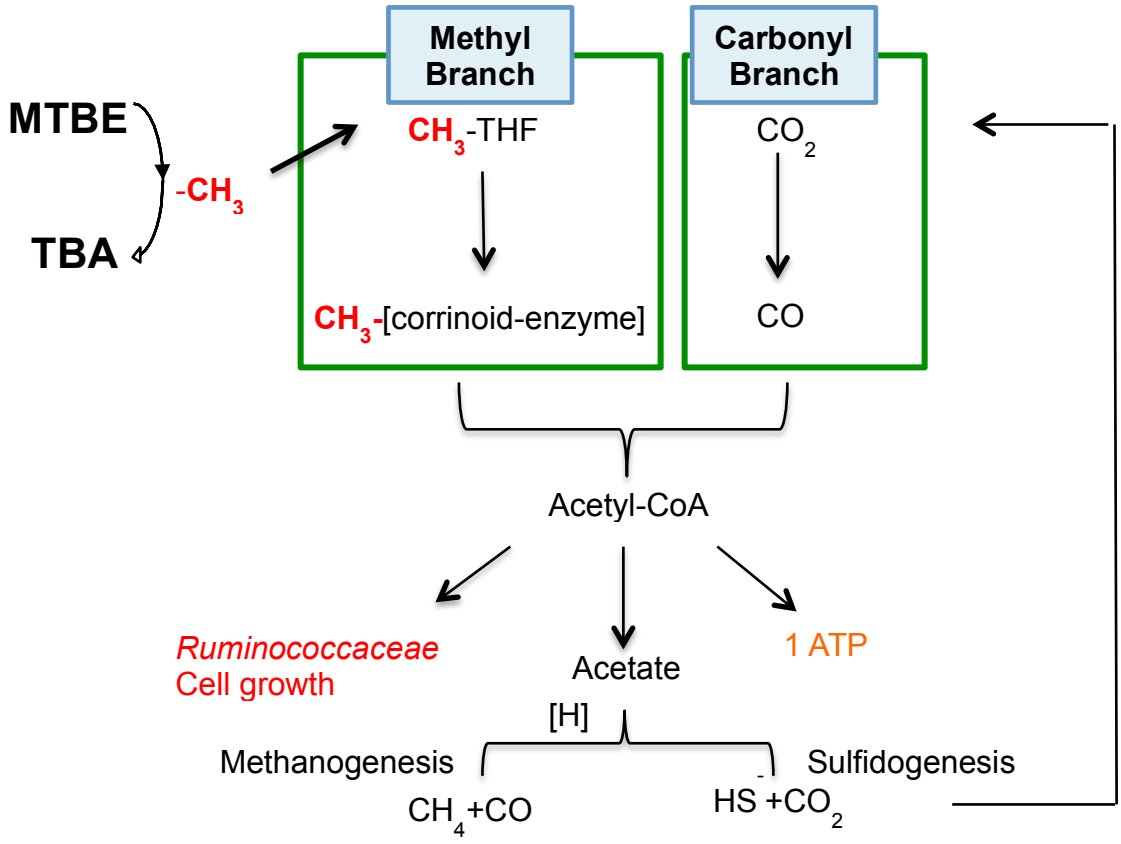


FIGURE 5.2. Proposed pathway for acetogenic metabolism of MTBE.

APPENDIX 1. List of anaerobic MTBE degrading cultures used

Original culture	Dilution	Experiment (reference)	Treatment
Arthur Kill Inlet methanogenic	10^{-10}	T-RFLP (Fig. 5.1a)	Portion of 10^{-9} dilution, transferred into a 150 ml culture at a 1:10 dilution
Arthur Kill Inlet methanogenic	10^{-11}	T-RFLP (SIP) (Fig. 2.2, 2.6); Pyrotag sequencing (Fig. 2.5)	Portion 10^{-10} dilution, subdivided into six 60 ml cultures at a 1:10 dilution
Arthur Kill Inlet methanogenic	10^{-13}	Raman microspectroscopy (SIP) (Fig. 4.2, 4.3)	Portion 10^{-12} dilution, subdivided into six 30 ml cultures at a 1:10 dilution, without adding resazurin
Arthur Kill Inlet sulfidogenic	10^{-10}	T-RFLP (Fig. 3.2a)	Portion of Arthur Kill Inlet eventually yielded a 150 ml culture at a 10^{-10} dilution of the original.
Arthur Kill Inlet sulfidogenic (1)	10^{-11}	T-RFLP (Fig. 3.2b, 3.5a 5.1b)	Portion of 10^{-10} dilution, transferred into three 60 ml culture at a 1:10 dilution
Arthur Kill Inlet sulfidogenic (2)	10^{-11}	T-RFLP (Fig. 3.2c) Pyrotag sequencing (Fig. 3.4)	Three consecutive MTBE feeding to Arthur Kill Inlet sulfidogenic (1)
Arthur Kill Inlet sulfidogenic	10^{-12}	T-RFLP (Fig. 3.2d, 3.3)	Portion of Arthur Kill Inlet sulfidogenic (2) subdivided into three 60 ml cultures at a 1:10 dilution of the original. Separated from culture 2-3 since 10^{-11}.
Arthur Kill Inlet sulfidogenic	10^{-13}	Raman microspectroscopy (SIP)	Portion 10^{-12} dilution, subdivided into six 30 ml cultures at a 1:10 dilution, without adding resazurin
New York Harbor sulfidogenic	10^{-9}	T-RFLP (Fig. 3.5b, 5.3c)	Portion of 10^{-8} dilution, transferred into a 150 ml culture at a 1:10 dilution

APPENDIX 2. Calculation of MTBE degradation kinetics

1. The initial cell density of AK methanogenic culture is $4*10^5$ cells/ml based on DAPI staining cell counting.

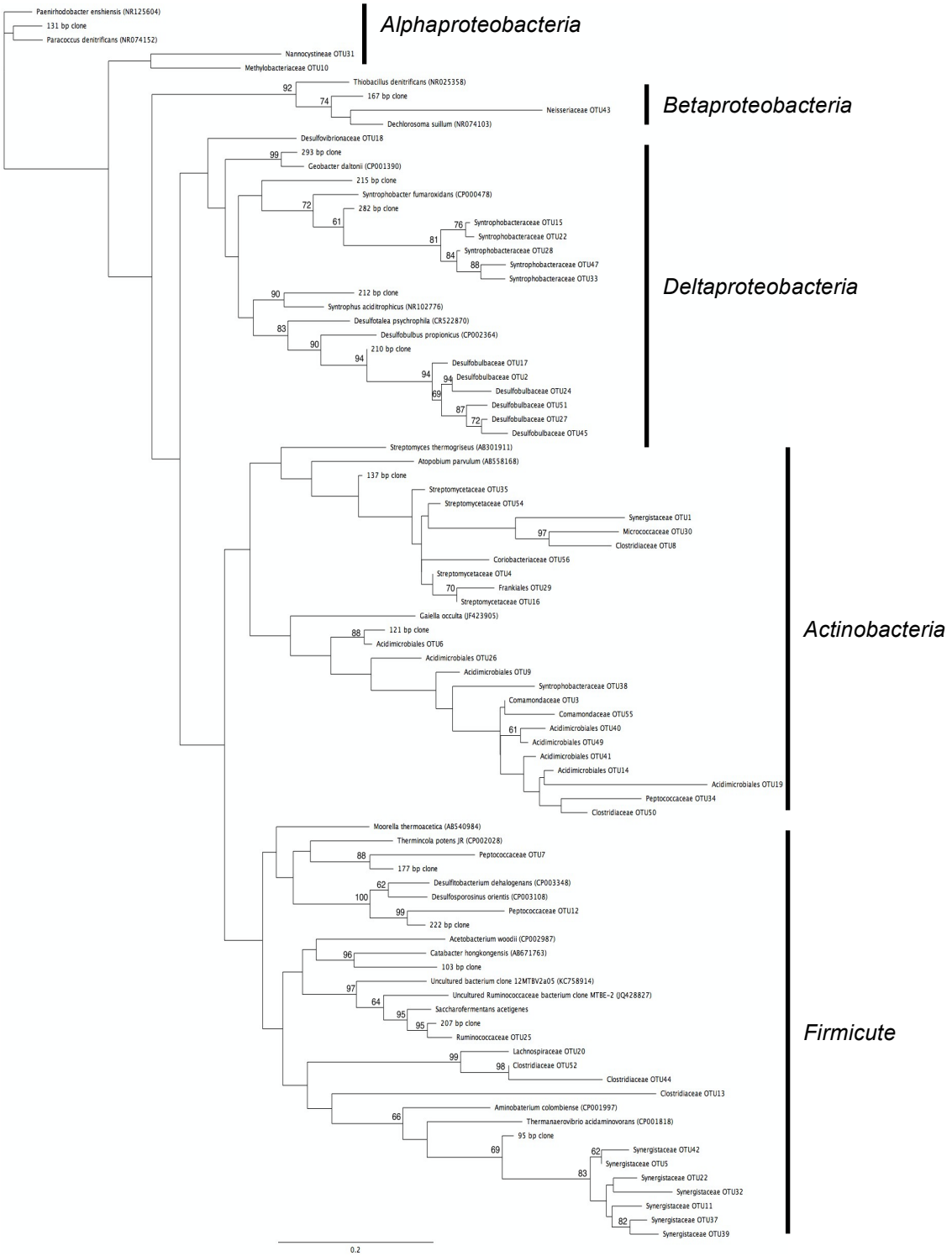
2. If 10% of the community is assumed to be archaeal cells, then the bacterial cell density is $4*10^5$ cells/ml * (1-10%) = $3.6*10^5$ cells/ml.

3. *Ruminococcaceae* species was active in AK methanogenic culture which assume to be the microorganisms that responsible for anaerobic MTBE biodegradation.

4. The relative abundance of the *Ruminococcaceae* species (207 bp T-RF) was 2~5% of the bacterial community, according to T-RFLP and pyrosequencing community analysis (Figs. 2.3 and 2.4). Therefore the range of cell density of *Ruminococcaceae* species is 2%*$3.6*10^5$ cells/ml ~ 5%*$3.6*10^5$ cells/ml = $7.2*10^3$ cells/ml~$1.8*10^4$ cells/ml.

5. MTBE degradation rate is nearly linear (Fig. 2.1, R=0.97, $y_{\text{MTBE conc.}}=-4.30x_{\text{days}}+94.45$), the kinetics is assumed to be zero order reaction. The degradation rate is 4.3 μM (MTBE)/day.

6. On single cell level, MTBE degradation rate is 2.11~5.26*10^{-5} μg/*Ruminococcaceae* cell/day.



APPENDIX 3. Maximum likelihood phylogenetic relationships of organisms detected in Arthur Kill MTBE-degrading methanogenic culture based on 16S rRNA genes sequenced from clone library by Sanger method and PCR products by Pyrotag method. 372 bp unambiguously aligned nucleotide positions were used analysis. Numbers at nodes indicate bootstrap values from 100 replications. Accession numbers for reference sequences are indicated in parenthesis.

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