THE APPLICATION OF MOLECULAR METHODS INCLUDING STABLE ISOTOPE PROBING TO IDENTIFY THE MICROORGANISMS INVOLVED IN TOLUENE AND MTBE DEGRADATION IN MIXED MICROBIAL SYSTEMS

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ABSTRACT

THE APPLICATION OF MOLECULAR METHODS INCLUDING STABLE ISOTOPE PROBING TO IDENTIFY THE MICROORGANISMS INVOLVED IN TOLUENE AND MTBE DEGRADATION IN MIXED MICROBIAL SYSTEMS

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Sites containing leaking underground storage tanks (LUST sites) are a national problem, with over 443 568 releases confirmed as of 2003, resulting in BTEX (benzene, toluene, ethylbenzene, xylenes) or oxygenate (e.g. MTBE or TBA) contamination. These chemicals are a threat to drinking water supplies because of their human health effects and relatively high aqueous solubilities. Bioremediation can be a cost-effective method to remove such groundwater contaminants either through natural attenuation or by advanced engineering methods. Understanding the microbial processes involved in the biodegradation of BTEX and MTBE has the potential to improve the efficiency of LUST site remediation. The overall aims of this project were to: 1) characterize the microorganisms able to degrade toluene under a range of redox conditions from a variety of soil and sediment sources using stable isotope probing (SIP); 2) investigate the diversity of the *bssA* and *bamA* genes in a wide range of anaerobic toluene-degrading consortia; and 3) identify the microorganisms able to transform MTBE anaerobically using SIP.

The first study in this research involved using SIP to identify the active members in an aerobic toluene degrading consortium. Specifically, SIP was used with terminal restriction fragment length polymorphism (TRFLP) and the results indicated that a 313 bp terminal restriction fragment (T-RF) incorporated the majority of the ^{13}C from ^{13}C labeled toluene. Sequencing of 16S rRNA genes from these communities indicated the organism represented by this T-RF was a

Polaromonas spp. Real-time PCR was also utilized to provide quantitative patterns in gradient fractions and document increases in *Polaromonas* populations as toluene was degraded. In the second study, SIP was applied to five toluene-degrading consortia under sulfate and nitrate amended conditions. In all, five different phylotypes were found to be responsible for toluene degradation and these included previously identified toluene degraders as well as novel toluene degrading microorganisms. In nitrate amended microcosms, inoculated from granular sludge, microorganisms classifying within the genus *Thauera* were the primary toluene degraders. Whereas in nitrate amended microcosms, inoculated from a different source (agricultural soil), microorganisms in the family *Comamonadaceae* (genus unclassified) were the key degraders. In one set of sulfate amended microcosms (agricultural soil), the primary degrader affiliated within the class *Clostridia* (genus *Desulfosporosinus*), while in other sulfate amended microcosms, the primary degraders affiliated with the class *Deltaproteobacteria,* classifying within the families *Syntrophobacteraceae* (digester sludge) or *Desulfobulbaceae* (contaminated soil) (genus unclassified for both). The third study involved an investigation into the diversity of anaerobic toluene-degrading functional genes (*bssA* and *bamA* genes) in a number of inocula sources. The results suggest that targeting the *bamA* and *bssA* genes in a quantitative or non-quantitative manner could be a productive approach for investigating toluene biodegradation potential over a range of samples and redox conditions. The final study involved using SIP to investigate the dominant degraders in an anaerobic MTBE degrading microcosms. These experiments indicated bacteria in the phyla *Firmicutes* (family *Ruminococcaceae*) and *Alphaproteobacteria* (genus *Sphingopyxis*) were the dominant MTBE degraders in a methanogenic MTBE-degrading consortium seeded from activated sludge.

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ABBREVIATIONS

bamA- the gene encoding 6-oxocyclohex-1-ene-1-carBoN1yl-CoA hydrolase

BSS - benzylsuccinate synthase, a glycyl radical enzyme catalyzing the first step in anaerobic toluene degradation

BssA- α-subunit of benzyl succinate synthase

bssA - the gene encoding BssA

BTEX - benzene, toluene, ethylbenzene and o-, p-, and m-xylenes

DNA - deoxyribonucleic acid

MTBE - methyl tertiary butyl ether

OTU - operational taxonomic unit

PCR - polymerase chain reaction

qPCR- quantitative PCR

rDNA - ribosomal DNA

TBA - tertiary butyl alcohol

TRFLP - terminal restriction fragment length polymorphism

CHAPTER 1

INTRODUCTION

MTBE and BTEX Contamination

The chemicals benzene, toluene, ethylbenzene and xylene (BTEX) and MTBE (a fuel oxygenate) are frequently the major pollutants at gasoline contaminated sites (leaking underground storage tank sites or LUST sites) (24). In the United states, 50 states have reported MTBE in groundwater, surface water and drinking water (29). Bioremediation can be a cost-effective method to remove groundwater contaminants either through natural attenuation or by advanced engineering methods. Understanding the microbial processes involved in the biodegradation of BTEX and MTBE has the potential to improve the efficiency of LUST site remediation.

Stable Isotope Probing (SIP)

Although many BTEX degraders have been identified through enrichment and isolation procedures, a question arises that culturing microorganisms may not fully reflect the microbial diversity (2, 3, 30). Stable isotope probing (SIP) is a novel molecular method which can link the function to active microbial members in environmental samples or directly in field studies. The application of SIP has the potential to increase our understanding of function in mixed microbial systems and its use has increased remarkably in recent years (9, 11, 20, 22, 23). The method consists of sample exposure to labeled substrates (e.g. ^{13}C, ^{15}N, ^{18}O), separation of labeled and

unlabeled nucleic acids (DNA or RNA) and analysis of separated heavy (labeled) or unlabeled (background) nucleic acids. Nucleic acids can be separated by needle extraction (25, 26) or by fractionation (9, 16, 21).

Functional Genes for Anaerobic Toluene Degradation

The gene encoding for the enzyme benzylsuccinate synthase has been linked to anaerobic toluene degradation under nitrate reducing (1, 15), sulfate reducing (32-34), ferric iron reducing (7, 10), and methanogenic enrichment cultures or environmental samples (31, 32). The enzyme benzylsuccinate synthase catalyzes the first step of anaerobic toluene degradation and involves the addition of toluene across the double bond of fumarate to produce (*R*)-benzylsuccinate. Benzylsuccinate is then oxidized to benzoyl-CoA and succinate via β-oxidation pathway. The final step is the recycling of the fumarate cosubstrate of benzylsuccinate synthase from succinate by succinate dehydrogenase. The overall result is an oxygen-independent reaction (6). Benzylsuccinate synthase, which has been identified in denitrifying bacteria, was characterized as a novel glycyl radical enzyme (19). The enzyme contains a big subunit, α-subunit (98 kDA) and two small subunits β (8.6 kDa) and γ (6.6 kDa); *bssA* encodes the large α subunits while *bssB* and *bssC* encode the other two small subunits, β and γ (13). A limited number of *bssA* sequences from pure cultures are available to date (1, 8, 10, 14, 19, 27, 28). Previous studies have developed PCR methodologies to target the *bssA* gene (5, 12) and the current work involved using these methods to amplify the functional gene.

The other functional gene investigated in this research was previously reported to be important for the degradation of aromatics. Anaerobic aromatic biodegradation typically involves the

channeling of aromatic growth substrates to the central intermediate benzoyl-coenzyme A (CoA) prior to dearomatization and ring cleavage (17). In *Thauera aromatica*, the metabolism of benzoyl-CoA comprises of several steps with ring cleavage action by 6-oxocylcohex-1-ene-1-carbonyl-CoA (6-OCH-CoA) hydrolase, which likely catalyzes the transformation of 6-OCH-CoA to 6-hydroxypimelyl-CoA (17, 18). The ring-cleaving hydrolase of the benzoyl-CoA pathway is encoded by the *bamA* gene. Recently, this pathway was studied in obligate anaerobes that use aromatic growth substrates (17).

Objectives

The overall objectives of this project were:

1) To characterize the microorganisms able to degrade toluene under a range of redox conditions (aerobic, nitrate and sulfate reducing and methanogenic) from a variety of soil and sediment sources using stable isotope probing (SIP) (Chapters 2 and 3).

2) To investigate the diversity of the *bssA* gene (encodes for benzylsuccinate synthase α unit) and *bamA* genes (encodes for the ring-cleaving hydrolase of the benzoyl-CoA pathway) in a wide range of anaerobic toluene-degrading consortia (Chapter 4);

3) To identify the organisms able to transform MTBE anaerobically using SIP (Chapter 5).

Chapter 2 describes the work targeting aerobic toluene degradation and also describes the SIP methodology used throughout the thesis. In summary, the work identified members of

Polaromonas as the key aerobic toluene-degrading bacteria. This conclusion was also supported by quantitative PCR (qPCR) results. A modified version of Chapter 2 was published in Applied and Environmental Microbiology.

The same SIP methodology was used in Chapter 3 to investigate diversity of microorganisms able to uptake carbon from toluene in a diverse number of samples under sulfate and nitrate amended conditions. In these studies, different microorganisms were identified as toluene degraders. Microorganisms classifying within the family *Comamonadaeae* and the genus *Thauera* were pinpointed in nitrate amended microcosms seeded from agricultural soil and granular sludge, respectively. Members of *Desulfosporosinus*, *Syntrophaceae, Desulfobulbaceae* were identified from agricultural soil, digester sludge and contaminated aquifer sediment as toluene degraders under sulfate amended conditions. A modified version of Chapter 3 was published in Applied and Environmental Microbiology.

The work described in Chapter 4 focuses on previously designed primer pairs to amplify the *bssA* and *bamA* genes. The *bssA* phylogenetic information was also related to the toluene degraders identified via SIP in Chapter 3. In Chapter 4, I tested a range of *bssA*- and *bamA*-primers on 16 toluene-degrading consortia. Two primer pairs, 7772f/8546r and SRBr/SRBf, exhibited good coverage for nitrate amended samples and sulfate amended samples, respectively. The *bamA* primer set (bam-sp9 and bam-asp1) produced a strong amplicon in DNA extracted from all except one microcosm. Partial *bssA* and *bamA* sequences were obtained for a number of samples (four *bssA* and six *bamA* sequences) and compared to those available in GenBank. The partial *bssA* sequences (from nitrate amended and methanogenic microcosms) were most similar

to *Thauera* sp. DNT-1, *Thauera aromatica*, *Aromatoleum aromaticum* EbN1 and *bssA* clones from a study involving sulfate reducing toluene degradation. The *bamA* sequences obtained could be placed into five clades.

In Chapter 5, SIP was utilized on a methanogenic MTBE-degrading consortium. The SIP experiments indicated bacteria in the phyla *Firmicutes* (family *Ruminococcaceae*) and *Alphaproteobacteria* (genus *Sphingopyxis*) were the dominant MTBE degraders. Previous studies on have suggested a role for *Firmicutes* in anaerobic MTBE degradation, however the *Alphaproteobacteria* phylotype represents a novel MTBE degrader. Two archaeal phylotypes (genera *Methanosarcina* and *Methanocorpusculum*) were also enriched in the heavy fractions and these organisms may be responsible for minor amounts of MTBE degradation or for the uptake of metabolites released from the primary MTBE degraders. The work described in Chapters 5 was published in Applied and Environmental Microbiology.

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CHAPTER 2

TOLUENE DEGRADATION IN CONTAMINATED SITE MICROCOSMS IS DIRECTLY LINKED TO A POLAROMONAS STRAIN

Introduction

Sites containing leaking underground storage tanks (LUST sites) are a national problem, resulting in BTEX (benzene, toluene, ethylbenzene, xylenes) or oxygenate (e.g. MTBE or TBA) contamination and risk to sensitive receptors such as drinking water supplies. A common remediation method for such sites involves *in situ* biological degradation of these contaminants. Aerobic bioremediation has been favored over other electron acceptors, such as nitrate, sulfate and ferric iron, because BTEX degradation is typically faster when oxygen is available. Although many aerobic BTEX isolates have been obtained in pure culture, less is known about the organisms responsible for *in situ* contaminant transformation. For example, toluene transformation has been particularly well studied in a number of isolates (e.g. *Pseudomonas putida* F1, *P. putida* mt-2, *P. mendocina* KR1, *P. stutzeri* OX1, *Burkholderia vietnamiensis* G4, *Burkholderia* sp. strain JS150 and *Ralstonia pickettii* PK01, for review see (14)), however, information on the organisms responsible for toluene degradation in a mixed community sample is lacking. In this study, we attempt to address this knowledge gap, focusing on toluene as a model BTEX contaminant. Identification of microorganisms responsible for BTEX degradation in mixed community environmental samples should result in a better understanding of microorganisms responsible for degradation *in situ*. It is hoped that such information can aid in the design of remediation approaches as well as in the prediction of contaminant removal rates.

To identify the dominant organism responsible for toluene transformation within a mixed community sample, an approach was adopted to result in less culture bias than would be produced using repeated enrichments or isolation. Therefore, the data produced should more accurately reflect the organisms responsible for *in situ* toluene transformation. To do this, stable isotope probing (SIP), a method that links *in situ* function with identity for mixed microbial systems (15), was adopted. The method involves sample exposure to labeled compounds, separation of heavy (label incorporated) and light (background) nucleic acids using ultracentrifugation, then gene sequencing (16S rRNA) to identify the label-consuming microorganisms. For this purpose, SIP has two key advantages over traditional microbiological methods for investigating contaminant removal, as follows, (1) identification of organisms able to assimilate carbon from the contaminant, therefore selectively pinpoints efficient degraders and (2) studies can be conducted on environmental samples, thus results are much more applicable to the field environment than traditional approaches.

The objective here was to identify the organisms responsible for aerobic carbon uptake from the environmental contaminant toluene in two soils obtained from a BTEX contaminated site. DNA based SIP was utilized to identify the active toluene degrader and following this, a real time PCR assay was designed to confirm these results. The results were also compared with those from previous *m*-xylene and benzene SIP studies on this soil (5). The research offers new insight into contaminant transformation in mixed communities.

MATERIALS AND METHODS

Experimental design, microcosm setup and chemical analyses

The gasoline-contaminated soil used in this study was collected from a gasoline-contaminated site located in Michigan. Two soil samples, obtained at different depths (3 - 4 ft and 5 - 6 ft deep) at the same site were used and are further referred to as soil 1 and soil 2. Microcosms were constructed with 6 g soil along with 20 mL phosphate-buffered mineral media (12) in a 150 mL serum bottle sealed with rubber stoppers and aluminum seals. Three sets of experiments were conducted, experiment 1 involved SIP to identify the dominant toluene degraders in both soils, experiment 2 focused on real-time PCR to confirm SIP results and investigate microorganism growth on toluene and experiment 3 involved simple biodegradation studies with other environmental contaminants (benzene, *m*-xylene and *cis*-dichloroethene).

The SIP study (experiment 1) was conducted on both soils 1 and 2 and involved triplicate abiotic controls, triplicate unlabeled toluene (1 µL, 99 %, Chem Service, West Chester, PA) and triplicate labeled toluene (1 µL ring-$^{13}C_6$ toluene, 99%, Cambridge Isotope Laboratories, Inc. Andover, MA) amended samples. Experiment 2 involved sixteen sample microcosms, each with 6 g soil 2 and 20 mL media (as above). Toluene (1 µL, 99 %, Chem Service, West Chester, PA) was added to eight of these and the other eight served as no-toluene controls (to determine if toluene was needed to cause an increase in *Polaromonas* sp. cell numbers). At each sampling time, two samples and two controls were sacrificed for DNA extraction and qPCR.

In experiment 3, for each contaminant investigated (benzene, *m*-xylene and *cis*-dichloroethene),

three sample microcosms and two autoclaved control microcosms were constructed using the supernatant of toluene degrading soil microcosms from soil 2. Specifically, following the depletion of toluene, 5 mL supernatant was transferred to a 150 mL serum bottle with 20 mL media (as above). Following this, either benzene (99.8%, Sigma Aldrich), *cis*-dichloroethene (purity not provided, Supelco) or *m*-xylene (99%, Sigma Aldrich) were added to a final solution concentration of approximately 45 mg L^{-1}. All microcosms were incubated at room temperature (~20 °C) with reciprocal shaking. Contaminant (toluene, benzene, *m*-xylene and *cis*-dichloroethene) concentrations in headspace gas samples (200 µL) were typically determined daily with a gas chromatograph (Perkin Elmer) equipped with flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53mm). Injector and detector temperature were set at 200 ^{o}C and the column temperature was 120^{o}C.

DNA extraction

The powersoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) was used for soil DNA extraction in experiments 1 and 2 according to the manufacturer's recommended procedure. Extraction times, number of samples and mass of soil extracted varied depending on the experiment. In experiment 1, DNA was extracted from the entire microcosm, whereas in experiment 2, DNA was extracted from 0.3 g soil. In experiment 1 (SIP study), for both soil 1 and soil 2, two labeled and two unlabeled microcosms were sacrificed for DNA extraction at day 7 (at this time, all toluene had been depleted). In experiment 2 (qPCR study), DNA was extracted from samples sacrificed at successive time points for the unlabeled toluene sample microcosms and no-toluene-amended control microcosms. Specifically, two sample and two control

microcosms were scarified at day 3, 4, 5, 6 when approximately 20 %, 50 %, 80 % and 100 % of the added toluene was transformed.

DNA ultracentrifugation

Approximately 10 μg DNA (quantified with Nanodrop, ND-1000) was added to Quick-Seal polyallomer tubes (13×51 mm, 5.1 ml, Beckman Coulter) along with a Tris-EDTA (TE, pH 8.0) /CsCl solution. Prior to sealing (cordless quick-seal tube topper, Beckman), the buoyant density (BD) was determined with a model AR200 digital refractometer (Leica Microsystems Inc.) and adjusted by adding small volumes of CsCl solution or Tris-EDTA buffer. The tubes were centrifuged at 178,000 *g* (20 ºC) for 48 h in a Stepsaver 70 V6 Vertical Titanium Rotor (8 x 5.1 ml capacity) within a Sorvall WX 80 Ultra Series Centrifuge (Thermo Scientific). Following centrifugation, the tubes was placed onto a fraction recovery system (Beckman) and fractions (150 μl) were collected. The BD of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation.

PCR and TRFLP

The ultracentrifugation fractions from replicates of labeled and unlabeled microcosms for both soils were PCR-amplified using 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescine) and 1492R (5'-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) as previously described (4). The presence of PCR products was confirmed by 1.5% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide. PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.), following the manufacturer's instructions and approximately 150 ng purified PCR products were digested with

Hae III (New England Biolabs) with a 6-hour incubation period. Additional digests (Hha I, Mse I, Bsp1286I, BsrB I) for TRFLP analyses in a number of heavy labeled fractions were included to correlate the TRFLP fragment lengths to the *in silico* cut sites of the cloned 16S rRNA gene sequences. DNA fragments were separated by capillary electrophoresis (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) at the Research Technology Support Facility (RTSF) at Michigan State University. Data were analyzed with GeneScan software (Applied Biosystems) and the percent abundance of each fragment was determined.

16S rRNA gene sequencing

Heavy fraction ^{13}C-DNA (BD value of 1.744 g mL^{-1}) was amplified as above except the forward primer was unlabeled and the final extension time was extended to 15 minutes. The PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.) and cloned into *Escherichia coli* TOP10 vector supplied with a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria-Bertani (LB) medium solidified with 15 g agar L^{-1} with 50 μg ampicillin L^{-1} for 16 h at 37 ºC. Colonies with inserts were verified by PCR with primers M13 F (5'-TGTAAAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.) and the insertions were sequenced at RTSF. The Ribosomal Database Project (RDP) (Center for Microbial Ecology, Michigan State University) analysis tool "classifier" was utilized to assign taxonomic identity.

Real-time PCR

A quantitative real-time PCR assay (qPCR) was developed targeting the 16S rRNA gene of the dominant toluene degrader identified above. The assay was developed to quantify the increase in the DNA BD resulting from label incorporation. In addition, the assay provided a rapid method to investigate cell numbers under mixed culture conditions as toluene was depleted. The assay was conducted in a Chromo 4 real-time PCR cycler (Bio-Rad) using the primer set PO313F (5'-AATGGATGGTACAGAGGGTC-3') and PO313R (5'-ATTACTAGCGATTCCGACTT-3') (Operon Biotechnologies), and produced a 114 bp PCR product. Primers were designed with NCBI primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) and checked for specificity using the Ribosomal Database Project (RDP) probe match tool. The forward primer matched 64 16S rRNA gene sequences (out of a total of 856,341) and of this, 32 belonged to the genus *Polaromonas*. The reverse primer matched 105,600 16S rRNA gene sequences and from this, 252 belonged to the genus *Polaromonas*. For the purposes of this study, this level of specificity was considered adequate. Both nucleic acid samples from density gradient fractions (experiment 1, SIP fractions) and total DNA extractions at successive time points (experiment 2, real-time PCR) were quantified with primers PO313F and PO313R using a SYBR green real-time PCR kit (Applied Biosystems) following the manufacturer's recommended recipe. Each 20 μL PCR reaction mixture containing 10 μL ABI real-time PCR kit , 0.25 μM of each primer, and 1 μL DNA template. The thermal protocol consisted of an initial denaturation (95 ºC, 15 s), 40 cycles of amplification (95 ºC, 15 s; 60 ºC, 20 s; 72 ºC 20 s) and a terminal extension step (72 ºC, 2 min). Melting curves were constructed from 55.0ºC to 95.0ºC, read every 0.6ºC for 2 s. For each gradient fraction, 1 μL was diluted with 3 μL water as template (to conserve the sample). For experiment 2, 1 μL total DNA was used directly as the template.

For both, samples were measured in triplicate. Cloned plasmid DNA was utilized as a standard for quantification and gene copies were determined as shown in the equation below, as previously described (16) (plasmid size was 5441 bp, including 1485 bp of insert).

$$\text{gene copies} = \left(\text{DNA concentration}, \frac{\text{ng}}{\mu\text{L}}\right) \times \left(\frac{1\text{g}}{1000^3\,\text{ng}}\right) \times \left(\frac{1\,\text{mol bp DNA}}{660\text{g DNA}}\right) \times \left(\frac{6.023 \times 10^{23}\,\text{bp}}{\text{mol bp}}\right) \times \left(\frac{1\,\text{copy}}{\text{plasmid size [bp]}}\right) \times (\text{volume}, \mu\text{L})$$

RESULTS AND DISCUSSION

Toluene removal (both labeled and unlabeled) occurred rapidly (~100 % removal in seven days) in triplicates of soil 1 and soil 2 microcosms, but was limited in the autoclaved controls (~20% decrease, likely due to sorption), confirming a biological removal mechanism (Table 2.1). Several molecular methods (SIP and qPCR) were employed to identify the dominant microorganism responsible for aerobic toluene removal in these microcosms. Following toluene depletion, DNA was extracted from replicate soil 1 and soil 2 microcosms (labeled and unlabeled toluene amended) and subjected to ultracentrifugation, followed by fractionation of the ultracentrifugation samples. TRFLP was then conducted on all fractions so that heavy fractions from the labeled and unlabeled samples could be compared and thus account for false positives (from contamination or high GC content microorganisms) in the sample heavy fractions. TRFLP profiles indicated one fragment (313 bp) was more dominant in the heavy fractions (> ~ 1.74 g mL^{-1}) of labeled toluene amended microcosms compared to the controls (unlabeled toluene) (Figure 2.1). This trend was observed in the replicates of both soils. Other TRFLP fragments were found in the heavy fractions from the labeled toluene amended samples, however, as these

were also found at similar levels in the heavy fractions of the controls, they were excluded from further analyses.

The identity of the organism producing the TRFLP 313 bp fragment was determined using two methods. Firstly, the fraction (BD value of 1.744 g mL^{-1}) with highest relative abundance of the 313 bp fragment was subject to cloning and sequencing. Sequences with a Hae III cut site of 313 bp were identified from this. Additionally, fractions with dominant 313 bp fragments were chosen for additional TRFLP analyses with four other enzyme (Hha I, Mse I, Bsp1286I, BsrB I). The dominant fragments obtained from these additional TRFLP digests were then compared to the clone library sequences to correlate actual cut sites with predicted cuts sites, thus determine the identity of the organism producing the Hae III 313 bp peak (Table 2.2). Only slight differences (1 to 2) between the predicted and actual lengths were seen, such differences have been noted by others (1, 13) and may be a result of variability within the TRFLP method. The clone sequence obtaining the five appropriate cut sites classified as a *Polaromonas* strain within the class *β Proteobacteria*. To our knowledge, this is the first report directly linking toluene degradation to the *Polaromonas* genus.

Two additional lines of inquiry provided further evidence that the *Polaromonas* strain was indeed responsible for toluene transformation within the mixed community sample. Firstly, the relative distribution of *Polaromonas* 16S rRNA genes, as determined via the developed qPCR assay, indicated an increase in DNA BD between the labeled and unlabeled toluene amended microcosms (Figure 2.2, soil 1). Maximum 16S rRNA gene abundance levels from labeled toluene amended microcosms were found at 1.742 g mL^{-1} (soil 1) and1.744 g mL^{-1}(soil 2), a

clear increase over the maximum abundance values in the unlabeled toluene amended samples (1.719 g mL^{-1} for soil 1 and 1.726 g mL^{-1} for soil 2). Thus, the BD differences between the peak abundance in unlabeled compared to the labeled were 0.023 g mL^{-1} (soil 1) and 0.018 g mL^{-1} (soil 2), respectively. The slight difference between the two soils is likely a result of analytical variability. As expected, the increase is less than has been seen with pure cultures exposed to higher concentrations of labeled substrates (e.g. an increase of 0.038 g mL^{-1} was noted in *E. coli* following exposure to 1.3 g L^{-1} ^{13}C lactate (3)), yet it is a large enough signal to indicate label uptake from toluene by the *Polaromonas* population.

The second line of evidence indicating the *Polaromonas* sp. was responsible for toluene degradation is provided by *Polaromonas* specific qPCR analyses of toluene degrading microcosms. Specifically, qPCR was utilized to monitor the total number of *Polaromonas* species at successive time points of toluene removal. The data collected indicated a clear increase in *Polaromonas* sp. cell numbers as toluene was depleted in the replicate live samples but not in the replicate no-toluene controls, indicating toluene was required for growth of these organisms (Figure 2.3). The qPCR assay provided a rapid investigative tool enabling cell populations to be determined and correlated with toluene removal in mixed culture, without the need for isolation. Again, to our knowledge, these data represent the first report of growth on toluene of by an organism in the *Polaromonas* genus.

Supernatant samples transferred from toluene degrading microcosms (following complete removal of toluene) were tested for their ability to transform other contaminants. Previous SIP

research indicated the same (based on 16S rRNA gene sequences) *Polaromonas* sp. was responsible for benzene transformation within a mixed community microcosm sample constructed from the same soil (5), therefore benzene degradation was tested to confirm this result. Another SIP study on *m*-xylene degradation with the same soil found that a microorganism other than the *Polaromonas* sp., was responsible for *m*-xylene degradation (5), therefore *m*-xylene was also added to the supernatant to further investigate this finding. Finally, given the importance of *cis*-dichloroethene (cDCE) as an environmental contaminant, and previous reports of *Polaromonas* sp. strain JS666 using cDCE as a sole energy and carbon source (2, 11), the supernatant was also tested for cDCE removal. As expected, benzene transformation occurred rapidly in the samples but not in the autoclaved controls, whereas neither *m*-xylene nor cDCE concentrations declined in either the samples or controls (Figure 2.4).

This research provides evidence that that a *Polaromonas* species survives and grows in the presence of toluene while in a mixed community sample. Research to date on both aerobic and anaerobic toluene degradation has typically focused on pure cultures, due to the technical difficulties associated with examining particular species while existing in mixed culture. The primary method of inquiry used here, SIP, allows function to be linked with identity in a mixed community sample, more realistically reflecting a real-world scenario. The method has been utilized once before to investigate toluene degradation in mixed community samples. In that study, another novel organism, belonging to the "candidate" phylum TM7 was responsible for toluene transformation in mixed community samples (10). Clearly, SIP provides a unique ability to open up the "black box" of the microbial world providing interesting contrasts to previous reports based on isolations. Stable isotope probing, in theory, avoids the biases involved in

conventional isolation procedures. In other words, microorganisms that do not grow well under typical isolation conditions can still be studied and identified if SIP is used. The results presented here confirm this, as others have indicated *Polaromonas* species have traits (e.g. oligotrophic and slow growing) that are likely to impede their isolation and characterization by standard methods (11).

Since the genus was first reported in 1996 (6), only a small number of other *Polaromonas* strains have been obtained. The type stain, *P. vacuolata,* was isolated in 1996 from Antarctic marine waters (6). Others, also obtained from interesting sources, were isolated more recently, including *P. aquatica* obtained from tap water in 2006 (9) and *P. hydrogenivorans*, a psychrotolerant hydrogen oxidizing bacterium isolated from soil over permafrost in Alaska in 2007 (17). More relevant to the research presented here are the remaining two *Polaromonas* strains previously reported, as both have been linked to the degradation of important environmental contaminants. The first, isolated in 2002 from granular activated carbon from a chlorinated solvent pump-and-treat plant in Germany, *Polaromonas* sp. strain JS666, is the only known microorganism able to grow using the environmental contaminant cDCE as a sole carbon and energy source (2, 11). The other, isolated in 2003 from coal-tar contaminated sediment, *P. naphthalenivorans* strain CJ2 uses naphthalene as a sole carbon and energy source (7). Interestingly, *P. naphthalenivorans* strain CJ2 was also first identified with the SIP method (8). The 16S rRNA gene of the toluene degrading *Polaromonas* strain described here was 96.6 % (1447/1498) and 97.6 % (1459/1495) similar to *P. naphthalenivorans* strain CJ2 and *Polaromonas* sp. strain JS666, respectively.

In conclusion, several lines of evidence indicated a *Polaromonas* species was responsible for toluene removal in mixed culture samples. Firstly, SIP illustrated that a 313 bp TRFLP fragment was more dominant in heavy fractions obtained from labeled microcosms compared to the heavy fractions of the unlabeled controls. This pattern suggests the microorganism represented by fragment 313 bp was responsible for ^{13}C uptake from toluene. The identity of this organism was determined by 16S rRNA gene sequencing as well as TRFLP with additional enzymes on heavy fractions. Additionally, qPCR targeted to the identified *Polaromonas* species indicated an increase in DNA BD over the gradient fractions between the labeled and unlabeled samples, further confirming label uptake occurred. A second line of evidence indicating the *Polaromonas* strain was responsible for growth on toluene in mixed culture was provided by qPCR, which illustrated an increase in cell numbers only when toluene was present. In addition, samples highly enriched with *Polaromonas* cells rapidly depleted benzene. These results contribute to the growing body of knowledge on the abilities of *Polaromonas* species to degrade and grow on key environmental organic pollutants and thus indicate these may be key species for use *in situ* contaminant removal. Further, we provided evidence that this particulate *Polaromonas* strain can thrive in mixed culture suggesting they will likely compete well at a contaminated site.

Tables and figures

Table 2.1. Average percent toluene remaining in control and sample microcosms (error bars represent standard deviations represent from triplicates).

		Toluene concentration (mg L^{-1})		
	Time (days)	Sterile controls	Unlabeled toluene (^{12}C) samples	Labeled toluene (^{13}C) samples
Soil 1	0	37.2 ± 1.9	38.0 ± 0.5	34.7 ± 4.8
	4	33.5 ± 2.4	23.8 ± 7.7	25.6 ± 7.6
	5	29.4 ± 3.3	9.0 ± 4.6	6.5 ± 7.1
	7	29.3 ± 0.5	0.1 ± 0.2	0.3 ± 0.2
Soil 2	0	31.3 ± 1.9	34.2 ±2.7	30.2 ± 4.1
	4	28.3 ± 0.8	22.6 ± 4.3	17.2 ± 4.8
	5	28.3 ± 0.8	10.8 ± 3.4	9.0 ± 3.6
	7	25.6 ± 1.6	0.1 ± 0.1	0.1 ± 0.1

Table 2.2. Comparison of dominant fragments in heavy fraction TRFLP to clone restriction enzyme cut sites predicted from sequence analyses.

Restriction enzyme	TRFLP	Clones
Hha I	204	203
Bsp 1286 I	119	117
BsrB I	214	212
Mse II	535	534

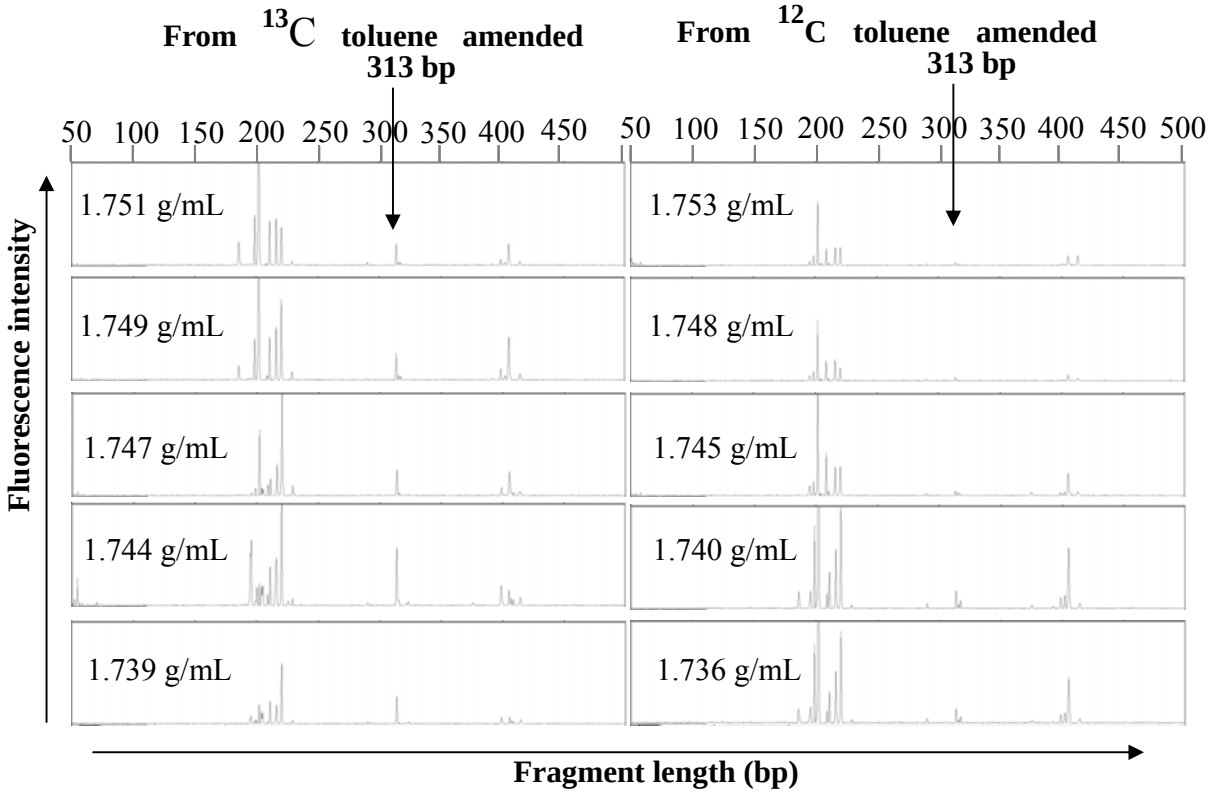

Figure 2.1. Comparison of TRFLP electropherograms of heavier fractions (>1.736 g mL^{-1}) between DNA obtained from labeled and unlabeled toluene amended microcosms, illustrating the dominance of TFRLP fragment 313 bp in fractions from the labeled toluene amended microcosms. A similar pattern was observed in the soil 1 replicate and in replicates of soil 2.

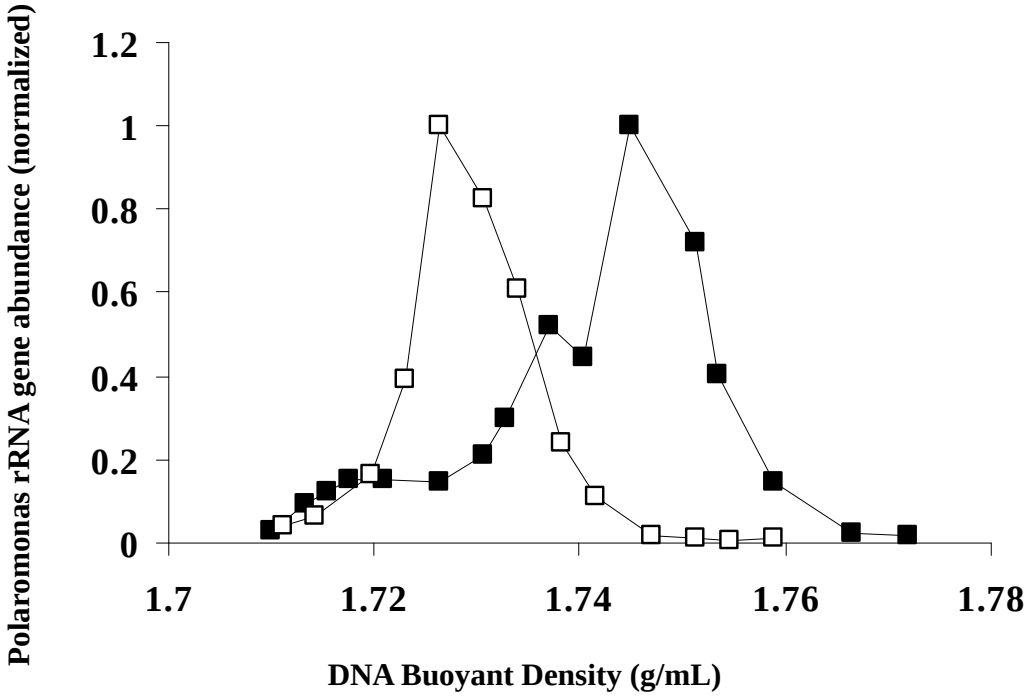

Figure 2.2. Difference between abundance of *Polaromonas* sp. rRNA gene copies in ultracentrifugation fractions from labeled (^{13}C toluene) and unlabeled toluene amended microcosms from soil 2 as determined via qPCR. Fractions obtained from soil 1 illustrated a similar trend.

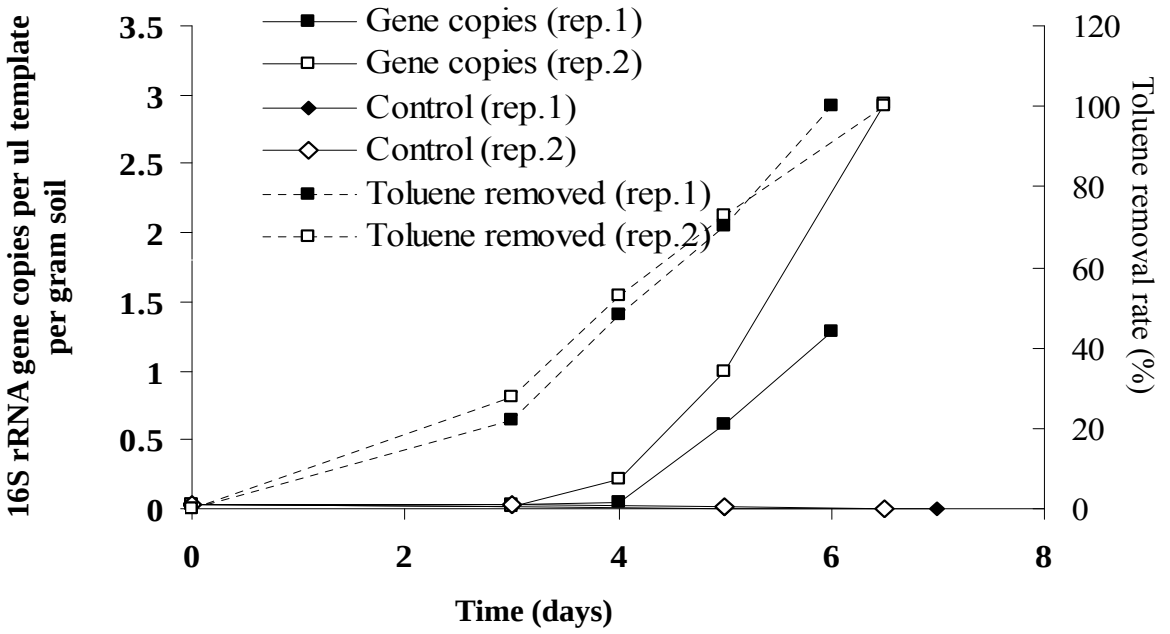

Figure 2.3. Correlation between *Polaromonas* sp. 16S rRNA gene copies (determined by qPCR) and toluene removal over time in live samples and no-toluene controls (microcosms constructed from soil 2).

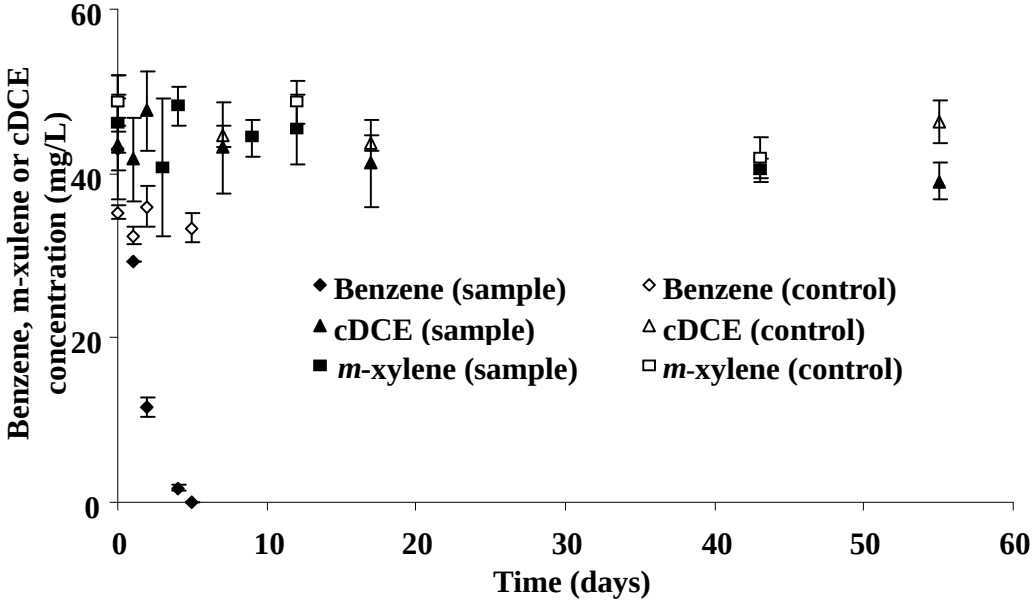

Figure 2.4. Benzene, *m*-xylene and *cis*-dichloroethene (cDCE) concentrations over time in supernatant of microcosms samples and in autoclaved controls (error bars represent standard deviations).

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CHAPTER 3

DIVERSITY OF TOLUENE DEGRADING MICROORGANIMS AND THE BSSA GENE IN FIVE NITRATE OR SULFATE AMENDED MICROBIAL COMMUNITIES INVESTIGATED USING STABLE ISOTOPE PROBING (SIP)

Introduction

Among the petroleum-related environmental contaminants, benzene, toluene, ethylbenzene and xylene (BTEX) are of particular concern because of their toxicity and easy migration in groundwater. Aerobic degradation of these chemicals is generally rapid (37, 53, 59, 60), however, because anaerobic conditions typically exist at contaminated sites, understanding anaerobic biodegradation is more relevant to site cleanup. Unfortunately, the fate of BTEX under anaerobic conditions is still difficult to predict because removal is slow (or non-existent), and therefore difficult to study, and the microorganisms responsible are still being identified. In addition, many factors, such as electron acceptor availability, substrate competition between potential indigenous biodegraders and co-contamination (e.g. ethanol), can affect anaerobic BTEX degradation. More knowledge on the diversity of degrading species in complex samples and the effect of electron acceptor availability on these species has the potential to enhance our understanding of the variability associated with anaerobic degradation.

From the BTEX contaminants, toluene degradation has been of great interest to many, with information available on pure and mixed cultures (based on the 16S rRNA gene) as well as the functional genes and enzymes involved. Toluene degradation has been observed over a range of electron accepting conditions with nitrate and sulfate being two important electron acceptors for

toluene degradation. Nitrate reduction may be an important mechanism for aromatic biodegradation because agricultural activity may result in high groundwater nitrate concentrations. In addition, relatively high levels of sulfate may occur naturally in some groundwater systems or may be added to enhance anaerobic degradation. A number of key toluene denitrifiers have been identified, affiliating within the genera of *Azoarcus, Aromatoleum, Magnetospirillum Pseudomonas, Dechloromonas* and *Thaurea* (5, 15, 23, 45, 48, 49, 54, 62). Similarly, microorganisms have also been linked to toluene degradation under sulfate reducing conditions, including, for example, microorganisms in the genera *Desulfobacula* (10), *Desulfocapsa* (39, 58), *Desulfotomaculum* (40), *Desulfotignum* (42), *Desulfovibrio* (3) and *Desulfosporosinus* (31, 57) (34).

Although numerous organisms have been linked to anaerobic toluene degradation in pure or mixed cultures, it is challenging to determine if these organisms are actually responsible for toluene degradation in complex samples. To address this, molecular methods have been developed to link function with identity in complex samples enabling a greater understanding of microbial communities involved in contaminant removal or other biological processes. A key molecular method for this has been stable isotope probing (SIP). This method has been used only recently to study anaerobic toluene degradation. To date, SIP has been applied in three different studies under sulfate reducing conditions. In 2010, SIP was used to identify the active toluene degraders in aquifer sediment from a former gasworks site in Germany (12, 41, 57), and also in a sulfate reducing consortium developed from a BTEX contaminated aquifer also in Germany (12, 41, 57). In 2011, SIP was also applied to contaminated sediment samples from Germany to identify active toluene degrading species (44).

In the current study, we expand on this knowledge by applying SIP to samples from a wider range of sources, including uncontaminated sites. SIP was applied over two electron accepting conditions (nitrate or sulfate amended) and time-series SIP (DNA extraction over time) was used to enable label cross-feeding between species to be investigated. SIP was applied to the ribosomal gene as well as the functional gene (*bssA* encoding for benzylsuccinate synthase) previously correlated to anaerobic toluene degradation. Benzylsuccinate synthase has been recognized as a key enzyme for anaerobic toluene biodegradation under nitrate reducing (2, 29), sulfate reducing (56-58), ferric iron reducing (13, 25), and methanogenic enrichment cultures or environmental samples (55, 56).

Here, the overall aim was to determine the diversity of active anaerobic toluene degraders and *bssA* genes across different habitats, both contaminated and uncontaminated samples, and compare these results to the current knowledge on pure cultures as well as previous SIP studies involving only contaminated site samples. This work represents the first study to use SIP to examine the diversity of active anaerobic toluene degraders across diverse sample sources.

Materials and Methods

Development of Toluene Degrading Microcosms

A wide range of inocula sources were investigated for toluene degrading potential. These sources included agricultural soils (MI), subsurface soil from BTEX-contaminated sites (MI), sediments from a former gas-compressor site (24) (OK), digester sludges from wastewater treatment plants

(MI) and anaerobic granular sludge (WA). Triplicates of ~10g (wet weight) were incubated in sterile 160 mL serum bottles containing 50 mL anaerobic basal media (61), sealed with rubber stoppers and aluminum seals. Microcosms were prepared under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratory Products INC, Grass Lake, MI). Potassium nitrate and magnesium sulfate were amended as electron acceptors. From approximately 38 incubations, only two nitrate-amended and three sulfate-amended microcosms exhibited toluene degradation and these were selected for the SIP experiments. The two active nitrate-amended microcosms were seeded from an agricultural soil (hereafter AgN) and anaerobic granular sludge from an UASB reactor in Washington (hereafter GSN). The three sulfate-amended toluene-degrading microcosms were inoculated from the same agricultural soil (hereafter AgS), contaminated sediments from a previous BTEX contaminated aquifer (hereafter CSS) (24) and digester sludge from a wastewater treatment plant in St. Clair, Michigan (hereafter DSS). For each microcosm, 3 g biomass (wet weight: enrichment cultures obtained in screening stage were inoculated for AgN, AgS and CSS; freshly sampled sludges were seeded for GSN and DSS) were anoxically incubated in 60 mL serum bottles containing 25 mL of anaerobic basal media as described above. Potassium nitrate and magnesium sulfate were added to a final concentration of 1 g L^{-1} NO_3^- and SO_4^{2-} . Each treatment involved triplicate abiotic controls, triplicate unlabeled toluene (1 μL, 99 %, Chem Service, West Chester, PA) and three triplicate labeled toluene (1 μL ring-^{13}C6 toluene, 99%, Cambridge Isotope Laboratories, Inc. Andover, MA) amended samples. These microcosms were incubated at room temperature (~20 °C) with reciprocal shaking.

Analytical Techniques

Toluene concentrations in headspace gas samples (200 μL) were typically determined weekly with a gas chromatograph (Perkin Elmer) equipped with flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53mm). Injector and detector temperature were set at 200 ^{o}C and the column temperature was 120^{o}C.

DNA Extraction and Ultracentrifugation

Microcosms were sacrificed for DNA extraction at various time points during toluene depletion for AgN, GSN, AgS and DSS to further understand the flow of carbon through these microbial communities. For CSS, an early time stage (50% toluene removal) was investigated due to limited availability of active microcosms. The powersoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) was used for total nucleic acids extraction from entire microcosms in each treatment according to the manufacturer's recommended procedure. Quantified DNA extracts (~10 μg) were loaded into Quick-Seal polyallomer tubes (13×51 mm, 5.1 ml, Beckman Coulter) along with a Tris-EDTA (TE, pH 8.0) /CsCl solution. Prior to sealing (cordless quick-seal tube topper, Beckman), the buoyant density (BD) was determined with a model AR200 digital refractometer (Leica Microsystems Inc) and adjusted by adding small volumes of CsCl solution or Tris-EDTA buffer with a final BD of 1.7300 mgL^{-1}. The tubes were centrifuged at 178,000 *g* (20 ºC) for 48 h in a Stepsaver 70 V6 Vertical Titanium Rotor (8 x 5.1 ml capacity) within a Sorvall WX 80 Ultra Series Centrifuge (Thermo Scientific). Following centrifugation, the tubes was placed onto a fraction recovery system (Beckman) and fractions (150 μl) were collected. The BD of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation.

PCR and TRFLP

The density-resolved fractions from ^{12}C and ^{13}C microcosms for each treatment were PCR-amplified using 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescine) and 1492R (5'-GGTTACCTTGTTACGACTT) (Operon Biotechnologies) as previously described (16). The presence of PCR products was confirmed by 1.5% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide. PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.), following the manufacturer's instructions and approximately 150 ng was digested with *HaeIII* (New England Biolabs) with a 6-hour incubation period. Additional digests (*HhaI, MseI, Bsp1286I, BsrBI* etc.) for TRFLP analyses in a number of heavy labeled fractions were included to correlate the TRFLP fragment lengths to the *in silico* cut sites of the cloned 16S rRNA gene sequences. DNA fragments were separated by capillary electrophoresis (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) at the Research Technology Support Facility (RTSF) at Michigan State University. Data were analyzed with GeneScan software (Applied Biosystems) and the percent abundance of each fragment was determined.

Presence of *bssA* in Microcosms and Enumeration in SIP Fractions

The presence of the benzylsuccinate synthase alpha-subunit gene (*bssA*) was investigated using different primers pairs (Table 3.1) on DNA extracted from each of the five treatments. A gradient PCR was performed with annealing temperature ranging from 45ºC to 58 ºC. Positive *bssA* amplicons were selected for cloning and sequencing (see below). Four treatments illustrated the

presence of partial *bssA* genes (AgN, GSN, DSS and CSS). For three of these (except CSS due to lack of sample), quantitative PCR (qPCR) was used to enumerate *bssA* gene copy numbers in gradient fractions. The assay was conducted in a Chromo 4 real-time PCR cycler (Bio-Rad) using the primer set 7772f/8546r for AgN and GSN, and SRBf/SRBr for DSS. Both ^{12}C and ^{13}C nucleic acid samples from density gradient fractions were subject to quantification. Each 20 μL PCR reaction mixture containing 10 μL SYBR green real-time PCR solution (Applied Biosystems), 0.25 μM of each primer, and 1 μL DNA template. The thermal protocol consisted of an initial denaturation (95 ºC, 15 min), 40 cycles of amplification (95 ºC, 15 s; 55 ºC, 20 s; 72 ºC 20 s) and a terminal extension step (72 ºC, 2 min). Melting curves were constructed from 55 ºC to 95 ºC, read every 0.6ºC for 2 s. For each gradient fraction, 1 μL solution was diluted with 3 μL water as template (to conserve the sample). Cloned plasmid DNA was utilized as a standard for quantification and gene copies were determined as shown below (plasmid size was 4730 bp, including 774 bp(7772f/8546r amplicons) and 97 bp(SRBf/SRBr amplicons) of insert).

$$\text{gene copies} = \left(\text{DNA concentration}, \frac{\text{ng}}{\mu\text{L}}\right) \times \left(\frac{1\text{g}}{1{,}000^3\,\text{ng}}\right) \times \left(\frac{1\,\text{mol bp DNA}}{660\text{g DNA}}\right) \times \left(\frac{6.023 \times 10^{23}\,\text{bp}}{\text{mol bp}}\right) \times \left(\frac{1\,\text{copy}}{\text{plasmid size [bp]}}\right) \times (\text{volume}, \mu\text{L})$$

Sequencing of Partial bssA and 16S rRNA Genes

Clone libraries of the 16S rRNA genes were constructed for each treatment using DNA extracted following toluene depletion. The DNA was amplified with 27F/1492R as above except the forward primer was unlabeled and the final extension time was extended to 15 minutes. To reduce sequencing redundancy, restriction fragment length polymorphism (RFLP) analyses was performed and specific operational taxonomic units (OTU) were selected for sequencing. In

addition to 16S rRNA sequencing, amplicons generated with *bssA* primer pairs were also prepared for cloning and sequencing. The PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.) and cloned into *Escherichia coli* TOP10 vector supplied with a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria-Bertani (LB) medium solidified with 15 g agar L^{-1} with 50 μg ampicillin L^{-1} for 16 h at 37 ºC. Colonies with inserts were verified by PCR with primers M13 F (5'-TGTAAAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.) and the insertions were sequenced at RTSF. The Ribosomal Database Project (RDP) (Center for Microbial Ecology, Michigan State University) analysis tool "classifier" was utilized to assign taxonomic identity. Phylogenetic trees for the partial *bssA* sequences along with the closest matches in Genbank were obtained by the neighbor-joining method using MEGA 4.1 software.

Results

Frequency of Toluene Degradation

Toluene degradation using various inocula sources, including agricultural soil, digester sludge, anaerobic granular sludge and contaminated soils and sediments, was examined under nitrate and sulfate amended conditions. Toluene biodegradation was observed in 2 from 18 and in 3 from 20 experiments set-ups, involving nitrate amendment and sulfate amendments, respectively. Active toluene microcosms were also tested for their benzene biodegradation, however, no biodegradation was found in any consortia. SIP was performed on the two nitrate amended (an agricultural soil and granular sludge) and on the three sulfate amended microcosms (an

agricultural soil, digester sludge and contaminated site soil). At various time points, DNA was extracted from the labeled and unlabeled toluene-amended microcosms, and was subject to ultracentrifugation, fractionation and TRFLP (on each fraction). The organisms responsible for ^{13}C assimilation were identified by the comparison of relative abundances of specific T-RFs between the control (unlabeled toluene amended) and the sample (labeled toluene amended) at selective time points for each fraction. The identities of each enriched T-RFs for each microcosm type were then determined using additional restriction enzyme digests and by comparison to predicted cut sites in each 16S rRNA gene clone library, as described below.

SIP on Agricultural Soil Nitrate Amended Microcosms (AgN)

In AgN, a 214 bp *HaeIII* T-RF fragment became the only dominant T-RF fragment (>80%) among labeled 'heavy' fractions (banding between 1.7523 to 1.7448 g ml^{-1}) in all three sampling points (33%, 75% and 100% toluene removal) (Figure 3.1 a). To identify the toluene-degrading bacteria based on the TRFLP results and to assign phylogenetic affiliation to distinct T-RFs, the16S rRNA clone library (Table 3.2) derived from total DNA was inspected. Three different *Burkholderiales*-related microorganisms classifying within the families *Alcaligenaceae*, *Comamonadaceae* and *Oxalobacteraceae* all exhibited a predicted *HaeIII* T-RF close to 214 bp. Specifically, from the analysis of the clone sequences, each had a predicted *Hae III* cut site of 219 bp (Table 3.2). Multiple digestions were then applied to determine which of these three sequences were actually responsible for the 214 bp *HaeIII* T-RF in the heavy fractions. From these digests (Table 3.3), the phylotype within the family *Comamonadaceae* were found to be directly correlated with carbon uptake from toluene and hence toluene degradation. Interestingly, the AgN

clone library (Table 3.2) contained a partial sequence related to the genus *Azoarcus*, which has previously been linked to toluene degradation under nitrate amended conditions (2, 8, 23, 63). However, no enrichment of the appropriate *Azoarcus* T-RF (77 bp) was noted in the heavy labeled fractions (Figure 3.1 b), indicated these organisms were likely not the major toluene degraders.

SIP on the Granular Sludge Nitrate Amended Microcosms (GSN)

Compared to AgN, the nitrate amended microcosms inoculated from anaerobic granular sludge exhibited a more diverse microbial community (Table 3.2). TRFLP revealed significant ^{13}C uptake for three T-RF (Figure 3.2), a fragment sized 72 bp dominated in labeled heavy fractions (BD> 1.7448 g ml^{-1}). Additional enzyme digestions (Table 3.4) in combination with the 16S rRNA clone library (Table 3.2) were utilized to further distinguish this *Hae III* T-RF. The putative toluene degrader classified within the genus *Thauera* (72 bp). *Thauera*-related phylotypes were strongly enriched in the heavy fractions in all three time points with relative abundances > 70% at first two sampling points and >34% at the last time point. The peak of relative abundance decreased over time, suggesting the ^{13}C-label assimilation by other microorganisms may have diluted the fraction of *Thauera*-related phylotypes in heavy fractions. The presence of *Thauera*-related *bssA* genes and the enrichment of these genes in ^{13}C heavy fractions (see below) as quantified by qPCR strengthens the hypothesis that the *Thauera* phylotype was responsible for toluene degradation in this complex microbial community.

SIP on the Agricultural Soil Sulfate Amended Microcosms (AgS)

In the sulfate amended agricultural soil microcosms (AgS), the majority (22 from 29 clones) of the microbial community classified within the class *Clostridia* (Table 3.5). Slight label assimilation was noted in two T-RFs in the DNA extracted from microcosms which consumed ~33% toluene, however, in the later two DNA extraction points, (~75% and ~100% toluene removal) label assimilation was more pronounced (Figure 3.3 a & b). Two TRFLP fragments, 77 bp and 213 bp, were enriched in the heavy ^{13}C-fractions during the course of biodegradation and no PCR products were found in the corresponding heavy ^{12}C-fractions. It is likely that both phylotypes were responsible for carbon uptake from toluene over the course of the incubations. The 16S rRNA clone library data (Table 3.5) in combination with additional restriction digests (Table 3.6) indicated that the two TRFLP fragments both affiliated with genus *Desulfosporosinus* (>98% sequence identity).

SIP on the Digester Sludge Sulfate Amended Microcosms (DSS)

In DSS, a significant proportion (20 from 59 clones) of the microbial community classified within the class *Deltaproteobacteria* (Table 3.5). The most abundant TRFLP fragment in ^{13}C-heavy fractions was a 204 bp T-RF. This fragment was highly enriched in heavy fractions at the three extraction times and the effect was most obvious for the last two time points (~75 % and ~100 % toluene depleted) (Figure 3.4). The maximum relative abundances of the 204 bp fragment was 27 % (at BD of 1.7360 g ml^{-1}), 43 % (at BD of 1.7480 g ml^{-1}) and 50 % (at BD of 1.7502 g ml^{-1}) for the three extraction points, indicating an increase in label uptake with time. In

contrast, the relative abundance of this fragment was less than 5 % in each unlabeled control gradient fraction (Figure 3.4). The BD values of these "heavy" peaks are very close to that of fully ^{13}C labeled *M. extorquens* (1.757 g ml^{-1})(36), indicating a high degree of label assimilation. The bacterial clone library generated (Table 3.5) indicated the 204 bp T-RF was assigned with family *Syntrophobacteraceae*. As described above, additional digestion on the heavy fractions were performed to confirm this correlation between 204 bp T-RF and the associated 16S rRNA sequence (Table 3.7).

SIP on Gas Compressor Site Soil Sulfate Amended Microcosms (CSS)

The third sulfate amended sample involved microcosms inoculated with sediment from a former gas compressor site (24). Previous research indicated sulfate was the major terminal electron acceptor at this site. The clone library for this treatment indicated the dominance (91 from 106 clones) of microorganisms classifying within the *Deltaproteobacteria* (orders *Desulfobacterales* and *Desulfuromonadales*). Unfortunately, because of sample limitations, SIP was performed only on an early toluene-degrading stage (~50% toluene removal). A 202 bp T-RF was enriched in the heavy ^{13}C- fractions (relative abundance as ~70% with a BD of 1.7469 g ml^{-1}). The clone library for this microbial community (Table 3.5) illustrated a dominance of *Desulfobulbaceae*-affiliated organisms (80 from 106 clones) which correlated with the 203 bp T-RF. Multiple enzyme digestions (Table 3.8) confirmed that the *Desulfobulbaceae*-affiliated 16S rRNA gene sequence was responsible for the enriched *Hae III* 203 bp T-RF. These data indicate the *Desulfobulbaceae* affiliated microorganisms were responsible for toluene degradation.

Partial Sequencing of the *bssA* Gene

A number of primers successfully amplified partial *bssA* genes from four of the five treatments (Table 3.9). Amplicons from AgN, GSN, DSS and CSS were selected for sequencing and phylogenetic trees were generated for each enrichment culture along with their closest matches in Genbank (Figure 3.6-3.9). Primer set 7772f/8546r, produced expected-size PCR products (~774 bp) within the two nitrate amended consortia (Figures 3.6 & 3.7). A total of 32 clones from agricultural soil and granular sludge nitrate amended enrichment cultures were digested and representative clones (as indicated by restriction digests) were selected for sequencing. All 64 clones showed the same OTU indicating sequence similarity, belonging to *Thauera*-related *bssA* genes (Figures 3.6 & 3.7). Primer pairs SRBf/SRBr displayed good coverage for the sulfate amended enrichment cultures (Table 3.9). Representative OTUs from the DSS and CSS were sequenced and three amplicons of each sample showed ~92% (90 bp out of 97 bp) similarity to partial sulfate-reducing bacterium PRTOL1 *bssA* gene (EU780921.1), which was congruent with the template sequences of the primer set. In addition, for CSS, longer partial *bssA* genes were obtained using primer pair 7772f/8828r and the sequences obtained were found to branch into two distinct lineages (Figure 3.9). Of these, 19 were closely related to *bssA* gene of strain TRM1 (99% sequence similarity)(58). Two clones were affiliated with uncultured bacterium clone Zz-ox_12 *bssA* gene, showing only 73% sequence similarity with the sulfate-reducing bacterium TRM1 *bssA* gene (22). Three clones could not be classified with any known *bssA* sequences. Unfortunately, unspecific PCR products or no PCR products were produced from the sulfate amended agricultural soil (AgS) from the primers tested (Table 3.1).

Quantification of *bssA* Genes in SIP Fractions

To further confirm anaerobic toluene degradation in the nitrate and sulfate amended samples, the *bssA* genes were quantified from ^{12}C and ^{13}C gradient fractions at the last time point using primer sets 7772f/8546r and SRBf/SRBr. Quantitative PCR analysis of gradient fractions detected separation of *bssA* genes in labeled and unlabeled samples (Figure 3.10, a-c). In GSN, quantitative label assimilation was very evident in the ^{13}C-fractions where bulk *bssA* gene moved to a heavier fraction with a BD of 1.7513 g mL^{-1}, compared to that of the ^{12}C-fractions (1.7208 g mL^{-1}) (Figure 3.10 a). In AgN, highly ^{13}C-labeled *bssA* genes was present at a BD of 1.7415 g mL^{-1}, while such 'heavy' *bssA* genes were not found in the ^{12}C-fractions but the peak occurred at a lighter fraction with a BD of 1.7099 g mL^{-1}. Also a tail of *bssA* gene formed in ^{13}C-lighter fractions with a BD of 1.7046 g mL^{-1} (Figure 3.10 b). In DSS, a separation of ^{12}C and ^{13}C peak was also seen (Figure 3.10 c). Quantitative PCR was not performed on the AgS fractions (no *bssA* primers were suitable for these microcosms) or the CSS fractions (limitation on sample available).

Discussion

Previous SIP studies on BTEX biodegradation have focused primarily on samples or biomass from former contaminated sites (20, 30, 57). To expand on this work and to discover novel toluene-degrading bacteria, the current study applied SIP to a wide range of inocula sources including agricultural soil, contaminated aquifer sediment and anaerobic granular and digester sludge. Noteably, the selection of inocula sources covers both uncontaminated and contaminated

sources, as well as natural and engineered microbial communities. These experiments involved the characterization of five toluene-degrading microbial consortia (through 16S rRNA clone libraries) and the identification of the active toluene degraders in each sample using time series DNA based SIP. For some of these samples, *bssA* gene sequences were also obtained and label uptake into the *bssA* gene was also documented.

Toluene biodegradation under nitrate reducing conditions has been studied extensively (4, 11, 14, 46). To the authors' knowledge, no detailed SIP study has not been conducted on nitrate-reducing, toluene-degrading mixed consortia. For the current study, a natural microbiota (agricultural soil) and an engineered microbial community (granular sludge) were investigated. These communities were targeted, in part, because it was likely that both had high levels of denitrifying microorganisms.

In the nitrate amended agricultural soil (AgN), microorganisms classifying within family *Comamonadaceae* were found to be responsible for toluene biodegradation. Although *Comamonadaceae*-related bacteria are often characterized as aerobic bacteria, others have reported members of the *Comamonadaceae* as denitrifying bacteria (17, 26). *Comamonadaceae*-related microorganisms have been correlated with cyclohexanol (38) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (26) biodegradation under nitrate reducing conditions. Depletion of nitrate was not measured in the current study, however, the following lines of evidence suggest the *Comamonadaceae*-related bacteria were degradating toluene under nitrate reducing conditions: 1) strict anaerobic methods were followed, 2) partial *bssA* genes were obtained from the micrososms and 3) the *bssA* genes were enriched in the heavy

fractions (qPCR data, Figure 10) indicating the *Comamonadaceae*-related bacteria (also enriched in the heavy fractions) were anaerobic (benzylsuccinate synthase is irreversibly inactivated in the presence of molecular oxygen (28)). These results are novel because the most commonly reported nitrate-reducing, toluene-degrading species (genera *Azoarcus, Thauera* and *Dechloromonas*) classify within another *Betaproteobacteria* family, the *Rhodocyclaceae*.

The AgN clone library (Table 3.2) also contained a 77 bp fragment belonging to genus *Azoarcus*, a common toluene degrader under nitrate reducing conditions (2, 8, 23, 63), however, no enrichment of this T-RF was noted in the heavy fractions, indicating these organisms were not responsible for ^{13}C-substrate assimilation. The enrichment of *bssA* and *Comamonadaceae* 16S rRNA genes in the heavy ^{13}C–fractions along with the presence of *Azoarcus* sp. in the clone library could indicate the *Comamonadaceae*-related biodegraders may have obtained the *Azoarcus bssA* gene by horizontal gene transfer. Horizontal gene transfer of such catabolic genes has been suggested or found previously (13, 48, 56, 58). These finding illustrates the importance of culture independent approaches, compared to culture dependent approaches, for understanding functions in mixed cultures.

In the nitrate amended, granular sludge treatments (GSN), a 72 bp T-RF associated with genus *Thauera* was enriched in all three time points. This is a reasonable conclusion because *Thauera* spp. have been reported by others to be an important toluene-degrading species under nitrate reducing conditions (6, 11, 32, 50). The toluene metabolic pathway in *Thauera* is known to be initiated by the formation of benzylsuccinate from toluene and fumarate(10). Sequencing of the

partial *bssA* genes revealed that all 32 clones were closely related with *Thauera* spp. *bssA* gene. The presence of *Thauera*-related *bssA* genes and the enrichment of these genes in ^{13}C heavy fractions as quantified by qPCR strengthen the hypothesis that the *Thauera* phylotype was responsible for toluene degradation in this complex microbial community.

In the sulfate amended agricultural soil (AgS), two T-RFs (77 bp and 213 bp), both belonging to the genus *Desulfosporosinus* exhibited strong label assimilation at the last two sampling points (Figures 3.3 a & b). The genus *Desulfosporosinus* has previously been linked to toluene degradation (31, 47). It was also identified as being able to assimilate ^{13}C-toluene in a recent SIP project (57). Since no other T-RFs are significantly enriched in the labeled heavy fractions over the three time points, it is likely that the *Desulfosporosinus* spp. were responsible for toluene degradation in the AgS microcosms.

In the sulfate-amended, digester sludge treatment (DSS), a 204 bp T-RF, representing a phylotype within the family *Syntrophobacteraceae*, was the only T-RF dominated in the ^{13}C-heavy fractions. The label assimilation of this T-RF intensified over time (Figure 3.4). This effect was most prominent at the last sampling point with more than 50 % relative abundance in the ^{13}C-heavy fractions. Notably, a 77-bp T-RF affiliated within the genus *Desulfovibrio*, was slightly enriched in the ^{13}C-heavy fractions (abundance around or less than 10%). The *Desulfovibrio* spp. may have been able to scavenge labelled metabolic by-products from the *Syntrophobacteraceae* related microorganisms. Members of *Syntrophobacteraceae* have previously been identified as sulfate reducing bacteria (18, 35) and can degrade long chain fatty

acids (51, 52) and propionate (7) under sulfate-reducing conditions. The closest relatives of the 204 bp T-RF with a validly published name is *Syntrophobacter wolinii* (19), sharing 91% sequence similarity. *S. wolinii* has been reported as a sulfate-reducing bacterium and its closest relatives are *Desulfomonile tiedjei* and *Desulfoarculus baarsii*. The 204 bp T-RF also shared a more distant similarity (88% 16S rRNA similarity) with sulfate reducing strain PRTOL1, a toluene sulfate reducer isolated from fuel-contaminated subsurface soil (9, 10). *Syntrophobacteraceae* were identified in a toluene-degrading sulfate-reducing bacterial consortium, but in that study, the *Desulfobulbaceae* were identified as key organisms of toluene degradation within the consortium (41). In another study, carbon stable isotope analysis in combination with whole-cell hybridization linked toluene degradation to the *Desulfobacter*-like populations, while *Synthrophobacter* was present in the microbiota but not responsible for toluene biodegradation (43). The *Syntrophobacteraceae* was also observed in a benzene-degrading *in situ* microcosm but was not linked with benzene biodegradation (21). To date, to the authors' knowledge, there has been no direct evidence to correlate members of the family of *Syntrophobacteraceae* with anaerobic toluene biodegradation. The presence of *bssA* genes similar to sulfate reducing strain PRTOL1-related *bssA* genes (Figure 3.8) in DNA extracted from the digester sludge treatment also confirmed the hypothesis that the novel *Syntrophobacteraceae* clade played an active role in toluene biodegradation in these sulfate amended samples.

The third sulfate amended sample involved microcosms inoculated with material originating from a former gas compressor site (CSS). SIP was only performed at one time point (~50% toluene removal), however, the 16S rRNA gene and *bssA* gene clone libraries support the SIP

results. Microorganisms classifying within the *Desulfobulbaceae* family appeared to be dominant in the ^{13}C-heavy fractions and in the clone library. *Desulfobulbaceae* have previously been classified as BTEX sulfate reducers (1, 27, 30, 33, 41). They were also reported in the DNA-SIP project described above (57) but were not identified as the primary toluene degrader. Interestingly, *Desulfosporosinus*-related microorganisms (identified as primary toluene degraders in AgS) were present in the CSS clone library (Table 5) but were not enriched in the ^{13}C-heavy fractions.

In summary, five distinct phylotypes were identified as the active toluene-degrading bacteria under either nitrate or sulfate amended conditions for five different microbial communities. For one microcosm type, syntrophic partners were also identified. For three treatments, the phylotypes were similar to previously identified toluene degraders (*Thauera, Desulfosporosinus* and *Desulfobulbaceae* related phylotypes), whereas two treatments produced novel toluene degraders (*Comamonadaceae* and *Syntrophobacteraceae* related phylotypes). The discovery of two novel toluene degraders indicates the importance of culture independent approaches for identifying the active microorganisms in complex samples. In addition, this study provided information on the diversity of *bssA* sequences and the utility of a number of primer pairs for detecting the *bssA* gene. Further, the work highlights the value of combining ribosomal and functional gene based SIP to link function with identity for complex microbial samples and adds to our understanding of the microbial ecology of toluene degrading communities from various environments.

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Tables and figures

Table 3.1. Primers used in this study to investigate the presence of the *bssA* gene

Primer	Sequence(5'-3')	Reference
6888f	AATTCATCGTCGGCTACCACG	(Winderl et al. 2007)
7772f	GACATGACCGACGCSATYCT	(Winderl et al. 2007)
8546r	TCGTCGTCRTTGCCCCAYTT	(Winderl et al. 2007)
8828r	AGCAGRTTGSCCTTCTGGTT	(Winderl et al. 2007)
SRBf	GTSCCCATGATGCGCAGC	(Beller et al. 2008)
SRBr	CGACATTGAACTGCACGTGRTCG	(Beller et al. 2008)
bssApd2f	CCTATGCGACGAGTAAGGTT	(Winderl et al. 2008)
bssApd2r	TGATAGCAACCATGGAATTG	(Winderl et al. 2008)
bssN2f	GGCTATCCGTCGATCAAGAA	(Winderl et al. 2008)
bssN2r	GTTGCTGAGCGTGATTTCAA	(Winderl et al. 2008)
bssAf	ACGACGGYGGCATTTCTC	(Beller et al. 2002)
bssAr	GCATGATSGGYACCGACA	(Beller et al. 2002)

Table 3.2. Phylogenetic affiliation of each 16S rRNA clone in nitrate amended toluene degrading microcosms as determined with the RDP analysis tool "classifier"

Soil/Fragment (*Hae III* digestion)	Enriched in ^{13}C heavy fractions	# of clones	Class	Order	Genus
Agricultural Soil, NO_3^- Amended					
39 bp	No	20	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonas*
219 bp	Yes	7	*Betaproteobacteria*	*Burkholderiales*	Unclassified
77 bp	No	8	*Betaproteobacteria*	*Rhodocyclales*	*Azoarcus*
219 bp	No	5	*Betaproteobacteria*	*Burkholderiales*	Unclassified
41 bp	No	4	*Gammaproteobacteria*	*Xanthomonadales*	*Dokdonella*
219 bp	No	3	*Betaproteobacteria*	*Burkholderiales*	Unclassified
39 bp	No	1	*Gammaproteobacteria*	*Xanthomonadales*	*Luteimonas*
250 bp	No	1	*Sphingobacteria*	*Sphingobacteriales*	*Ferruginibacter*
250 bp	No	1	*Clostridia*	*Clostridiales*	*Sedimentibacter*
269 bp	No	1	*Acidobacteria_Gp3*		*Gp3*
315 bp	No	1	*Betaproteobacteria*	*Burkholderiales*	*Polaromonas*
Granular Sludge, NO_3^- Amended					
77 bp	Yes	25	*Betaproteobacteria*	*Rhodocyclales*	*Thauera*
77 bp	Yes	14	*Deltaproteobacteria*	*Syntrophobacterales*	*Smithella*
217 bp	Yes	12	*Betaproteobacteria*	*Burkholderiales*	*Simplicispira*
82 bp, 205 bp, 271 bp	No	8	*Deltaproteobacteria*	*Syntrophobacterales*	Unclassified
260 bp	No	8	*Bacteroidia*	*Bacteroidales*	
39 bp	No	7	*Flavobacteria*	*Flavobacteriales*	
226 bp	No	7	*Epsilonproteobacteria*	*Campylobacterales*	*Arcobacter*
180 bp	No	5	*Nitrospira*	*Nitrospirales*	*Magnetobacterium*
180 bp, 245 bp	No	5	*Planctomycetia*	*Planctomycetales*	*Planctomyces*
39 bp	No	4	*Bacteroidia*	*Flavobacteria*	Unclassified
260 bp	No	4	*Bacteroidia*	*Bacteroidales*	Unclassified
318 bp	No	4	*Clostridia*	*Clostridiales*	*Fusibacter*

Table 3.2 (cont'd)

951 bp	No	4	*Epsilonproteobacteria*	*Campylobacterales*	*Sulfurospirillum*
189 bp	No	2	*Synergistia*	*Synergistales*	Unclassified
191 bp	No	2			OP3
37 bp	No	1	*Flavobacteria*	*Flavobacteriales*	Unclassified
202 bp	No	1	*Thermotogae*	*Thermotogales*	*Kosmotoga*
318 bp	No	1	*Clostridia*	*Clostridiales*	*Fusibacter*
951bp	No	1	*Epsilonproteobacteria*	*Campylobacterales*	*Sulfurospirillum*

Table 3.3. Comparison of fragment length of dominant T-RF in heavy fractions to predicted fragment length from *in silico* sequence analyses (nitrate amended agricultural soil microcosms)

	Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Comamonadaceae affiliated sequence	*Hae III*	214	219
	Msp I	485	490
	Hha I	205	207
	RSA I	424	429
	Mse I	534	538
	BsrBi	211	216

Table 3.4. Comparison of fragment length of dominant T-RFs in heavy fractions to predicted fragment lengths from *in silico* sequence analyses (nitrate amended granular sludge microcosms).

	Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Enriched fragment 1 *Thauera* affiliated sequence	*Hae III*	72	77
	Msp I	77	82
	Hha I	204	210
	RSA I	470	476
	Mse I	537	541
	BSP 1286 I	119	123
Enriched fragment 2 *Smithella* affiliated sequence	*Hae III*	73	77
	Msp I	508	507
	Hha I	89	95
	RSA I	240	244
	BSP 1286 I	625	623
Enriched fragment 3 *Simplicispira* affiliated sequence	*Hae III*	213	217
	Msp I	489	488
	Hha I	205	205
	RSA I	472	471
	Mse I	537	536

Table 3.5. Phylogenetic affiliation of each 16S rRNA clone in sulfate amended toluene degrading microcosms as determined with the RDP analysis tool "classifier"

Soil/Fragment	Enriched in heavy ^{13}C fractions	#of clones	Class	Order	Genus
Agricultural Soil, SO_4^{2-} Amended					
		2			
82bp,214 bp	Yes	5	*Clostridia*	*Clostridiales*	*Desulfosporosinus*
252 bp	No	4	*Gammaproteobacteria*	*Pseudomonadales*	*Acinetobacter*
39 bp	No	1	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonas*
223 bp	No	1	*Bacilli*	*Bacillales*	*Paenibacillus*
228 bp	No	1	*Clostridia*	*Clostridiales*	*Gracilibacter*
228 bp	No	1	*Clostridia*	*Clostridiales*	*Gracilibacter*
249 bp	No	1	*Clostridia*	*Clostridiales*	*Sedimentibacter*
262 bp	No	1	*Sphingobacteria*	*Sphingobacteriales*	*Toxothrix*
299 bp	No	1	*Clostridia*	*Clostridiales*	*Proteiniborus*
316 bp	No	1	*Clostridia*	*Clostridiales*	*Centipeda*
Digester Sludge, SO_4^{2-} Amended					
		1			
77bp, 209bp	Yes	0	*Deltaproteobacteria*	*Syntrophobacterales*	Unclassified
		1			
77bp, 214bp	No	0	*Deltaproteobacteria*	*Deltaproteobacteria*	Unclassified
258bp, 298 bp	No	9	*Bacteroidia*	*Bacteroidales*	
203bp	No	8	*Thermotogae*	*Thermotogales*	*Kosmotoga*
238bp, 299bp	No	6	*Caldisericia*	*Caldisericales*	*Caldisericum*
220 bp	No	4	*Anaerolineae*	*Anaerolineales*	Unclassified
914 bp	No	4			Bacterium Eub 3
324 bp	No	3			OP9
214 bp	No	2	*Spirochaetes*	*Spirochaetales*	Unclassified
267 bp	No	2	*Clostridia*	*Clostridiales*	Unclassified

Table 3.5 (cont'd)					
251 bp	No	1	*Planctomycetia*	*Planctomycetales*	*Planctomyces*
Contaminated Soil, SO_4^{2-} Amended					
		8			
205 bp	Yes	0	*Deltaproteobacteria*	*Desulfobacterales*	Unclassified
209 bp, 275 bp	No	6	*Deltaproteobacteria*	*Desulfobacterales*	Unclassified
206 bp	No	5	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacter*
220 bp	No	5	*Actinobacteria*	Unclassified	
206 bp	No	4	*Clostridia*	*Clostridiales*	
318 bp	No	3	*Clostridia*	*Clostridiales*	*Fusibacter*
171 bp	No	1	*Nitrospira*	*Nitrospirales*	*Magnetobacterium*
214 bp	No	1	*Clostridia*	*Clostridiales*	*Desulfosporosinus*
220 bp	No	1	*Clostridia*	*Clostridiales*	*Acetivibrio*

Table 3.6. Comparison of fragment length of dominant T-RF in heavy fractions to predicted fragment lengths from *in silico* sequence analyses (sulfate amended agricultural soil microcosms).

	Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Desulfosporosinus affiliated sequence	*HaeIII*	213	216
	MspI	136	140
	BsrBI	52	56
	MseII	178	180

	Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Desulfosporosinus affiliated sequence	*HaeIII*	77	82
	MspI	53	62
	BsrBI	N/A	N/A
	MseII	223	227

Table 3.7. Comparison of fragment length of dominant T-RF in heavy fractions to predicted fragment lengths from *in silico* sequence analyses (digester sludge sulfate amended microcosms).

	Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Syntrophobacteraceae affiliated sequence	*HaeIII*	204	209
	MspI	160	168
	MseII	626	628
	RsaI	238	230
	HhaI	91	99

Table 3.8. Comparison of fragment length of dominant T-RF in heavy fractions to predicted fragment lengths from *in silico* sequence analyses (sulfate amended contaminated site soil microcosms).

		Restriction Enzyme	TRFLP (bp)	Sequence Data (bp)
Desulfobulbaceae	affiliated sequence	*HaeIII*	202	205
		MspI	158	162
		RsaI	222	226
		HhaI	92	93

Table 3.9. Success (+) or failure (-) of primers sets for *bssA* gene amplification.

Inocula sources	Forward Reverse	bssAf bssAr	SRBf SRBr	BssN2f BssN2r	7772f 8546r	6888f 8546r	7772f 8828r
Granular sludge (nitrate amended)		-	-	-	+(cloned)	+	+
Agricultural soil (nitrate amended)		-	-	-	+(cloned)	+	+
Agricultural soil (sulfate amended)		-	-	-	-	-	-
Digester sludge (sulfate amended)		-	+(cloned)	-	-	-	-
Contaminated soil (sulfate amended)		-	+(cloned)	-	-	-	+(cloned)

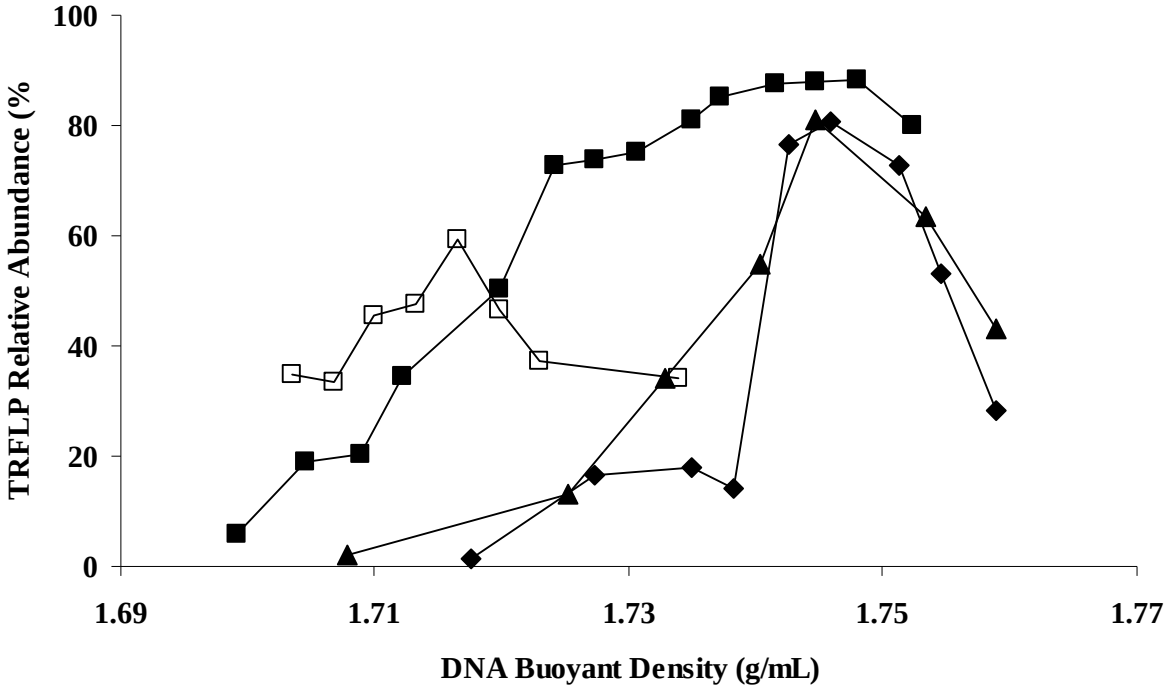

Figure 3. 1. Percent relative abundance of fragments (digested by Hae III) assigned to Comamonadaceae within buoyant density gradients of DNA extracted from the nitrate amended agricultural soil microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

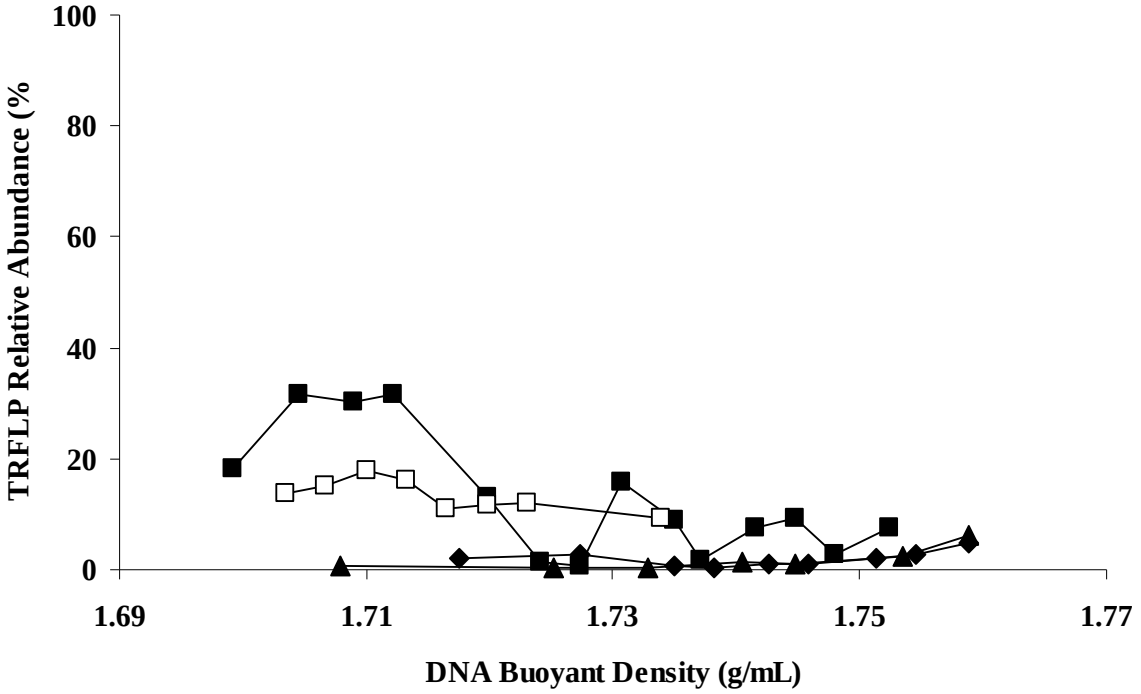

Figure 3.2. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Azoarcus* within buoyant density gradients of DNA extracted from the nitrate amended agricultural soil microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

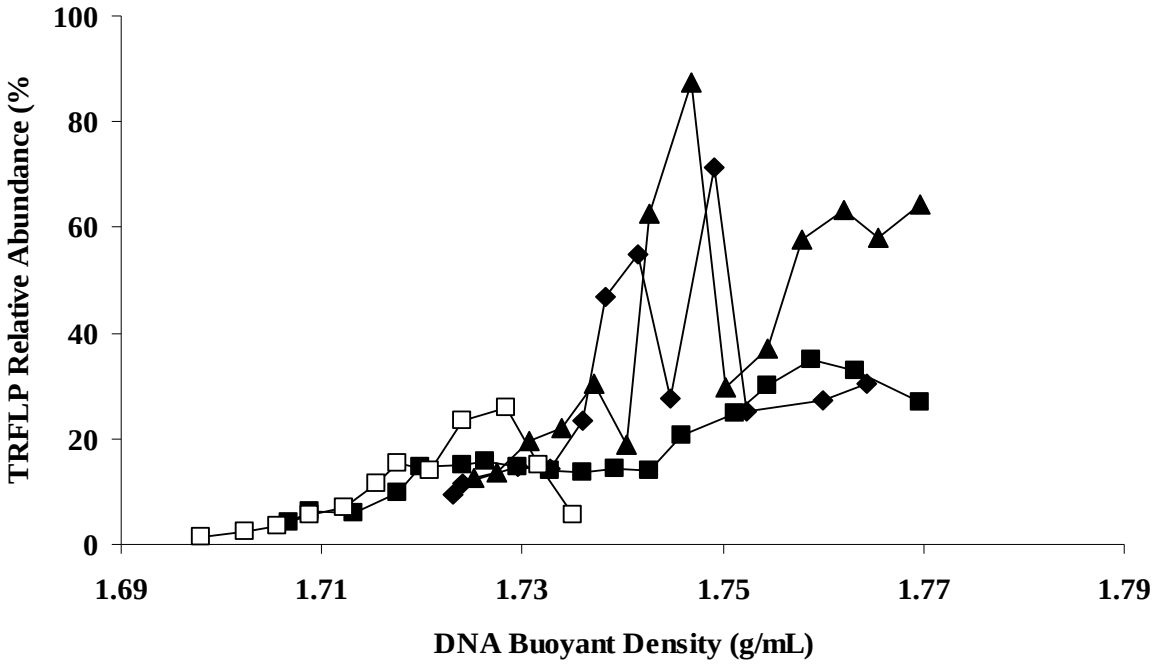

Figure 3.3. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Thauera*, within buoyant density gradients of DNA extracted from the nitrate amended granular sludge microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

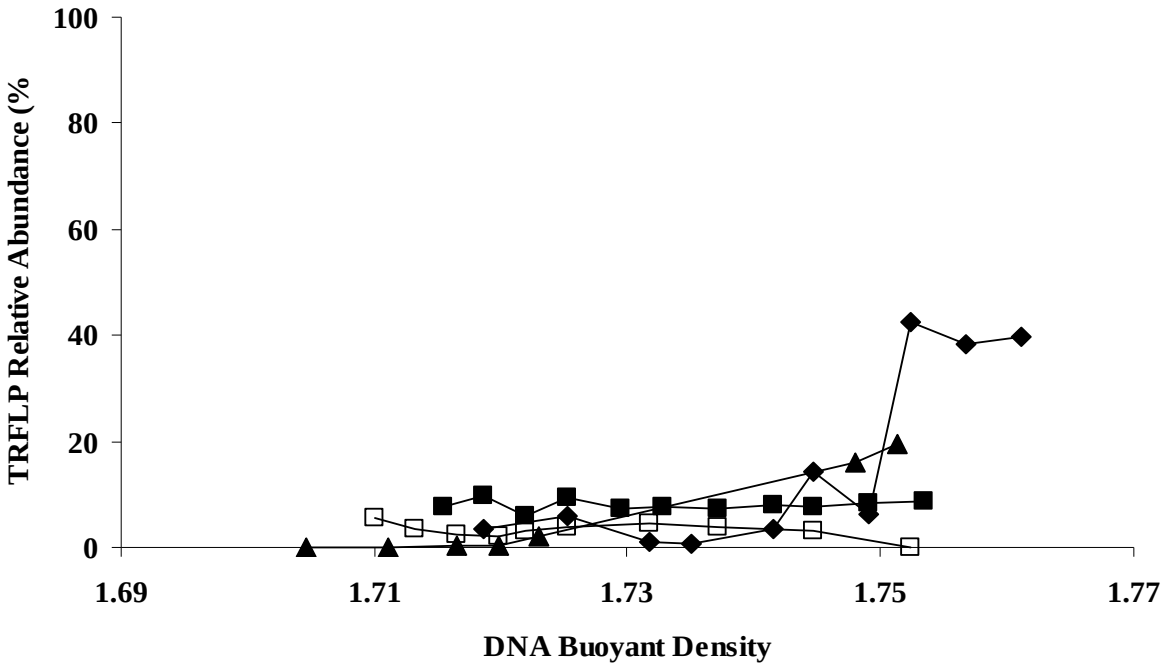

Figure 3.4. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Desulfosporosinus* with T-RF 77 bp within buoyant density gradients of DNA extracted from the agricultural soil sulfate amended microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

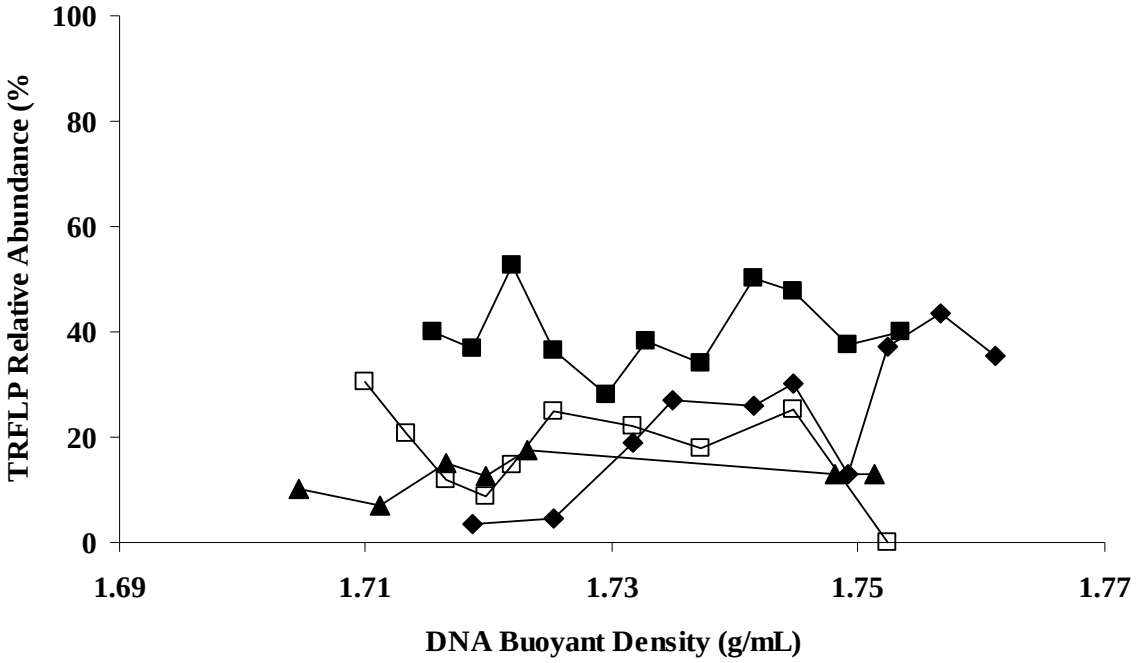

Figure 3.5. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Desulfosporosinus* with T-RF 213 bp within buoyant density gradients of DNA extracted from the agricultural soil sulfate amended microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

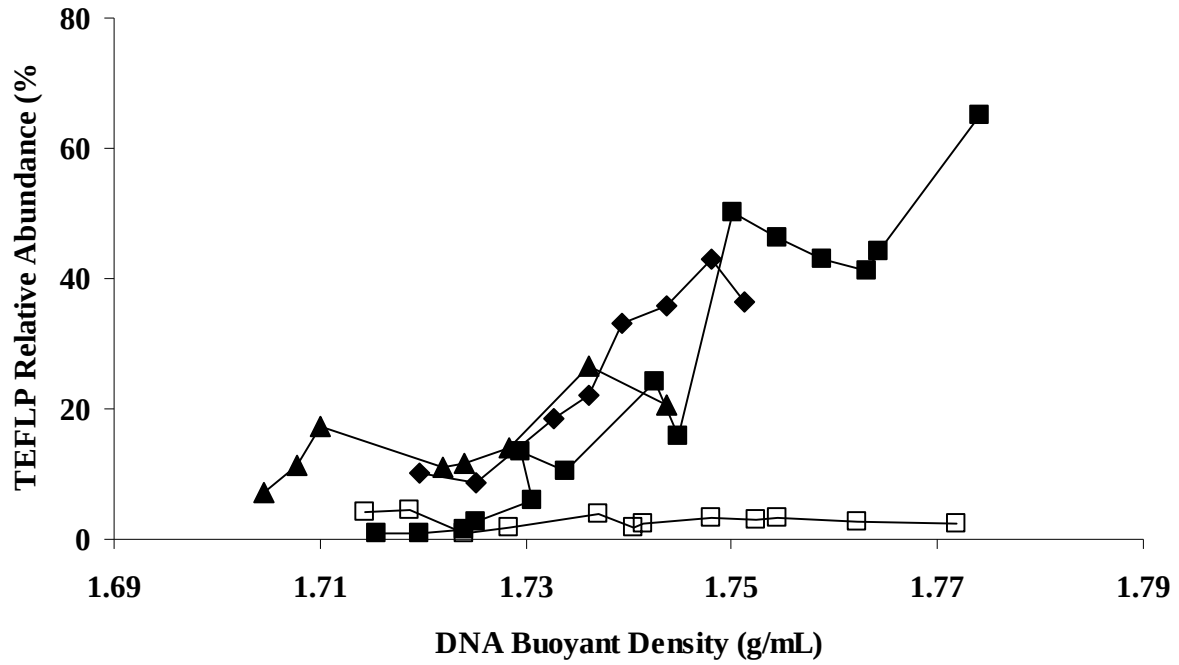

Figure 3.6. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Syntrophobacteraceae* within buoyant density gradients of DNA extracted from the digester sludge sulfate amended microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

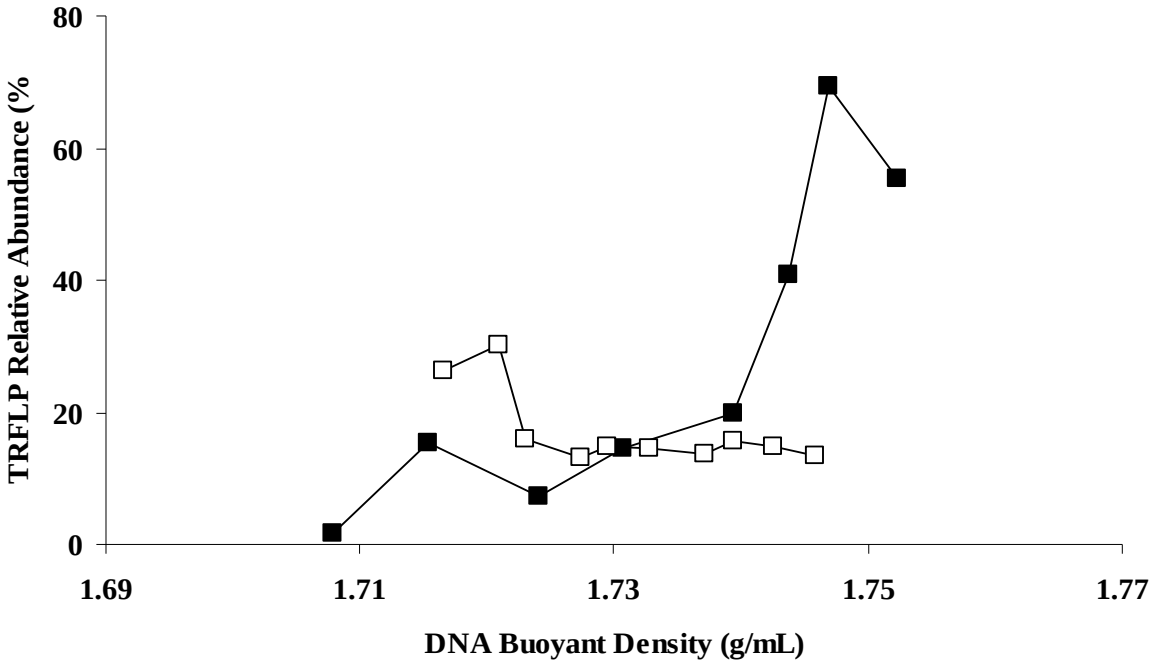

Figure 3.7. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Desulfobulbaceae* within buoyant density gradients of DNA extracted from the contaminated site, sulfate amended microcosms. Figure symbols: ▲ ^{13}C-toluene (~33% toluene degraded); ♦ ^{13}C-toluene (~75% toluene degraded); ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

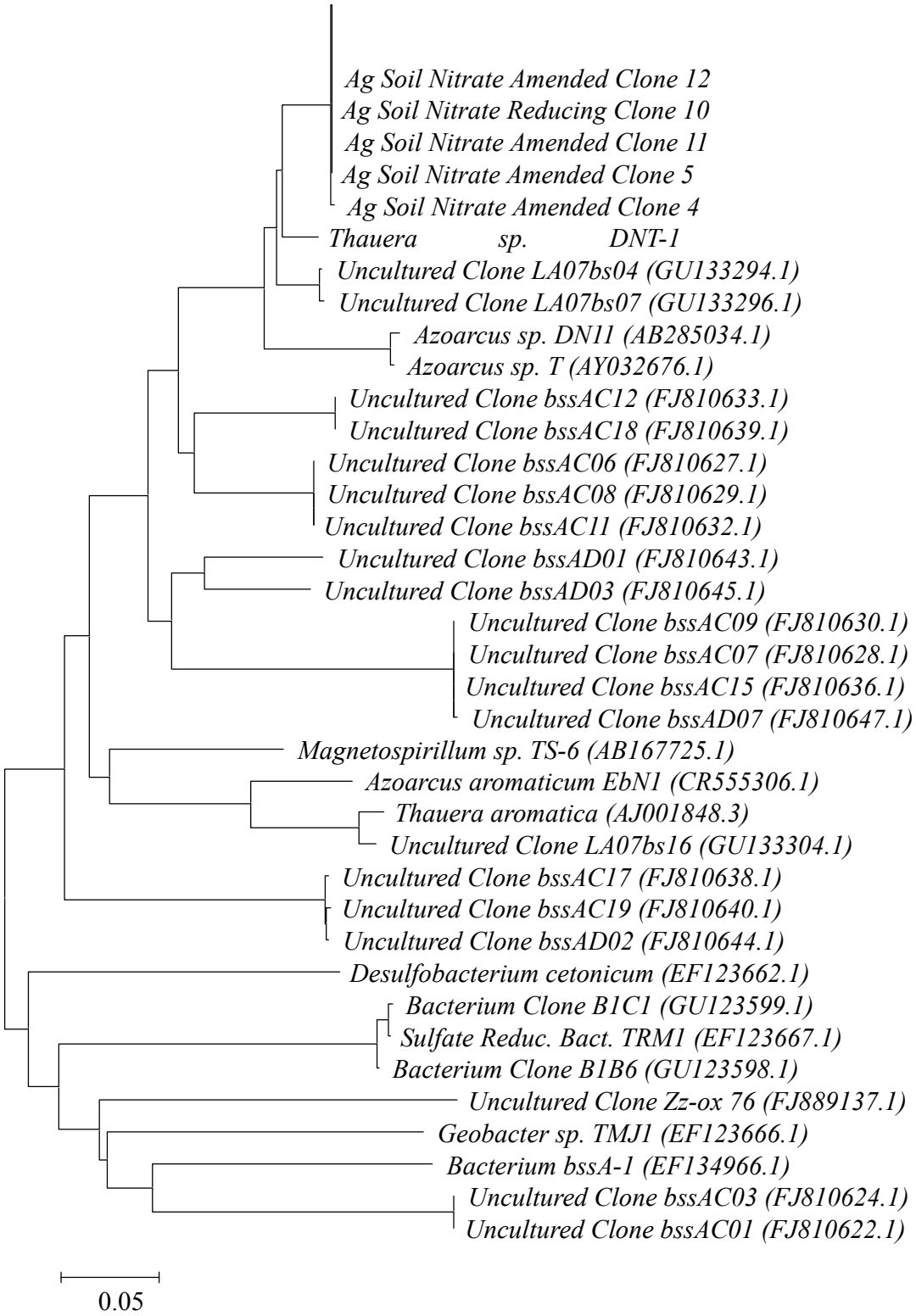

Figure 3.8. Phylogenetic tree of *bssA* partial sequences (722 bp) from nitrate amended agricultural soil microcosms (using the primer set 7772f/8546r) along with the closest matches in GenBank, constructed with MEGA 4.1 software using the neighbor-joining method.

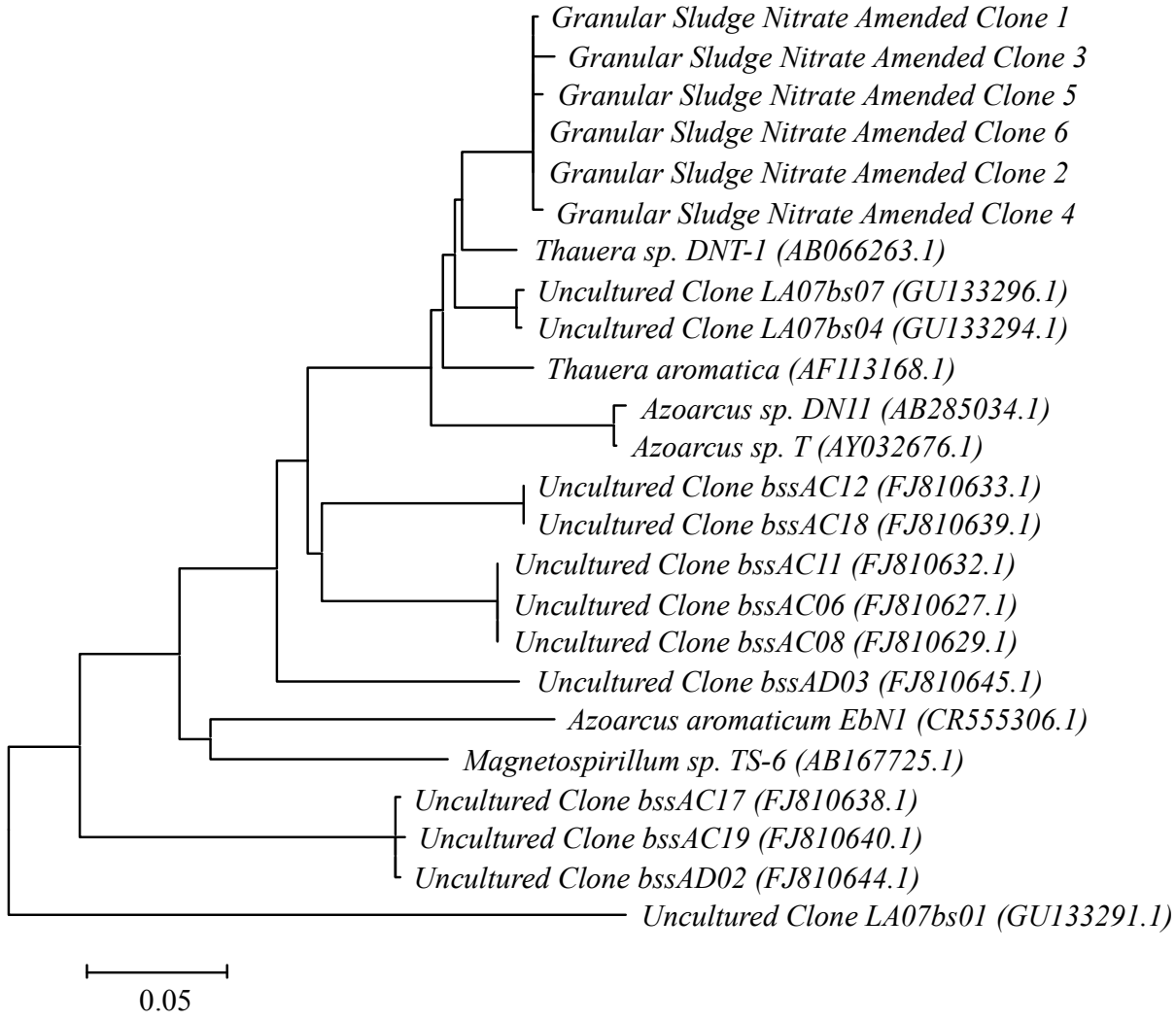

Figure 3.9. Phylogenetic tree of *bssA* partial sequences (722 bp) from nitrate amended granular sludge microcosms (using the primer set 7772f/8546r) along with the closest matches in GenBank, constructed with MEGA 4.1 software using the neighbor-joining method.

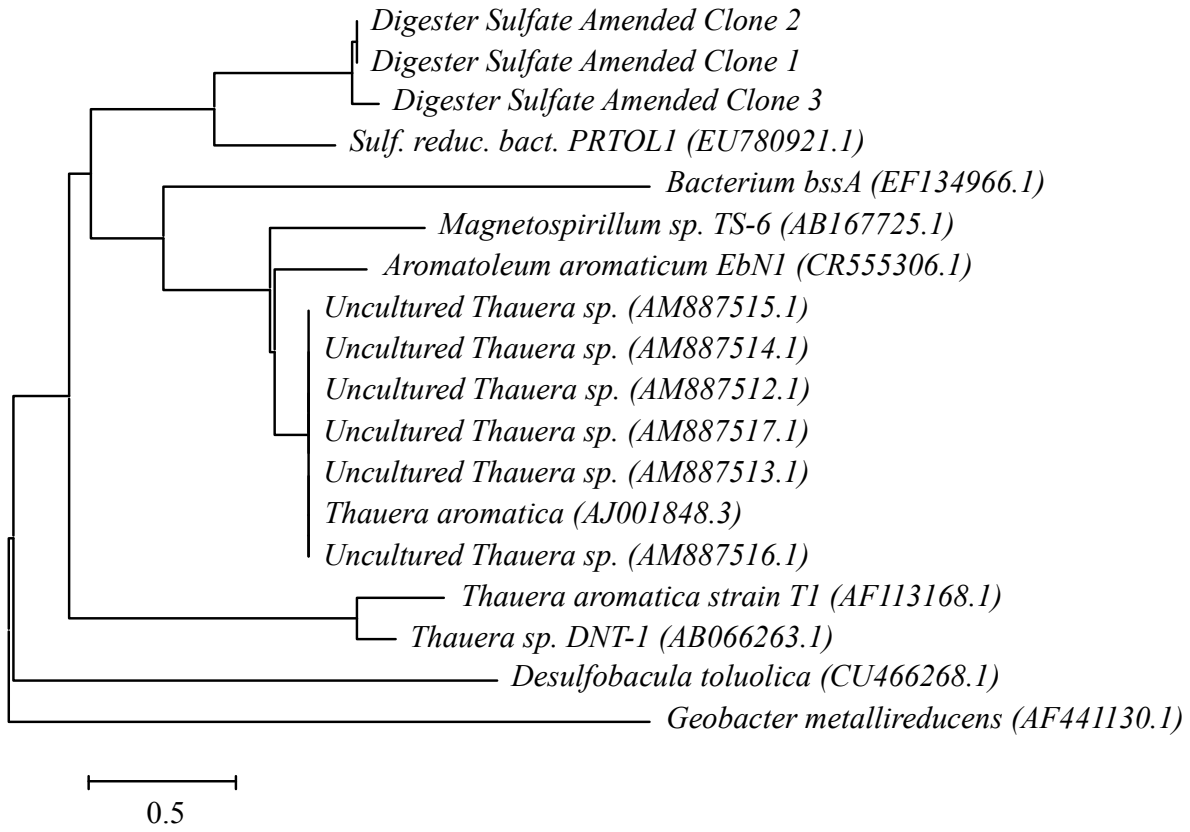

Figure 3. 10. Phylogenetic trees of *bssA* partial sequences (97 bp) from sulfate amended digester sludge microcosms (using the primer set SRBf/SRBr) along with the closest matches in GenBank, constructed with MEGA 4.1 software using the neighbor-joining method.

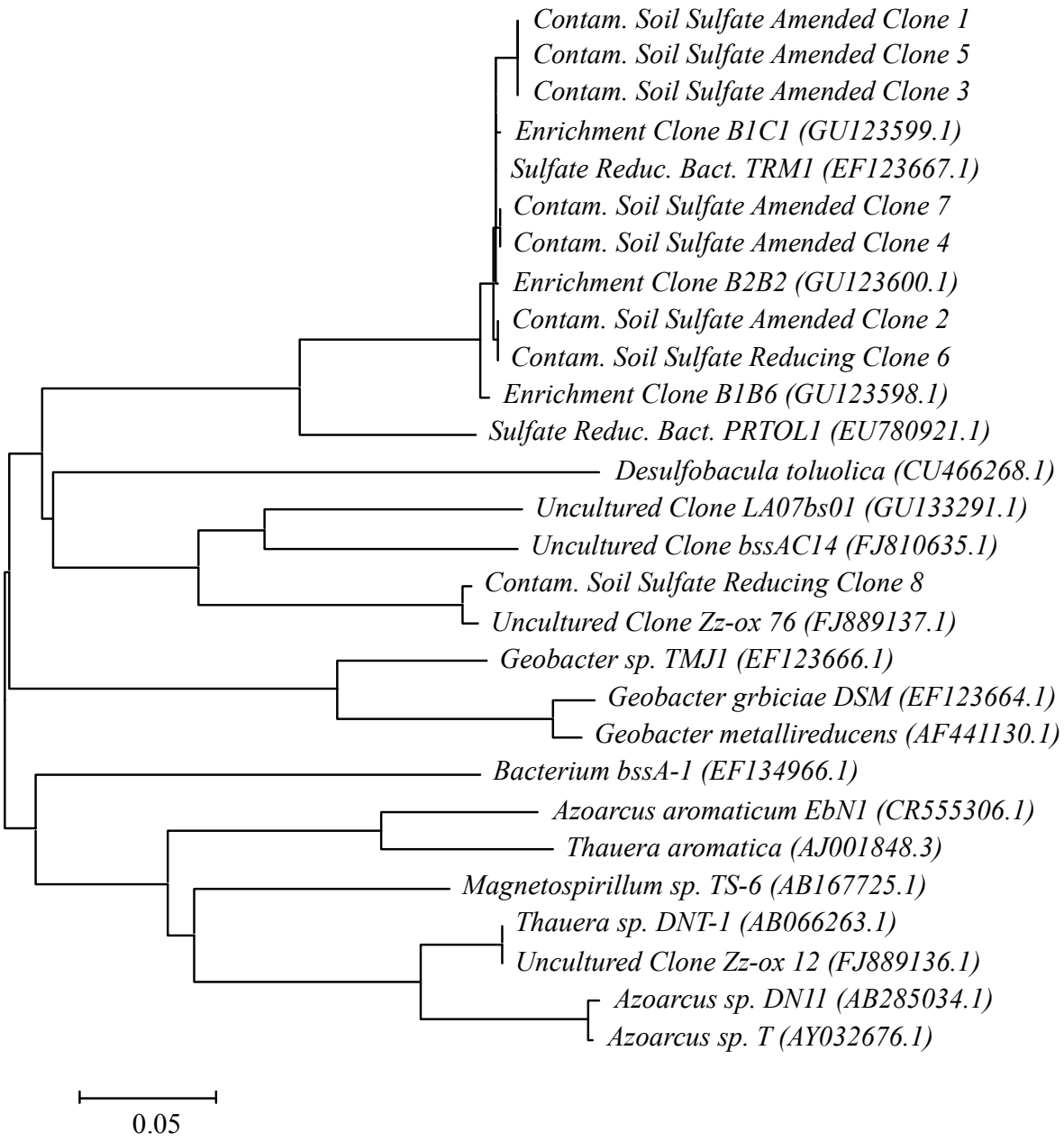

Figure 3.11. Phylogenetic trees of *bssA* partial sequences (637 bp) from sulfate amended contaminated soil microcosms (using the primer set 7772f/8828r) along with the closest matches in GenBank, constructed with MEGA 4.1 software using the neighbor-joining method.

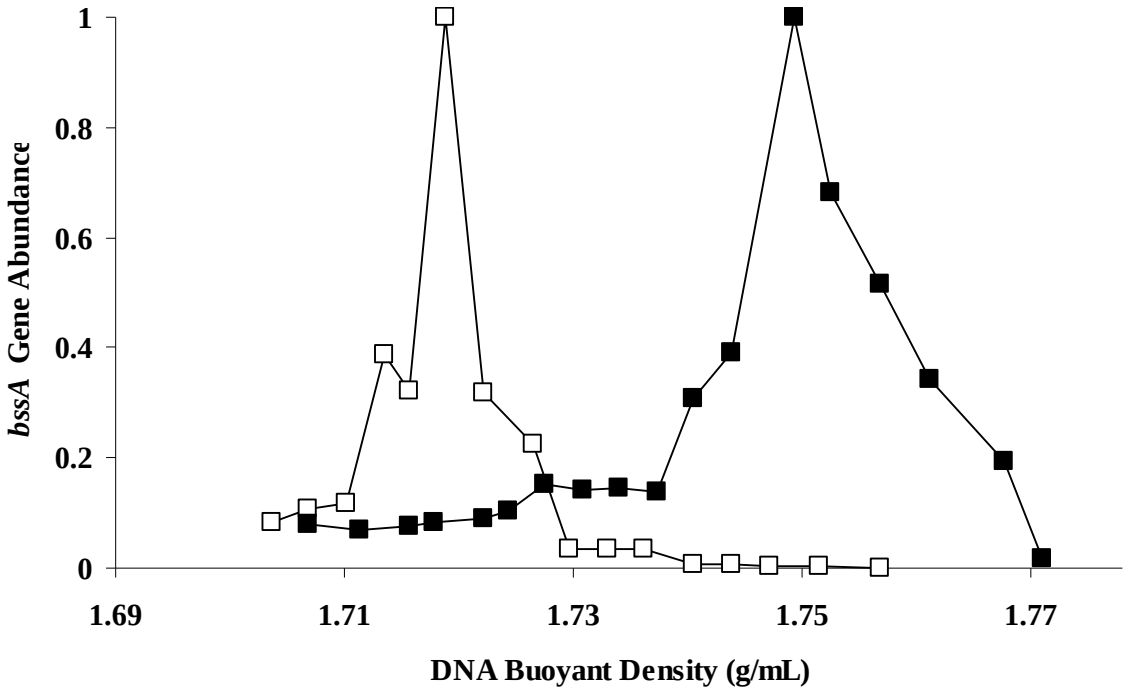

Figure 3.12. Difference between abundance of *bssA* gene copies in ultracentrifugation fractions from labeled (^{13}C toluene) and unlabeled toluene amended microcosms from the granular sludge nitrate amended microcosms as determined via qPCR. Figure symbols: ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

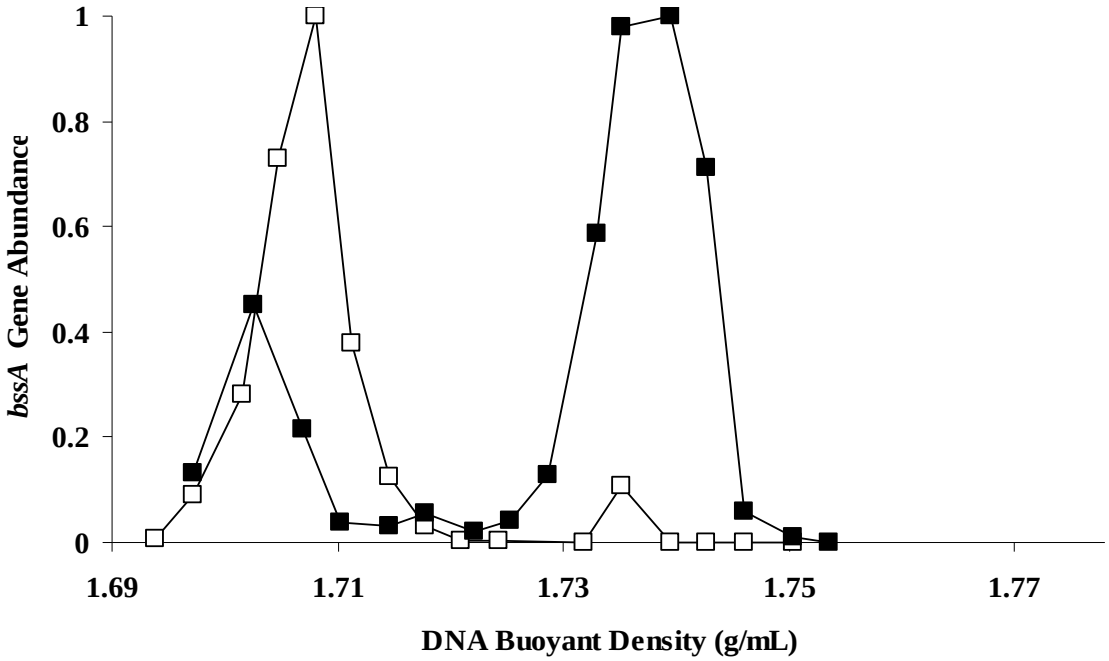

Figure 3.13. Difference between abundance of *bssA* gene copies in ultracentrifugation fractions from labeled (^{13}C toluene) and unlabeled toluene amended microcosms from the agricultural soil nitrate amended microcosms as determined via qPCR. Figure symbols: ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

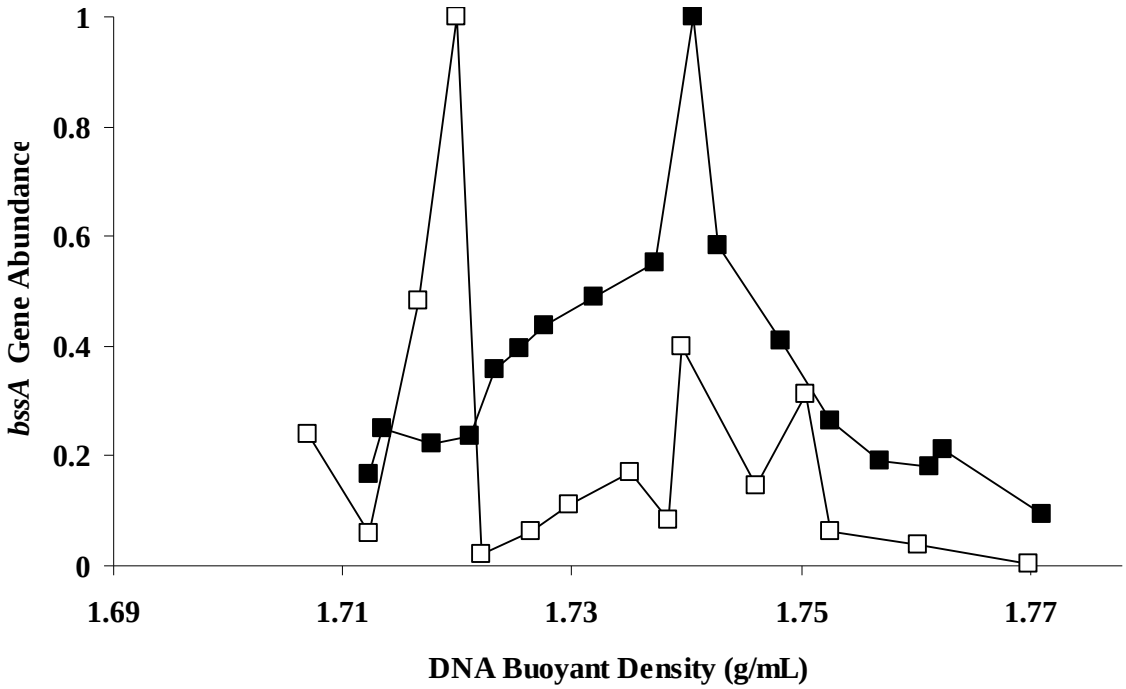

Figure 3.14. Difference between abundance of *bssA* gene copies in ultracentrifugation fractions from labeled (^{13}C toluene) and unlabeled toluene amended microcosms from the digester sludge sulfate amended microcosms as determined via qPCR. Figure symbols: ■ ^{13}C-toluene (~100% toluene degraded); □ ^{12}C-toluene (~100% toluene degraded).

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CHAPTER 4

PRESENCE,DIVERSITY AND THE ENUMERATION OF TOLUENE DEGRADING FUNCTIONAL GENES (*BSSA* AND *BAMA*) ACROSS A RANGE OF REDOX CONDITIONS AND INOCULUM SOURCES

Introduction

An understanding of the biodegradation pathways for aromatic compounds is important for the remediation of many contaminated environments. Biodegradation under anaerobic conditions is especially critical to comprehend because under these conditions, which are typical at many contaminated aquifers, removal rates are slow and therefore time for site closure may be prolonged. A common approach for understanding biodegradation pathways involves an analysis of the microbial community at the molecular level. Although an investigation into 16S rRNA genes can provide useful indicator data, it is now well recognized that more information can be gained by studying key functional genes. Recent research in this area involves the design and application of molecular assays targeted to the benzoyl-CoA degradation pathway to investigate the diversity of microorganisms involved in anaerobic aromatic biodegradation (9, 10, 14).

Anaerobic aromatic biodegradation typically involves the channeling of aromatic growth substrates to the central intermediate benzoyl-coenzyme A (CoA) prior to dearomatization and ring cleavage(9). In *Thauera aromatica*, the metabolism of benzoyl-CoA comprises of several steps with ring cleavage action by 6-oxocylcohex-1-ene-1-carbonyl-CoA (6-OCH-CoA) hydrolase, which likely catalyzes the transformation of 6-OCH-CoA to 6-hydroxypimelyl-CoA (9, 11). Enzymes similar to those in the benzoyl-CoA pathway in *T. aromatica* are reported to be present in most other aromatic degrading facultative anaerobes(5, 12). The ring-cleaving

hydrolase of the benzoyl-CoA pathway is encoded by the *bamA* gene. Recently, this pathway was studied in obligate anaerobes that use aromatic growth substrates (9). The researchers expressed genes putatively encoding for 6-OCH-CoA hydrolases from the obligately anaerobic *Geobacter metallireducens* and *Syntrophus aciditrophicus* in *Escherichia coli* and identified the products as 6-OCH-CoA hydrolases and found these genes to be highly conserved in all anaerobic bacteria using aromatic growth substrates (9). The researchers also designed primers to amplify the gene of 6-OCH-CoA in a number or obligate and facultative anaerobes (*G. metallireducens* GS-15, *T. aromatic* K172, *Azoarcus* EbN1, *A. evansii* KB740, *Desulfococcus multivorans*, *S. aciditrophicus* SB, *Magnetospirillum* species TS-6 and CC-26) as well as from two sediment-free sulfate reducing mixed cultures degrading toluene or *m*-xylene.

To date, there have only been two applications of the primers developed from this first study, the *bamA* assay was modified and used to investigate *bamA* gene sequences in two recent field studies(10, 14). One study investigated the diversity of *bamA* sequences in microcosms incubated at benzene contaminated aquifers (10). In the other study, the authors investigated the diversity of mono-aromate-degrading microorganisms by targeting the functional genes encoding benzylsuccinate synthase α-subunit (*bssA*) as well as *bamA* (14). Benzylsuccinate synthase is a key enzyme for anaerobic toluene biodegradation under nitrate reducing (1, 8), sulfate reducing (18-20), ferric iron reducing (4, 7), and methanogenic enrichment cultures or environmental samples (17, 18). In this second field study, the site was a leachate contaminated aquifer near the Banisveld landfill (the Netherlands) under iron reducing conditions. The work involved analysis of groundwater samples along a pollution plume in 1999 and 2004, which enabled the researchers to correlate site conditions (e.g. ferrous iron concentrations and dissolved organic

matter) with sequence diversity.

The overall objective of the current study was to expand on the three *bamA* studies to further investigate the diversity of *bamA* sequences across different redox conditions and inoculum sources. For this, we studied a number of toluene-degrading mixed microbial communities seeded from a wide range of sources, including contaminated soil from different sites, uncontaminated soil, anaerobic granular and digester sludge and aerobic activated sludge. The work is novel because it is the first to provide an in depth investigation of *bamA* diversity in microcosms over a range of redox conditions and inoculum sources. In addition, quantitative PCR (qPCR) assays to both *bamA* and *bssA* were developed and applied to a sub-set of these samples. In summary, this research involved the following specific objectives 1) to determine the diversity of *bamA* sequences in toluene degrading microcosms over a range of redox conditions, 2) to investigate the diversity of *bamA* sequences in microcosms from different sources including contaminated and non-contaminated sites and 3) to determine if qPCR targeted to the *bamA* and *bssA* genes could provide a reliable indicator for growth related toluene degradation.

Materials and Methods

Screening of Toluene Degrading Microcosms

A wide range of inoculum sources were examined for toluene biodegradation. The inocula were taken from pristine agricultural soils (MI), previous gasoline contaminated soils (MI), sediments from a former gas-compressor site (6) (OK), digester sludges from two wastewater treatment plants (MI), activated sludge (MI) and anaerobic granular sludge (WA). Triplicates of ~10g (wet

weight) biomass were incubated in sterile 160 mL serum bottles containing 50 mL anaerobic basal media (21), sealed with rubber stoppers and aluminum seals. Microcosms were prepared under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratory Products INC, Grass Lake, MI). Potassium nitrate and magnesium sulfate were added to a final concentration of 1 g L^{-1} NO_3^- and SO_4^{2-}.From approximately fifty three incubations, six nitrate-amended, five sulfate-amended and five methane generating (methanogenic) microcosms exhibited toluene degradation and these were selected for DNA extraction. In addition, three nitrate amended, one sulfate amended and two methanogenic microcosms were selected for qPCR. In this experimental set, triplicate microcosms were set up for each treatment. For each microcosm, 3 g biomass was anoxically incubated in 60 mL serum bottles containing 25 mL of anaerobic basal media as described above. The microcosm inoculum sources and the experiments conducted on each microcosm type are summarized in table 3.1. Toluene concentrations in headspace gas samples (200 μL) were typically determined weekly with a gas chromatograph (Perkin Elmer) equipped with flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53mm). Injector and detector temperature were set at 200 ^oC and the column temperature was 120^oC.

DNA Extraction and PCR

For the functional gene diversity studies (see below), microcosms exhibiting toluene biodegradation were sacrificed for DNA extraction when toluene was depleted in each microcosm. For the qPCR studies, DNA was extracted from samples (0.3g soil, wet weight) sacrificed at successive time points (typically 0, 50, and 100%) to enumerate the *bamA* genes and

bssA genes during the course of toluene depletion. The powersoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) was used for total nucleic acids extraction according to the manufacturer's recommended procedure. The presence of the benzylsuccinate synthase alpha-subunit gene (*bssA*) and 6-oxocyclohex-1-ene-1-carBoN1yl-CoA hydrolase (*bamA*) was investigated with a number of previously reported primers pairs (Table 4.1). A gradient PCR was performed with annealing temperature ranging from 45ºC to 58 ºC. The presence of PCR amplicons were determined using gel electrophoresis. A number of positive amplicons were selected for cloning and sequencing (see below).

Quantitative PCR

Three nitrate amended consortia, one sulfate amended consortium and two methanogenic consortia were selected for enumerating *bssA* and *bamA* genes during toluene biodegradation (Table 4.1). The quantification assays were conducted in a Chromo 4 real-time PCR cycler (Bio-Rad) using the primer sets 7772f/8546r (AgN, GSN, BoN11, HaM, ASM), 7772f/8828r (DUKE), (*bssA*) or Bam-sp9 and Bam-asp1 (*bamA*) (Table 4.1). Each 20 µL PCR reaction mixture containing 10 µL SYBR green real-time PCR solution (Applied Biosystems), 0.25 µM of each primer, and 1 µL DNA template. The thermal protocol consisted of an initial denaturation (95 ºC, 15 min), 40 cycles of amplification (95 ºC, 15 s; 55 ºC, 20 s; 72 ºC 20 s) and a terminal extension step (72 ºC, 2 min). Melting curves were constructed from 55 ºC to 95 ºC, read every 0.6ºC for 2 s. For each gradient fraction, 1 µL solution was diluted with 3 µL water as template (to conserve the sample). Cloned plasmid DNA was utilized as a standard for quantification.

Sequencing of Partial *bssA* and *bamA* Genes

Representative *bssA* and *bamA* amplicons were prepared for cloning and sequencing. The PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.) and cloned into *Escherichia coli* TOP10 vector supplied with a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria-Bertani (LB) medium solidified with 15 g agar L^{-1} with 50 μg ampicillin L^{-1} for 16 h at 37 ºC. Colonies with inserts were verified by PCR with primers M13 F (5'-TGTAAAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.) and the insertions were sequenced at RTSF. Phylogenetic trees for the partial *bamA* and *bssA* sequences along with the closest matches in Genbank were obtained by the neighbor-joining method using MEGA 4.1 software.

Results

This research builds on a previous study that involved a community analysis of five anaerobic toluene degrading consortia. In that research, the active toluene degraders were identified using stable isotope probing. The putative toluene degraders were classified within the genus *Thauera* (nitrate amended, GSN), the family *Comamonadaceae* (nitrate amended, AgN), the genus *Desulfosporosinus* (sulfate amended AgS), the family *Syntrophobacteraceae* (sulfate amended, DSS) and the family *Desulfobulbaceae* (sulfate amended, DUKE). In addition, partial *bssA* sequences were obtained from four of the five consortia. The current study expands on this research to investigate a larger number of toluene degrading samples (sixteen), includes methanogenic microcosms, targets the presence and diversity of the *bamA* gene and

quantitatively investigates the presence of the *bamA* and *bssA* genes in six of these samples.

bssA and *bamA* Gene Amplification

Primer sets 7772f/8546r(20) and bssAf/bssAr(2) produced strong specific amplicons for all six and for five of the six (except DSN) of the nitrate amended microcosms, respectively. The three other *bssA* primer pairs produced strong amplicons in only a small number of these microcosms (Table 4.1). In the sulfate amended microcosms, SRBf/SRBr(3) produced strong specific amplicons in four of the five (except BoS1) tested microcosms. Again, other primer pairs produced specific amplicons in only a small number of the microcosms. The primer set 7772f/8828r(20) produced a strong specific amplicon for DUKE and was therefore used for the qPCR assay on this sample. In the methanogenic microcosms, 7772f/8546f(20) performed well in two (HaM and ASM) of the five microcosms tested and was used for the qPCR assays. Only one other primer pair (bss1500f/bss2100r(13)) produced a strong amplicon in the methanogenic microcosms, and this was true for only one of the five methanogenic microcosms (SM). Overall, the success of *bssA* gene amplification was greatest in the nitrate amended microcosms and least in the methanogenic microcosms. This reflects the limited *bssA* sequence information (and therefore primers) on toluene degradation under methanogenic conditions compared to under nitrate reducing conditions.

A number of the partial *bssA* sequences amplified were cloned and sequenced. This work is an extension of a previous study on these samples. Previously, we obtained partial *bssA* sequences from four of these samples (AgN, GSN, DSS and DUKE). The partial *bssA* sequences from the nitrate amended microcosms (AgN and GSN) were found to be most similar to the *bssA* gene

from *Thauera* sp. DNT-1. In contrast, the majority (except one) of the previously obtained *bssA* sequences from the sulfate amended samples (DSS and DUKE) were most similar to the *bssA* sequence from sulfate reducing bacterium PRTOL1. One sequence from the contaminated site soil (DUKE) classified closest to an uncultured clone (Zz-ox 76) submitted to GenBank from an unpublished study entitled characterization of anaerobic xylene biodegradation by two dimensional isotope fractionation analysis. The current study adds to this growing database on *bssA* gene diversity. Specifically, *bssA* sequences were determined from two additional nitrate amended microcosms constructed from another agricultural soil (BoN11) and from digester sludge (DSN). Also, partial *bssA* sequences were obtained from methanogenic microcosms constructed from agricultural soil (HaM) or contaminated soil (SM).

The results regarding the amplification of *bamA* displayed a higher level of success. The primer pair (bam-sp9 and Bam-asp1(9)) targeting the *bamA* gene produced strong specific amplicons in all toluene-degrading microcosms tested except for one (contaminated soil, SM, methanogenic microcosm). To examine the diversity of the *bamA* gene, amplicons were sequenced from six of these samples (GSN, BoN1, DSS, DUKE, HaM and ASM). These samples were selected to represent two of each redox condition. The sequences obtained were compared to the GenBank database.

***bssA* and *bamA* Gene Quantification**

Quantitative PCR was used to examine the applicability of targeting both functional genes as a potential rapid screening method for toluene degradation potential. Microcosms from each redox condition were selected for qPCR, three for nitrate amended samples (AgN, GSN, BoN1), one

for sulfate amended samples (DUKE) and two for the methanogenic samples (HaM and ASM). In each case, the number of both genes increased over time as toluene was degraded (Figure 4.1-4.6).

In the nitrate amended samples (Figure 4.1-4.3), DNA was extracted three or three times during the toluene degradation period. For each experiment, a clear increase in *bssA* and *bamA* gene numbers was seen and the raise occurred simultaneously with the course of toluene degradation. The gene numbers of the *bssA* in GSN, AgN and BoN1 were approximately 145, 10200 and 400 times higher than those in the original sampling point. Similarly, the gene numbers of *bamA* were 3.62, 394 and 236 higher at the end compared to the beginning of the study for GSN, AgN and BoN1, respectively. In the one sulfate amended (DUKE) and two methanogenic (ASM and HaM) microcosms (Figure 4.4-4.6), DNA was extracted two times during the toluene degradation period. In all three, gene numbers of *bssA* and *bamA* increased with time as toluene was degraded. Interestingly, the final gene number for both genes differed considerably between the range of redox conditions. In the nitrate amended samples, the final *bssA* gene numbers were between 10^8 and 10^{10} per gram of soil. Whereas in the sulfate amended and methanogenic microcosms, *bssA* gene numbers were between 10^7 and 10^8 per gram of soil. A similar trend was noted for the *bamA* gene. In the nitrate amended microcosms, final *bamA* gene numbers were in the range of 10^8 and 10^9 per gram of soil. In the sulfate amended and methanogenic microcosms, *bamA* gene numbers were between 10^7 and 10^9 per gram of soil. These patterns suggest the toluene degrading population is higher under nitrate amended conditions.

Discussion

The partial *bssA* and *bamA* sequences obtained in the current study provide an interesting picture of the diversity of these sequences across redox conditions and different environments. Although a significant number of partial *bssA* sequences are available in the literature, only three studies have investigated the *bamA* gene (9, 10, 14). Here, we combine an investigation into both genes, investigating their diversity across samples types. In addition, we provide data to indicate qPCR targeted to these genes correlated well with toluene biodegradation. These results indicate the qPCR assay has the potential for providing evidence of toluene degradation potential at contaminated sites.

The study illustrated the difficulty associated with amplifying the *bssA* gene in sulfate amended and methanogenic toluene degrading microcosms. As stated above, we previously obtained four partial *bssA* sequences from nitrate and sulfate amended samples. In the current study, we expand on this and identified four additional *bssA* gene sequences. These sequences were obtained from two nitrate amended microcosms, one constructed from agricultural soil (BoN1) and the other from digester sludge (DSN). In addition, two partial sequences were obtained from methanogenic microcosms, one concentrated from agricultural soil (HaM) and the other from a contaminated site (SM). The agricultural soil (BoN1) under nitrate amended conditions contained two different partial *bssA* sequences with one being similar to *Thauera* sp. DNT-1 (87%). Interestingly, the other illustrated a high degree of similarity (83% identity) to *bssA* clones obtained from a study involving sulfate reducing toluene degradation from a tar-oil-contaminated aquifer at a former coal gasification plant (19). In the other nitrate amended samples (DSN), only one clone was found and this had the highest similarity to *Thauera*

aromatica (82%), a clone (clone LA07bs16) from the sulfate reducing study (81%) and *Aromatoleum aromaticum* EbN1 (80%). For the methanogenic microcosms, only one dominant sequence was obtained and these illustrated the greatest similarity to the clones described above from the study on toluene degradation from a tar-oil-contaminated aquifer (85% and 83% for HaM and SM, respectively). Several of these sequences were previously referred to as the "F2"-cluster *bssA* (19).

In contrast to the difficultly in amplifying *bssA*, amplifying *bamA* was highly successful in all except one toluene degrading microcosm (methanogenic SM). These results were unexpected, because previous studies (10, 14) modified the primer pair originally proposed in 2008 (9) and used in the current study. As discussed above, only three studies have investigated the *bamA* gene in relation to aromatic degradation. The first manuscript developed the primer pair bam-sp9 and bam-asp (9) and these primers were modified for use in the two field studies (10, 14). One study investigated the diversity of *bamA* sequences in microcosms incubated at benzene contaminated aquifers (10). The other involved a landfill leachate contaminated aquifer under iron reducing conditions (14). These two field studies are discussed below, followed by a comparison between the *bamA* sequences obtained in the current study and those found in the three previous studies.

The *bamA* assays used on DNA from the benzene contaminated sites involve the primers designed previously (9) as well as the development of two additional reverse primers (to produce ~700 and ~800-bp amplicons) to amplify the GMT (*Geobacter, Magnetospirillum* and *Thauera*) cluster and the SA (Gram-negative/Gram positive sulfate reducing bacteria and *Syntrophus,*

Azoarcus and *Aromatoleum*) cluster. The PCR assays were performed on *in situ* microcosms incubated at two different tar oil and BTEX contaminated anoxic aquifers (called Gneisenau and benzene production plant or BPP). The Gneisenau site had low concentrations of organics and previous work had demonstrated the potential for benzene and toluene degradation (15). The site had elevated concentration of total dissolved iron, indicating Fe (III) to Fe (II) reduction (10). At this site, one *bamA* clone showed the highest sequence identity to *Geobacter metallireducens* (88%). Five other clones had the highest similarity to *bamA* from *Geobacter daltonii* (85-88%). Interestingly, the authors did not obtain any appropriate amplicons using the SA cluster targeting *bamA* assay. The BPP site had a high concentration of benzene and the total dissolved iron concentrations indicated Fe (III) reduction. Again the authors used both *bamA* assays. Using the SA cluster targeting *bamA* assay, they found 8 clones with highest similarities to *bamA* genes from species of the genera *Azoarcus* and *Aromatoleum* (75-78% identity). Using the GMT cluster targeting *bamA* assay, they found two sequences similar to *bamA* sequences from species of the genera *Magnetospirillum* and *Thauera* (80% identity).

The other field study involved a leachate contaminated aquifer near the Banisveld landfill (the Netherlands) under iron reducing conditions (14). In this case, both the *bamA* and *bssA* genes were investigated. This field study produced the most extensive collection of *bamA* sequences to date. The sequences obtained were placed into seven clades: *bamA*-clade 1, *bamA*-clade 2, *bamA*-clade 3, *Thauera/Magnetospirillum*-clade, *Geobacter*-clade, *Syntrophus*-clade and a *Georgfuchsia/Azoarcus* clade (9). The work involved analysis of groundwater samples along the pollution plume in 1999 and 2004, which enabled the researchers to correlate site conditions e.g. ferrous iron concentrations and dissolved organic matter, with sequence diversity. The

researchers used qPCR to obtain a ratio of functional genes to bacterial 16S rRNA genes. On average, 1.8 *bssA* copies and 33.7 *bamA* copies were present per 10,000 16S rRNA copies in polluted samples in 2004. They found that species containing *bssA* sequences closely affiliated with *Georgfuchsia toluolica*. In contrast, *bamA* genes closely related to *Geobacteraceae* were dominant and only <2% of *bamA* genes were related to *Georgfuchsia*. From 64 partial *bssA* sequence variants, 57 *bssA* sequences clustered to the *bssA* gene of *Georgfuchsia toluolica* G5G6. A minor number of other *bssA* sequences affiliated with *Geobacter* spp., *Magnetospirillum* sp. TS-6 and *Aromatoleum aromaticum* sp. EbN1. In contrast, samples from 1999 illustrated a high occurrence of *Aromatoleum* related *bssA* sequences which coincided with denitrification being a dominant redox process. The 188 partial *bamA* sequence variants showed a high gene diversity and these sequences were placed into seven clades. A small fraction (1.5%) strongly grouped with sequences from *Georgfuchsia toluolica* sp. G5G6, *Azoarcus* sp. and *Aromatoleum aromaticum* sp. EbN1. A larger percentage (43.2%) contained sequences highly related to *Geobacter* spp., and these were referred to as the '*Geobacter*-clade'. A number of sequences (13.4%) showed similarity to *Syntrophus aciditrophicus* sp. SB and were called the '*Syntrophus*-clade'. A small number of sequences (5 of 188) were placed with the '*Thauera/Magnetospirillum*-clade'. Two clades (*bamA*-clade 1 and 2) did not contained reference genes from isolates and one clade (*bamA*-clade 3) had low similarity to *Azoarcus* and *Syntrophus aciditrophicus* sp. SB.

The *bamA* sequences obtained in the current study affiliated with five of these seven clades described above. The *bamA* sequences from the nitrate amended samples were placed within three clades (*bamA*-clade 1, *Georgfuchsia/Azoarcus* and *Magnetospirillum/Thauera* clades). In

contrast, the *bamA* sequences obtained from the sulfate amended microcosms were placed within two different clades (*Syntrophus* and *Geobacter* clades). The *bamA* sequences from the methanogenic microcosms were all placed within the *Syntrophus* clade. The details for each are described below.

In the nitrate amended agricultural soil microcosms, two different *bamA* sequences were obtained. Three sequences has the greatest similarity to *bamA* clone 0c_1999(1C) from the landfill study (97% identity) and the *bamA* sequence from *Azoarcus toluvorans* strain Td21 (97% identity). These three sequences could be placed in the *Georgfuchsia/Azoarcus* clade. The other sequence type had the greatest similarity to landfill clone -200b_2004(1B) (92% identity) and could be placed inside the *bamA*-clade 1. This was the only sequence obtained in the current study to be placed in this clade. This clade does not contain reference genes from isolates (9). The other nitrate amended microcosms (inoculated with digester sludge) contained a different set of *bamA* genes. This time, the sequences were most similar to *Magnetospirillum magneticum* (92% identity) and could be placed with the *Magnetospirillum/Thauera* clade. The placement of the *bamA* sequences from nitrate amended samples within clades containing known nitrate reducing toluene degrading species (*Azoarcus, Georgfuchsia, Magnetospirillum* and *Thauera*) suggests the *bamA* gene is highly conserved. Interestingly, one sequence was placed in a clade with no reference genes from isolates, indicating gene diversity under nitrate reducing conditions cannot be not completely accounted for by known isolates.

The *bamA* sequences obtained from the sulfate amended samples were placed into two clades. All clones from the digester sludge inoculated microcosms were placed into the *Syntrophus* clade

and had the greatest similarity to the landfill leachate plume clones (89% identity). In contrast, the *bamA* clone from microcosm inoculated with the contaminated site sediment was placed with the *Geobacter* clade and had the greatest similarity (84% identity) to *bamA* clones from the Gneisenau site (10). This site is located near a former coking plant and contained iron and sulfate as potential electron acceptors. All of the sequences obtained from the methanogenic samples could be placed within the *Syntrophus* clade. The *bamA* clones from the methanogenic activated sludge samples had to highest similarity to the *bamA* sequence from the obligately anaerobic bacteria *Syntrophus aciditrophicus* (87% identity) and to *bamA* sequences from the landfill leachate plume (9). The *bamA* sequences from the agricultural soil under methanogenic conditions were also most similar to *Syntrophus aciditrophicus* (84% identity) and the landfill leachate plumes. Again, these data indicate *bamA* sequence diversity to be strongly correlated with electron accepting conditions.

In the current study, *bssA* and *bamA* gene numbers were obtained for a select number of microcosms during toluene degradation. The only other published data quantifying these genes together exists in the landfill leachate study (14). In that case, the authors reported on average 1.8 *bssA* copies and 33.7 *bamA* copies were present per 10,000 16S rRNA copies in polluted samples in 2004. Although the current study did not quantify 16S rRNA gene numbers, a ratio of *bamA/bssA* can be calculated to compare the new data to the field data described above. In the nitrate amended microcosms, the *bamA/bssA* ratios are ~0.06, 1 and 0.25 for AgN, BoN1 and GSN, respectively. These ratios are much lower than those described above. In the sulfate amended sample (DUKE), the rate is ~11 and in the methanogenic microcosms the ratios are ~2.7 (HaM) and ~1.6. It is not clear why the ratios are lower in the laboratory studies. It is

possible that the presence of a diverse range of contaminants at the field site favors *bamA*, whereas microcosms exposed to toluene only favors *bssA*.

In summary, this work provides the first in-depth study of the diversity of *bamA* gene sequences across redox conditions and in microcosms constructed from different inoculum sources. Since 2008, to our knowledge, only three studies have investigated the *bamA* gene in anaerobic aromatic degrading cultures (9, 10, 14). The research also examined the presence and diversity of the *bssA* gene and documented the utility of a range of primers for *bssA* detection. Further, qPCR was targeted to these two functional genes and the results provided evidence that toluene degradation can be correlated with *bamA* and *bssA* gene numbers. Therefore, the qPCR assays have the potential for use at field sites to document toluene degradation.

Tables and figures

Table 4.1. Success of *bssA* and *bamA* primers, amplicons cloned and the consortia targeted for qPCR.

Consortia	Inoculum Source	Likely TEA	7772f/ 8546r(20)	SRBf/ SRBr(3)	bss1500f/ bss2100r(13)	Bamr/ Bamf(9)	qPCR
AgN	Ag soil	Nitrate	++* **X**	+*	-	++	**yes**
GSN	Granular sludge	Nitrate	++* **X**	-*	-	++ **X**	**yes**
ASN	Activated sludge	Nitrate	++	-	-	++	no
BoN1	Ag soil	Nitrate	++ **X**	-	++	++ **X**	**yes**
BoN2	Ag soil	Nitrate	++	-	++	++	no
DSN	Digester sludge	Nitrate	++ **X**	-	++	++	no
DSS	Digester sludge	Sulfate	+*	++* **X**	++	++ **X**	no
DUKE	Contaminated soil	Sulfate	+*	++* **X**	-	++ **X**	**yes** #
AgS	Ag soil	Sulfate	+*	++*	-	++	no
BoS1	Ag soil	Sulfate	-	-	-	++	no
StS	Digester sludge	Sulfate	+	++	++	++	no
SM	Contaminated soil	CO_2	+	+	++ **X**	+	no
HaM	Ag soil	CO_2	++ **X**	-	+	++ **X**	**yes**
ASM	Activated sludge	CO_2	++	-	+	++ **X**	**yes**
DSM	Digester sludge	CO_2	-	-	-	++	no
KRM	Ag soil	CO_2	+	+	-	++	no

++ strong specific amplicon + unspecific amplicon(s) - no amplicon

X – amplicons sequenced

- the primer set 7772f/8828r (20) was used to target *bssA* for qPCR

* Previous study in our group(16)

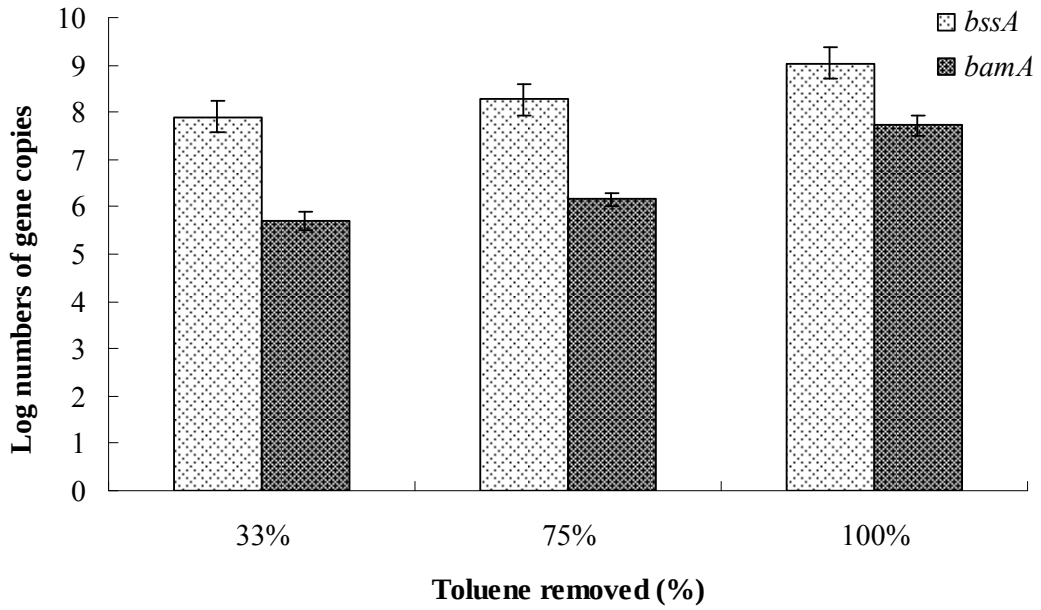

Figure 4. 1. Gene numbers of *bssA* and *bamA* during toluene degradation in nitrate amended microcosms AgN. The error bars represent standard deviations form triplicate qPCR samples.

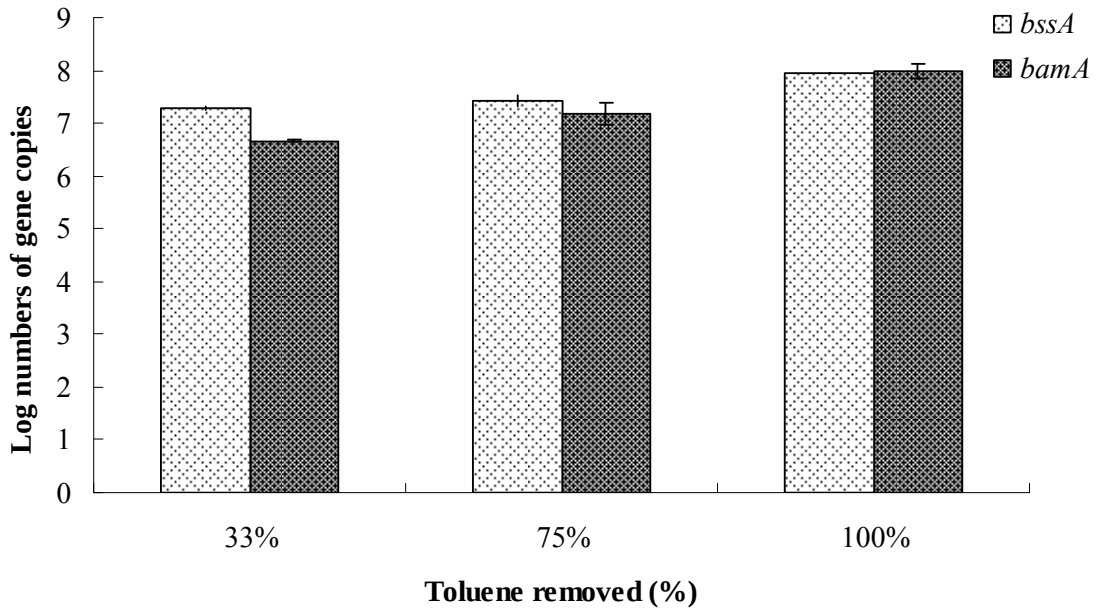

Figure 4.2. Gene numbers of *bssA* and *bamA* during toluene degradation in nitrate amended microcosms BoN1. The error bars represent standard deviations form triplicate qPCR samples.

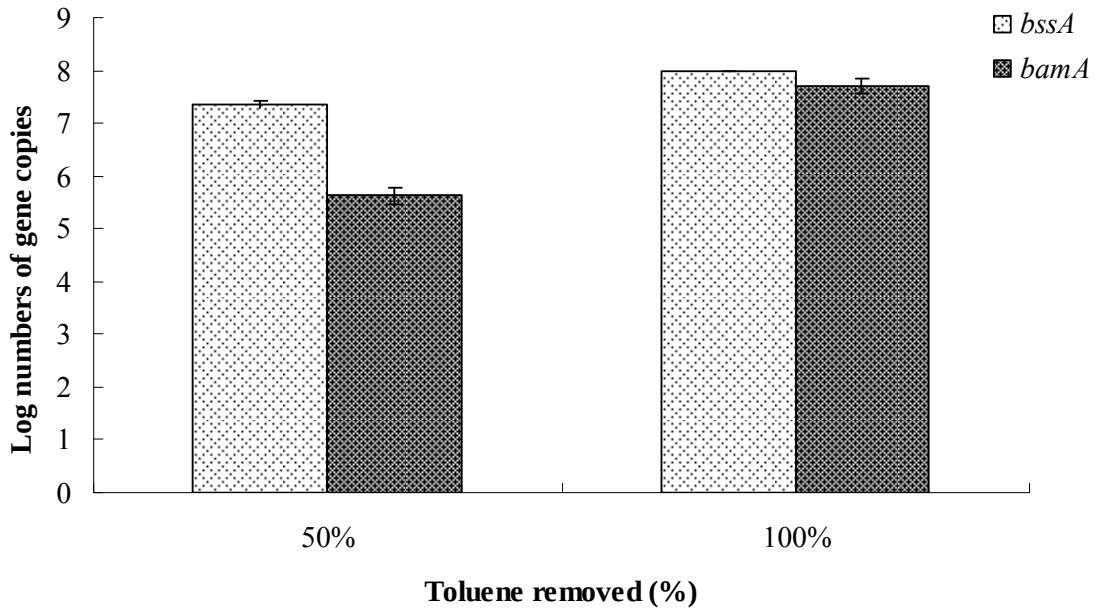

Figure 4.3. Gene numbers of *bssA* and *bamA* during toluene degradation in nitrate amended microcosms GSN. The error bars represent standard deviations form triplicate qPCR samples.

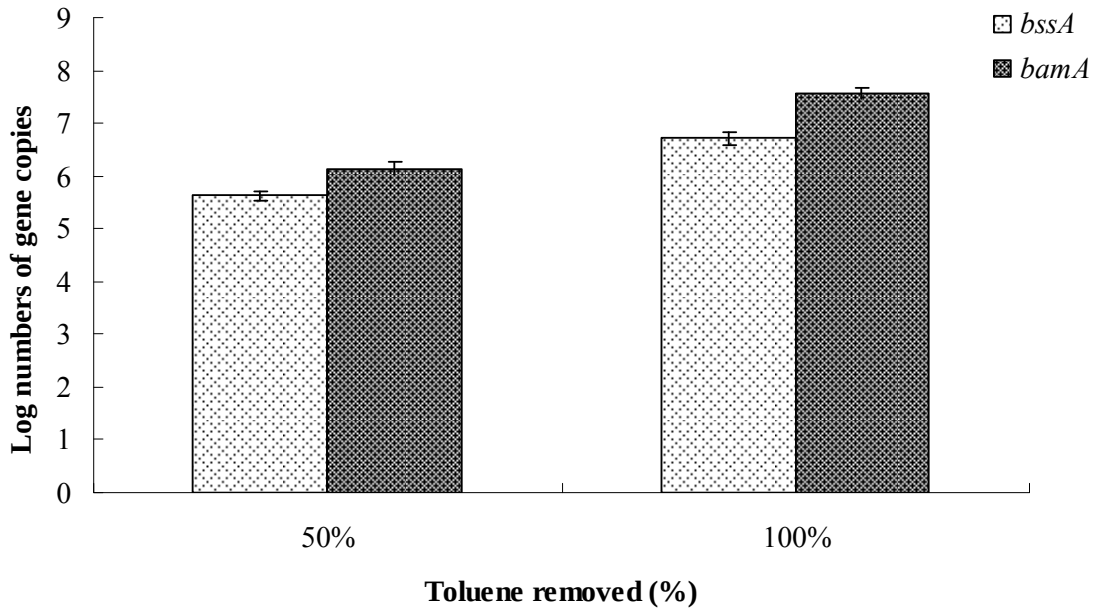

Figure 4.4. Gene numbers of *bssA* and *bamA* during toluene degradation in a sulfate amended microcosm, DUKE.The error bars represent standard deviations form triplicate qPCR samples.

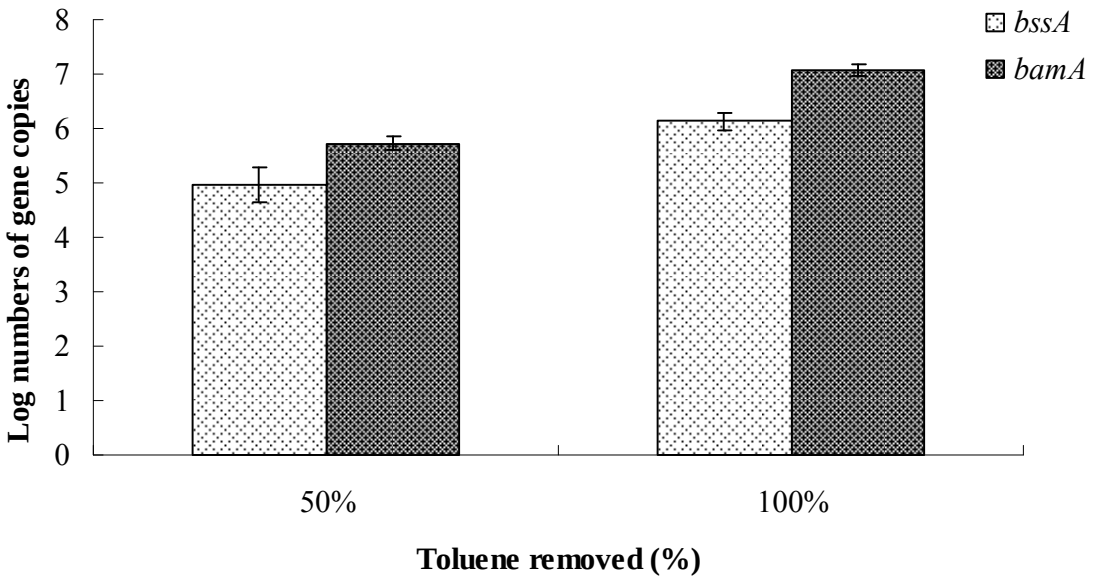

Figure 4.5. Gene numbers of *bssA* and *bamA* during toluene degradation in a methaongeic microcosm, ASM. The error bars represent standard deviations form triplicate qPCR samples.

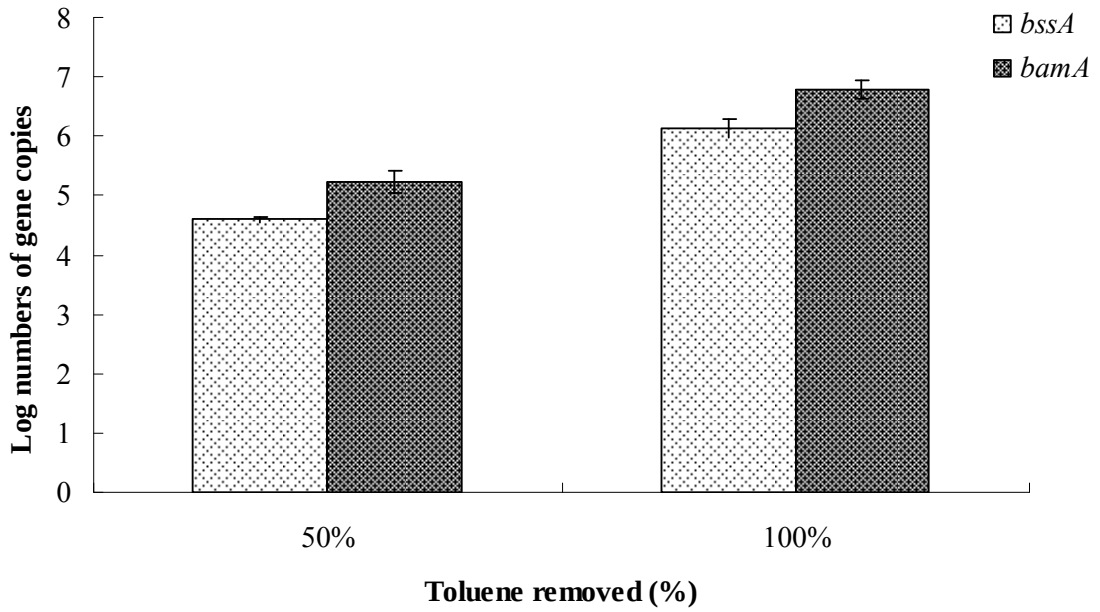

Figure 4.6. Gene numbers of *bssA* and *bamA* during toluene degradation in a methaongeic microcosm, HM. The error bars represent standard deviations form triplicate qPCR samples.

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CHPATER 5

ANAEROBIC MTBE DEGRADING MICROORGANISMS IDENTIFIED IN WASTEWATER TREATMENT PLANT SAMPLES USING STABLE ISOTOPE PROBING

Introduction

Methyl *tert*-butyl ether (MTBE) is a synthetic organic compound that was added to gasoline in the late 1970s, following the phase out of tetraethyl lead. Later, the implementation of the Clean Air Act Amendments (1990) caused a significant increase in MTBE use. In 1970, MTBE was the 39th highest produced US organic chemical whereas by 1998 it ranked 4th. During this time the aggregate production of MTBE was 60 million metric tons (11). The large scale use, combined with MTBE's physiochemical properties, has resulted in severe contamination. MTBE has a high water solubility (51 g L^{-1}), strongly partitions to water from air (dimensionless Henry's Law constant 0.02) (14), has a low sorption partition coefficient (K_{oc} is 1.035-1.091(15)) and, thus, is highly mobile in water (51). MTBE contamination has been reported in surface waters (3, 4, 9, 11, 15, 26, 46), groundwater (11, 15, 28, 29, 34, 38, 40, 53, 59, 64), and drinking water sources (1, 6, 11, 20, 35, 42, 48, 52, 56). MTBE contamination in drinking water has been reported in 36 states, with removal estimates being $25-33.2 billion (5).

Biological degradation is becoming increasing common as a remediation method for groundwater contaminants, either through natural attenuation or enhanced bioremediation. There have been numerous studies on aerobic MTBE biodegradation (32, 45, 57, 58) and microorganisms capable of degrading MTBE under aerobic conditions have been isolated (16, 39,

47, 49). Anaerobic MTBE biodegradation has also been documented (10, 21-23, 36, 41, 43, 54), however, much less is known about the microorganisms involved and, to date, no MTBE degrading isolates have been obtained. This knowledge gap is a significant limitation to *in situ* MTBE bioremediation because many contaminated sites are anaerobic. More information on the microorganisms capable of anaerobic MTBE degradation could result in the application of molecular methods to investigate their presence and abundance, and therefore the potential for MTBE degradation at different sites.

The microbial composition of anaerobic MTBE degrading enrichment cultures has only recently been investigated. In 2009, the microbial communities of three anaerobic MTBE degrading cultures derived from MTBE-contaminated aquifer material were examined using 16S rDNA based amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing (17). The cultures were maintained with anthroquinone-2,6-disufonate (AQDS), sulfate or fumarate as electron acceptors and the authors found that the microbial diversity varied under these different conditions. In another recent study, other researchers characterized the community composition of anaerobic enrichment cultures originating from three different contaminated sediments (63). Interestingly, terminal restriction fragment length polymorphism (TRFLP) profiles indicated substantially different community profiles from MTBE degrading microcosms established from different sediment sources. A third group investigated the microbial community present (16S rRNA gene sequencing) when MTBE degradation occurred under sulfate or iron reducing conditions or when both electron acceptors were present together and identified five to eight microorganisms in the three consortia (43).

The current study expands on these investigations by applying DNA based stable isotope probing (SIP) to determine which organisms are responsible for ^{13}C label uptake from MTBE in anaerobic MTBE degrading microcosms under methanogenic conditions. The SIP method is unique in that it can directly identify the microorganisms responsible for contaminant degradation and therefore offers more targeted information than community analysis alone (e.g. TRFLP, ADARA, 16S rRNA clone libraries). To date, SIP has yet to be used to investigate MTBE degradation in anaerobic MTBE degrading microcosms. The SIP method involves exposure of mixed cultures to the labeled compounds of interest (e.g., ^{13}C MTBE) and DNA extraction over time. The DNA is then subject to ultracentrifugation, fractionation (to separate label incorporated DNA from the unlabeled DNA) and TRFLP on each fraction. Any TRFLP fragment illustrating an increase in relative abundance in the heavy fraction of the samples (exposed to labeled substrate) compared to the controls (exposed to unlabeled substrate) is identified as the putative degrader. The method has been used to identify the microorganisms involved in the degradation of numerous contaminants (2, 13, 27, 30, 31, 37, 55, 60, 61). In the current study, a range of sources (contaminated site sediment, agricultural soils and wastewater treatment samples) were examined for anaerobic MTBE degradation potential. In the active anaerobic MTBE degrading microcosms, SIP targeted to both bacteria and archaea was applied to identify the putative MTBE degrading microorganisms.

Methods

Microcosm Construction and Analytical Techniques

A range of sources were tested for their potential to degrade MTBE (Table 5.1), including

agricultural soil, contaminated site soil and wastewater treatment samples. From all samples tested only one source (WWTP sample) demonstrated MTBE degradation and was further investigated. Microcosms were prepared under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratory Products INC, Grass Lake, MI). For each microcosm, ~6g sample (wet weight) was anoxically incubated in 60 mL serum bottles containing 25 mL of anaerobic basal media (62). Each treatment involved triplicate abiotic controls, triplicate unlabeled MTBE (1 μL, 99 %, Sigma Aldrich, St. Louis, MO) and three triplicate labeled MTBE ($^{13}C_5$-MTBE 1 μL Sigma Aldrich, St. Louis, MO) amended samples. These microcosms were incubated at room temperature (~20 °C) with reciprocal shaking. MTBE concentrations in headspace gas samples (200 μL) were determined with a gas chromatograph (Perkin Elmer) equipped with flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53mm). Injector and detector temperature were set at 200 ^{o}C and the column temperature was 120^{o}C.

DNA Extraction and Ultracentrifugation

Microcosms were sacrificed for DNA extraction at two time points (~30% and ~70% MTBE removal) during MTBE depletion to understand the flow of carbon through these microbial communities. The powersoil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) was used for total nucleic acids extraction according to the manufacturer's recommended procedure. Quantified DNA extracts (~10 μg) were loaded into Quick-Seal polyallomer tubes (13×51 mm, 5.1 ml, Beckman Coulter) along with a Tris-EDTA (TE, pH 8.0) /CsCl solution. Prior to sealing (cordless quick-seal tube topper, Beckman), the buoyant density (BD) was determined with a model AR200 digital refractometer (Leica Microsystems Inc) and adjusted by adding small

volumes of CsCl solution or Tris-EDTA buffer with a final BD of 1.7300 mgL^{-1}. The tubes were centrifuged at 178,000 *g* (20 ºC) for 48 h in a Stepsaver 70 V6 Vertical Titanium Rotor (8 x 5.1 ml capacity) within a Sorvall WX 80 Ultra Series Centrifuge (Thermo Scientific). Following centrifugation, the tubes was placed onto a fraction recovery system (Beckman) and fractions (150 µl) were collected. The BD of each fraction was measured, and CsCl was removed by glycogen-assisted ethanol precipitation.

PCR, TRFLP and Sequencing of 16S rRNA Genes

The density-resolved fractions from ^{12}C and ^{13}C microcosms for each treatment were PCR-amplified using 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG, 5' end-labeled with carboxyfluorescine) and 1492R (5'-GGTTACCTTGTTACGACTT) for generating bacterial amplicons and A109F—FAM (5'- ACKGCTCAGTAACACGT) and A934R (5'-GTGCTCCCCCGCCAATTCCT) for archaeal amplicons (Operon Biotechnologies). The presence of PCR products was confirmed by 1.5% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide. PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.), following the manufacturer's instructions and approximately 150 ng was digested with *HaeIII* (New England Biolabs) with a 6-hour incubation period. Additional digests (*HhaI, MseI, Bsp1286I, BsrBI* etc.) for TRFLP analyses in a number of heavy labeled fractions were included to correlate the TRFLP fragment lengths to the *in silico* cut sites of the cloned 16S rRNA gene sequences. DNA fragments were separated by capillary electrophoresis (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) at the Research Technology Support Facility (RTSF) at Michigan State University. Data were analyzed with

GeneScan software (Applied Biosystems) and the percent abundance of each fragment was determined. Clone libraries of the 16S rRNA genes were constructed using DNA amplified with 27F/1492R , A109F/A934R as above except the forward primer was unlabeled and the final extension time was extended to 15 minutes. To reduce sequencing redundancy, restriction fragment length polymorphism (RFLP) analyses was performed and specific operational taxonomic units (OTU) were selected for sequencing. The PCR products were purified with QIAquick PCR purification kit (Qiagen Inc.) and cloned into *Escherichia coli* TOP10 vector supplied with a TOPO TA cloning kit (Invitrogen Corporation). *E. coli* clones were grown on Luria-Bertani (LB) medium solidified with 15 g agar L^{-1} with 50 μg ampicillin L^{-1} for 16 h at 37 ºC. Colonies with inserts were verified by PCR with primers M13 F (5'-TGTAAAACGACGGCCAGT-3') and M13 R (5'-AACAGCTATGACCATG-3'), plasmids were extracted from the positive clones with a QIAprep miniprep system (Qiagen, Inc.) and the insertions were sequenced at RTSF. The Ribosomal Database Project (RDP) (Center for Microbial Ecology, Michigan State University) analysis tool "classifier" was utilized to assign taxonomic identity.

Results and Discussion

From twenty-two experimental set-ups, anaerobic MTBE biodegradation was noted in microcosms constructed from only one source and only under methanogenic conditions (Table 5.1). Microcosms for the SIP experiment were constructed using freshly sampled material from the wastewater treatment plant (WWTP). MTBE degradation occurred in both labeled MTBE amended and unlabeled MTBE amended samples, but not in the abiotic controls (Figure 5.1). At

two time points during the time period for MTBE biodegradation (~30% and 70% removal), DNA was extracted from the labeled and unlabeled MTBE-amended microcosms, and was subject to ultracentrifugation, fractionation and TRFLP. Bacterial microbial communities were profiled by TRFLP when 30% and 70% MTBE was degraded, whereas archaeal communities were only profiled when 70% was degraded. Assimilation of ^{13}C labeled MTBE was detected by comparing TRFLP profiles of DNA derived from labeled treatments with DNA from unlabeled treatments. Specifically, the organisms responsible for ^{13}C assimilation were identified by the comparison of relative abundances of specific terminal restriction fragments (T-RFs) between the labeled and unlabeled gradient-fractions.

Labeled bacterial DNA was successfully amplified in the fractions with buoyant density (BD) values of 1.7154 to 1.7719 g ml^{-1} (~70% degraded) and 1.7306 to 1.7653 g ml^{-1} (~30% degraded). PCR products were seen in the fractions with BDs of 1.7024 to 1.7589 g ml^{-1} in the unlabeled treatment (~70% degraded). Two bacterial T-RFs (67 bp and 215 bp) were highly enriched in the heavy ^{13}C fractions while such enrichment was not seen in the corresponding fractions with similar buoyant density (Figure 5.2A and B). A high (>30%) relative abundance (RA) of both T-RFs was noted in the heavier fractions and their RA increased over time, as MTBE was degraded (30% and 70% MTBE degraded). The RA of 215 bp T-RF was > 20% among the heavy fractions (banding between 1.7545 to 1.7719 g ml^{-1}) with the maximum RA (46.6%) in the fraction with BD of 1.7676 g ml^{-1} (~70 % degraded) (Figure 5.2A). The RA of the 67 bp T-RF was generally > 30% in labeled heavy fractions (banding between 1.7480 to

1.7719 g ml^{-1}) under both time points with the maximum RA (48.4%) in the fraction with BD of 1.7643 g ml^{-1} (~70% degraded) (Figure 5.2B). An analysis of the archaeal SIP TRFLP profiles (from ~70% MTBE degraded) indicated two T-RFs (132 bp and 162 bp) were relatively more dominant in the labeled fractions compared to the controls (Figure 5.3 A and B).

Clone libraries for both bacteria (Table 5.2) and archaea (Table 5.3) were generated to investigate the diversity of the MTBE degrading microcosms and to identity the putative MTBE degraders represented by the T-RFs discussed above. The most dominant bacterial phylum was the *Proteobacteria* (59 from 122 clones or 48.4%), which contained *Alphaproteobacteria* (22/59), *Betaproteobacteria* (21/59), *Gammaproteobacteria* (10/59) and *Deltaproteobacteria* (6/59). The second most dominant phylum was the *Firmicutes* (29/122 or 23.8%), which primarily consisted of Clostridia (26/29). Other minor phyla included *Verrucomicrobia* (9/122), *Nitrospira* (6/122), *Bacteroidetes* (5/122), OP10 (5/122), *Lentisphaerae* (4/122), *Tenericutes* (3/122) and *Acidobacteria* (1/122). The genera of each clone determined from the RDP are also shown, with a significant number (13/26) from being unclassified to the genus level (Table 5.2). The archaeal community was less diverse with only 7 different phylotypes (Table 5.3), all within the class *Methanomicrobia* (phylum *Euryarchaeota*). Two phylotypes could not be classified at the genus level.

The bacterial and archaeal sequences were digested in silico to identify the T-RFs enriched in the heavy fractions as discussed above. The two bacterial T-RFs dominant in the heavy fractions were identified as Clostridia (215 bp) and *Alphaproteobacteria* (67 bp). The Clostridia-related phylotype could be classified to the family level (*Ruminococcaceae*) and was most similar to an

uncultured *Acetivibrio* spp. (95% 16S rRNA gene sequence similarity: GenBank accession number EF613411.1). The Alphaproteobacteria-related clone classified to the genus *Sphingopyxix* and was most similar to an uncultured bacterium clone reservoir-30 (99% of 16S rRNA gene sequence similarity: GenBank accession number JF697411.1). The archaeal 132 bp and 162 bp T-RFs belonged to the genera *Methanosarcina* and *Methanocorpusculum*, respectively. The putative identifies of the bacterial T-RFs were confirmed with additional digests on the heavy fractions (Table 5.4).

The SIP data indicate the primary MTBE degraders in the methanogenic enrichment are bacteria and belong to the phyla Clostridia (family *Ruminococcaceae*) and *Alphaproteobacteria* (genus *Sphingopyxis*). As the label enrichment level was low in the archaeal phylotypes in the heavy fractions, it is unlikely that these organisms (genera *Methanosarcina* and *Methanocorpusculum*) are the dominant degraders. It is possible that the identified archaeal phylotypes are responsible for minor amounts of MTBE degradation or they are consuming metabolites produced by the primary bacterial degraders. Interestingly, other researchers have identified *Clostrida* as dominant organisms in their anaerobic MTBE degrading enrichments. Specifically, from the three enrichments (AQDS, sulfate or fumarate reducing) developed from MTBE contaminated aquifer material, the sulfate reducing enrichment contained 19.3% assigned to the order *Clostridiales* and the fumarate reducing enrichment contained a dominant clone (related to *Clostridium* sp. Kw12) (22.8%) also belonging to the phylum *Firmicutes* (17). Similarly, following continual enrichment of an anaerobic MTBE degrading consortium, researchers reduced the community to three dominant phylotypes belonging to *Deltaproteobacteria*, *Chloroflexi* and *Firmicutes* (63). In addition, *Clostridia* were found in anaerobic MTBE

degrading consortia under sulfate and iron reducing conditions (44). These previous studies, combined with the data in the current study indicate organisms in the phylum *Firmicutes* are important for the anaerobic degradation of MTBE.

Microorganisms associated with the family of *Ruminococcaceae* within the phylum *Firmicutes* are predominant members of mammalian gut microbial flora. The presence of these organisms in the enrichments is therefore not surprising given the source of the inocula (WWTP sample). Members of *Ruminococcaceae* isolated from human gut were correlated with biodegradation of complex polysaccharides such as starch or xylan (19). *Ruminococcaceae* isolated from rumen or human guts were proven to be able to degrade cellulose (7, 8, 18). Chassard et al. reported *Ruminococcaceae* were responsible for cellulose biodegradation in the fecal samples of methane-excreting subjects while the main cellulose-degrading bacteria belong essentially to *Bacteroidetes* in non-methane-excreting subjects (7), indicating a possible link between methane production and *Ruminococcaceae*-associated cellulose biodegradation. This observation is consistent with the current study in that no MTBE biodegradation under the sulfate- and nitrate-amended conditions (no methane was produced) although they were seeded from the same inocula. *Ruminococcaceae* were also linked with 2,4,6-trinitrotoluene (TNT) degradation in a recent study (12). The most similar isolate to the putative MTBE degrader (family *Ruminococcaceae*) identified in the current study is uncultured *Acetivibrio* sp. clone ZZ-S2G3 (95% 16S rRNA similarity; Genbank accession number EF613411.1) which was found in a sulfate reducing benzene-degrading microbial community but was not correlated with benzene biodegradation (33).

In contrast to the putative MTBE degrader (Firmicutes phylotype, TR-F 215 bp) discussed above, the other putative MTBE degrader (phylum *Alphaproteobacteria*, genus *Sphingopyxis* TR-F 67 bp), has no obvious previous links to anaerobic MTBE biodegradation. The 67-bp T-RF showed a high level of 16S rRNA similarity (98%) to an uncultured *Alphaproteobacteria* bacterium (GeneBank CU926829.1) detected in anaerobic digestion of sludge (50). The data obtained from the current study indicate this phylotype may be a novel anaerobic MTBE degrader and should be further investigated.

The presence of two enriched T-RFs suggests more than one microorganism may be responsible for MTBE biodegradation. Youngster et al. (24) suggested that anaerobic MTBE biodegradation required the interaction of a consortium. The exact mechanism of MTBE anaerobic biodegradation in the current study has yet to be elucidated. Others have reported tert-butyl alcohol (TBA) as a MTBE degradation metabolite (22, 25, 49), indicating the cleavage of ether bond is the initial step of MTBE biodegradation. Interestingly, the –C–O–C– bond found in MTBE also occurs in cellulose, therefore one might hypothesize that *Ruminococcaceae* which can degrade cellulose may be responsible for the initial step of MTBE biodegradation.

Tables and figures

Table 5. 1. The sources and conditions investigated for anaerobic MTBE degradation potential.

Inoculum type	Incubation time	Electron acceptor	Degr	Comment
Contaminated soil 1	1 year	sulfate	No	Samples obtained from a previously MTBE contaminated LUST site
Contaminated soil 1	1 year	nitrate	No	Same soil as above
Contaminated soil 2	1 year	sulfate	No	Samples obtained from a previously MTBE contaminated LUST site
Contaminated soil 2	1 year	nitrate	No	Same soil as above
Agricultural soil 1	1 year	sulfate	No	Samples obtained from a farm located at Michigan,
Agricultural soil 1	1 year	nitrate	No	Same soil as above
Agricultural soil 1	1 year	carbon dioxide	No	Same soil as above
Agricultural soil 2	1 year	sulfate	No	Samples obtained from a farm located at Michigan, the crop is soybean
Agricultural soil 2	1 year	nitrate	No	Same soil as above
Agricultural soil 2	1 year	carbon dioxide	No	Same soil as above
Agricultural soil 2	1 year	sulfate	No	Same soil as above
Agricultural soil 3	1 year	sulfate	No	Samples obtained from a farm located at Michigan, the crop is corn
Agricultural soil 3	1 year	nitrate	No	Same soil as above
Agricultural soil 3	1 year	carbon dioxide	No	Same soil as above
Granular sludge	1 year	sulfate	No	Samples obtained from a UASB reactor in Washington
Granular sludge	1 year	nitrate	No	Same sludge as above
Granular sludge	1 year	carbon dioxide	No	Same sludge as above
Activated sludge	1 year	sulfate	No	Samples obtained from East Lansing WWTP. The sludge was taken from the aeration tank of the WWTP.
Activated sludge	1 year	nitrate	No	Same sludge as above
Activated sludge	2 months	carbon dioxide	Yes	Same sludge as above,~90% MTBE removal, Significant methane production
Digester sludge	1 year	sulfate	No	Samples obtained from St.Clair WWTP.
Digester sludge	1 year	nitrate	No	Same sludge as above
Digester sludge	1 year	carbon dioxide	No	Same sludge as above

Table 5.2. Phylogenetic affiliation of bacterial 16S rRNA clone in methanogenic MTBE degrading microcosms as determined with the RDP analysis tool "classifier"

Soil/Fragment (Hae III digestion)	Enriched in heavy fractions	Number of clones	Phylum	Class	Order	Genus
39 bp	*N*	*2*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	**unclassified**
39 bp	*N*	*6*	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Dokdonella*
39 bp	*N*	*3*	*Proteobacteria*	*Alphaproteobacteria*	**unclassified**	
39bp,76 bp	*N*	*3*	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	*Kofleria*
75,251	*N*	3	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Haliscomenobacter*
71bp,227 bp	*Y*	*18*	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingopyxis*
190 bp	*N*	*1*	*Proteobacteria*	*Alphaproteobacteria*	**unclassified**	
198 bp	*N*	3	*Proteobacteria*	*Betaproteobacteria*	**unclassified**	
200 bp	*N*	*4*	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Aquimonas*
212bp	*N*	3	*Firmicutes*	*Clostridia*	*Clostridiales*	
217 bp	*N*	16	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Simplicispira*
217 bp,224 bp,266 bp,349bp	*Y*	*12*	*Firmicutes*	*Clostridia*	*Clostridiales*	**unclassified**
219 bp	*N*	2	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Methylibium*
221 bp	*N*	*3*	*Tenericutes*	*Mollicutes*	*Acholeplasmatales*	*Acholeplasma*
224 bp	*N*	4	*Verrucomicrobia*	*Subdivision5*	**unclassified**	*Subdivision5*
231 bp	*N*	5	*OP10*	**unclassified**		*OP10*
240 bp,318 bp	*N*	*2*	*Firmicutes*	*Clostridia*	*Clostridiales*	
248 bp	*N*	*4*	*Lentisphaerae*	*Lentisphaeria*	*Victivallales*	*Victivallis*
255 bp	*N*	*3*	*Firmicutes*	*Erysipelotrichi*	*Erysipelotrichales*	**unclassified**
264 bp	*N*	*6*	*Nitrospira*	*Nitrospira*	*Nitrospirales*	*Nitrospira*
265 bp	*N*	*3*	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	**unclassified**
268 bp	*N*	*1*	*Acidobacteria*	*Acidobacteria_Gp3*	**unclassified**	*Gp3*
290 bp	*N*	*5*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Sporacetigenium*
293bp	*N*	1	*Proteobacteria*	**unclassified**		

Table 5.3. Phylogenetic affiliation of Archaeal 16S rRNA clone in methanogenic MTBE degrading microcosms as determined with the RDP analysis tool "classifier"

Soil/Fragment(Hae III digestion)	Enriched in ^{13}C heavy fractions	Number of clones	Phylum	Class	Order	Genus
135 bp	*N*	*4*	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	
138 bp	*Y*	*12*	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcina*
135 bp	*Y*	*15*	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanoculleus*
400 bp	*N*	*3*	*Euryarchaeota*	*Methanomicrobia*		
162 bp	*Y*	*18*	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanocorpusculum*
28 bp,135 bp	*N*	*11*	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillum*
150 bp	*N*	*9*	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaeta*

Table 5.4. Comparison of fragment length of dominant T-RF in heavy fractions to predicted fragment length from *in silico* sequence analyses

Microorganism	Restriction enzyme	TRFLP	Sequence data
Ruminococcaceae	HaeIII	215	217
	MspI	199	204
	MSEI	588	583
	RsaI	453	450

Microorganism	Restriction enzyme	TRFLP	Sequence data
Sphingomonadaceae	HaeIII	67	71
	MspI	147	150
	MSEI	418	422
	RsaI	507	506

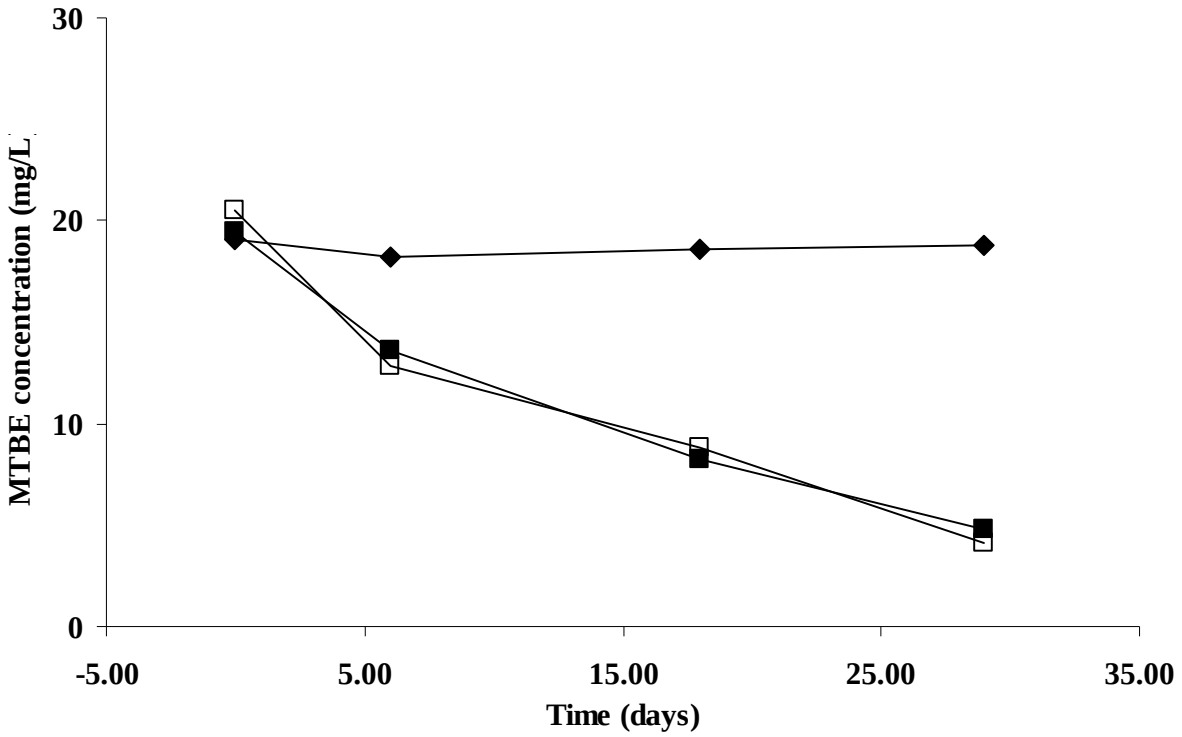

Figure 5.1. MTBE concentration over time in ^{12}C-MTBE amended abiotic controls (♦), ^{13}C-MTBE (■) and ^{12}C-MTBE (□) amended samples. The arrows indicate when DNA was extracted. The error bars represent standard deviations from triplicate microcosms.

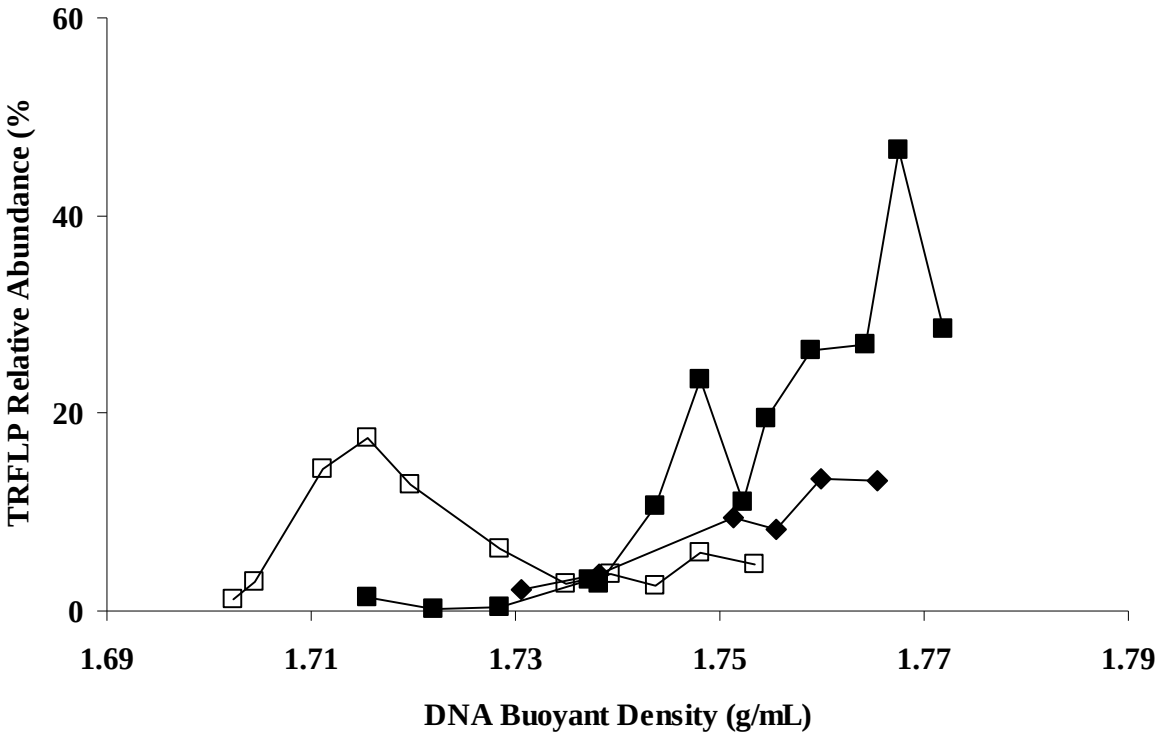

Figure 5.2. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Clostridiales* within buoyant density gradients of Bacterial DNA extracted from the methanogenic activated sludge microcosms. Figure symbols: ♦ ^{13}C-MTBE (~30% toluene degraded); ■ ^{13}C-MTBE (~70% toluene degraded); □ ^{12}C-MTBE (~70% toluene degraded).

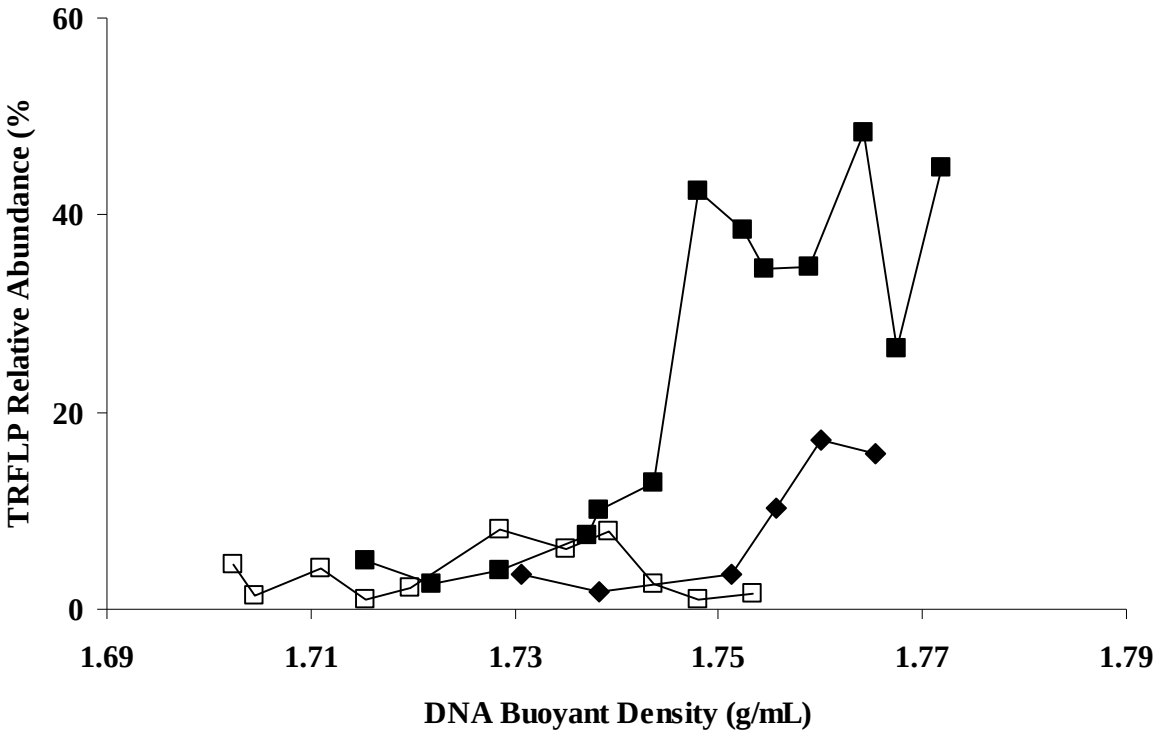

Figure 5.3. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Sphingomonadales* within buoyant density gradients of Bacterial DNA extracted from the methanogenic activated sludge microcosms. Figure symbols: ♦ ^{13}C-MTBE (~30% toluene degraded); ■ ^{13}C-MTBE (~70% toluene degraded); □ ^{12}C-MTBE (~70% toluene degraded).

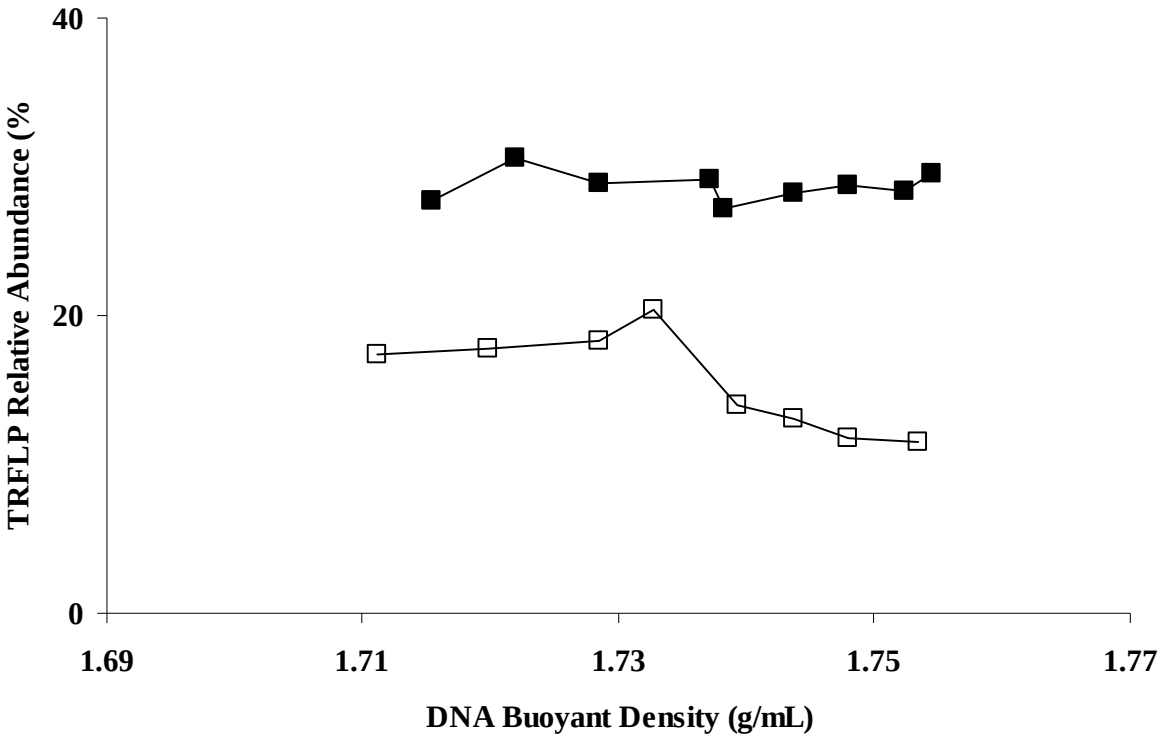

Figure 5.4. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Methanosarcina* within buoyant density gradients of Archaeal DNA extracted from the methanogenic activated sludge microcosms. Figure symbols:■ ^{13}C-MTBE (~70% toluene degraded); □ ^{12}C-MTBE (~70% toluene degraded).

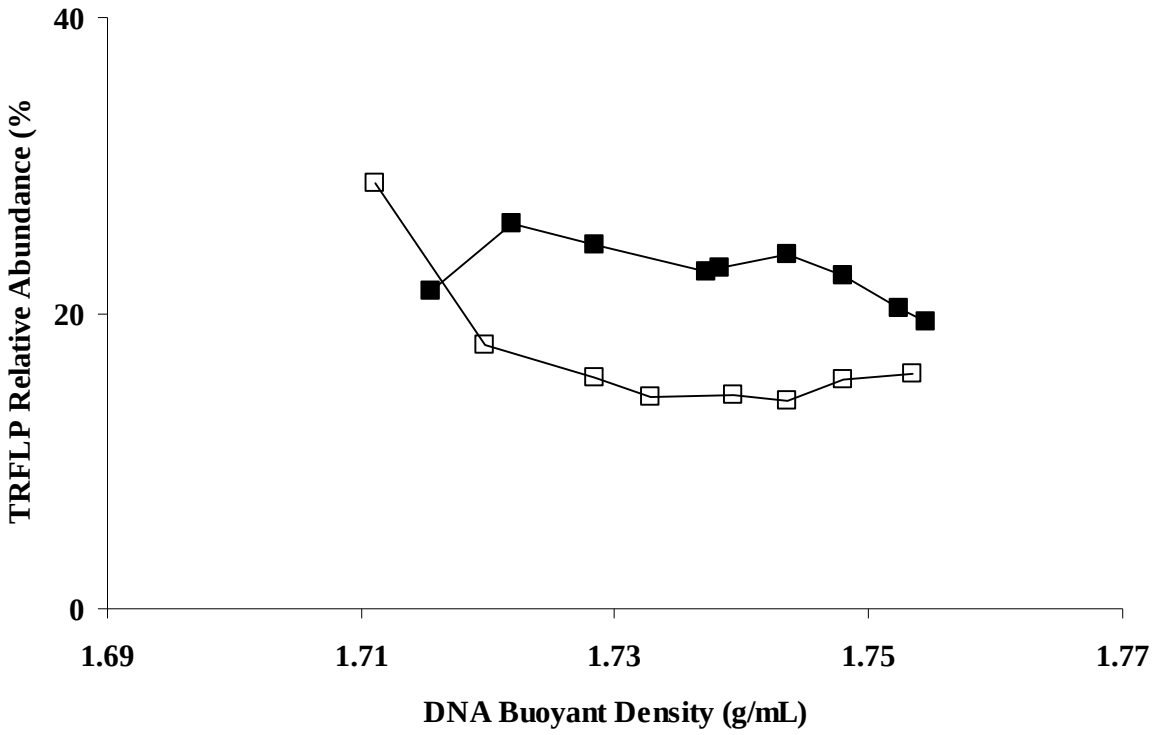

Figure 5.5. Percent relative abundance of fragments (digested by *Hae III*) assigned to *Methanocorpusculum* within buoyant density gradients of Archaeal DNA extracted from the methanogenic activated sludge microcosms. Figure symbols:■ ^{13}C-MTBE (~70% toluene degraded); □ ^{12}C-MTBE (~70% toluene degraded).

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