ABSTRACT

Title of Dissertation: ISOLATION AND CHARACTERIZATION OF POLYCYCLIC AROMATIC HYDROCARBON-DEGRADING MICROORGANISMS UNDER METHANOGENIC CONDITIONS

Youngsoon Um, Doctor of Philosophy, 2004

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Polycyclic aromatic hydrocarbons (PAHs) are among the most widely distributed organic contaminants in aquatic sediments due to their presence in coal and petroleum**.** While it has been demonstrated that PAHs are degraded under anaerobic conditions, little is known about the microorganisms responsible for PAH degradation. This study demonstrates not only the first isolations of naphthalene (NAP)- and phenanthrene (PHE)-degrading microorganisms under methanogenic conditions by utilizing modified plating methods but also the first identification and isolation of a fermentative bacterium responsible for initiating a syntrophic PHE-degradation. Molecular characterization of PAH-degrading methanogenic cultures via comparative 16S rDNA sequence analysis was employed to monitor the microbial community structure and consequently to design isolation strategies for the possible microbial species responsible for PAH-degradation. To isolate PAH-degrading microbes under anaerobic conditions, a modified plating method was first developed for detecting

microorganisms degrading solid PAHs on the agar-overlay plate. It was also verified that this method was not only applicable for the isolation of both aerobic and anaerobic PAH-degrading microorganisms but also effective to solve problems existing with other previous isolation methods. By employing the modified plating method, PHE-degrading microorganisms under methanogenic conditions were successfully isolated from the enrichment cultures. The degradation of PHE was partially inhibited by 2-bromoethanesulfonic acid; however, no $^{14}CH_4$ was detected when [9-^{14}C] PHE was employed, indicating partial mineralization of PHE. One species of bacterium was isolated and identified as an initial microbial catalyst for PHE-degradation. NAP-degrading microorganisms under methanogenic conditions were also isolated by employing an agar-overlay containing evenly dispersed fine particles of NAP. One species of the bacteria was identified to be the same microorganism as a fermentative bacterium initiating a syntrophic PHE-degradation, and the other one showed a syntrophic relationship with methanogen species. The results presented here will likely contribute to the development of the isolation techniques and the identification of microbial consortia for the biodegradation of PAHs under anaerobic conditions.

ISOLATION AND CHARACTERIZATION OF
POLYCYCLIC AROMATIC HYDROCARBON-DEGRADING
MICROORGANISMS UNDER METHANOGENIC CONDITIONS

By

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Chapter 1 Introduction

1.1 Motivation

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds containing two or more fused aromatic hydrocarbon rings. Numerous PAHs have been listed as priority pollutants by United States Environmental Protection Agency due to their carcinogenic and mutagenic properties (Keith and Telliard, 1979). They are produced in a variety of processes, such as by the incomplete combustion of fossil fuels and as a byproduct of coke production and petroleum refining (McNally *et al*, 1998). In the last few decades, the concentration of PAHs in the environment has reached unprecedented levels due to increased industrialization and coal utilization. Particularly, the low aqueous solubility and the high hydrophobicity of PAHs often result in their accumulation in soils and sediments to levels several orders of magnitude above aqueous concentration. Cerniglia (1992) reported PAH concentration ranges in soil from 0.005 μg/g in an undeveloped area to 1800 μg/g at an oil refinery. Additionally, the concentration of PAHs in harbor sediments can exceed 100ug/g by shipping activity, fuel spills, and runoffs (Johnson and Larsen, 1985). Due to the chemical stability of PAHs, these compounds tend to accumulate and persist in the environment, increasing the potential for human exposure.

The possible fates and transport pathways of PAHs in the environment include volatilization, photooxidation, chemical oxidation, bioaccumulation, adsorption to soil and sediment, and microbial degradation (Riser-Roberts, 1998). Recent studies have shown that microbiological degradation of PAHs is the major process for the

decontamination of sediment and soil (Cerniglia, 1992). There have been considerable research efforts to understand microbial degradation of PAHs in contaminated sites. Until recently, research on the biodegradation of PAHs has focused primarily on aerobic processes (Langenhoff *et al*., 1996); however, aerobic processes associated with soil and sediment leads to rapid depletion of dissolved oxygen, resulting in favorable growth environments for anaerobic microorganisms such as nitrate-reducing, sulfate-reducing, iron-reducing and methanogenic organisms. These anaerobes may play key roles in the degradation of PAHs in oxygen-depleted environments. Although PAHs generally had been thought to be recalcitrant to biodegradation without oxygen (Bauer and Capone, 1985; Evans and Fuchs, 1988), recent studies have demonstrated PAH degradation under nitrate- and sulfate-reducing conditions (Mihelcic and Luthy, 1988a; Mihelcic and Luthy, 1988b; Coates *et al.*, 1996; Coates *et al.*, 1997; Langenhoff *et al.*, 1996; Zhang and Young, 1997). The depletion of electron acceptors such as nitrate, sulfate and iron results in methanogenic conditions in soil and sediment. However, there has been no report demonstrating the degradation of PAHs under methanogenic conditions except one published result from our previous study (Chang *et al.*, 2001). Very recently, methanogenic conditions have drawn remarkable attention on the degradation of hexadecane, which was known to be a non-degradable compound under methanogenic conditions (Zengler *et al.*, 1999; Parkes, 1999; Anderson and Lovley, 2000), supporting the possibility of biodegradation of other recalcitrant compounds under methanogenic conditions. While it has been demonstrated that PAHs are degraded under anaerobic conditions, little information is available on anaerobic microorganisms in pure cultures responsible for the degradation (Phelps *et al.*, 1998).

This lack of isolates has limited the understanding of the mechanisms and the factors affecting anaerobic PAH degradation (Rockne *et al.*, 2000).

1.2 Project Scope

In this study, the isolation and characterization of anaerobic PAH-degrading microbes will be demonstrated with highly enriched PAH-degrading methanogenic cultures utilizing Baltimore Harbor (Baltimore, MD) sediment. Chapter 2 provides the background on anaerobic PAH biodegradation, the isolation of PAH-degrading microbes and metabolism of PAH biodegradation. Chapter 3 describes the identification of the microbial consortia for PAH-degrading enrichment cultures via comparative sequence analysis of 16S rDNA. In Chapter 4, a modified plating method is developed to isolate both aerobic and anaerobic PAH-degrading microbes. It is also verified that this isolation method successfully solves problems existing with other isolation methods utilizing clear zones on the agar plate. Chapter 5 describes the isolation of phenanthrene (PHE)-degrading consortium under methanogenic conditions by utilizing the modified plating method developed in Chapter 4. Further, the identification of a fermentative bacterium initiating the transformation of PHE, the inhibitory effect of 2-bromoethanesulfonic acid on PHE degradation and the study with radiolabeled PHE are presented. Finally, Chapter 6 illustrates the isolation of naphthalene (NAP)-degrading microorganisms under methanogenic conditions by utilizing an agar-overlay containing evenly dispersed fine particles of NAP. The characterization of the isolated NAP-degrading colony is also described.

Chapter 2 Background

2.1 Anaerobic biodegradation of PAHs

Our understanding of anaerobic biodegradation of PAHs has increased over the last few decades. Mihelcic and Luthy (1988a) were the first to demonstrate NAP biodegradation under nitrate-reducing conditions. With excess nitrate, NAP was degraded to undetectable levels (0.02mg/L) in less than 9 weeks in soil-water systems. Similarly, Langenhoff *et al*. demonstrated that NAP degradation occurred in the presence of nitrate after addition of the growth substrate, sodium benzoate (Langenhoff *et al*. 1996). More recently, McNally *et al.* (1998) utilized three microorganisms in pure culture to investigate three- and four-ring PAH degradation under nitrate-reducing conditions. All three strains were pseudomonads capable of growing on PAHs aerobically and under nitrate-reducing conditions. The rate of anaerobic NAP degradation (complete degradation of 3mg/l NAP in 6 to 8 h) was comparable to or only slightly slower than aerobic degradation rates. In contrast, Rockne and Strand (1998) reported anaerobic specific PAH biodegradation rates that were an order of magnitude slower than other published aerobic biodegradation rates. They used a fluidized-bed reactor (FBR) to enrich NAP, biphenyl, dibenzofuran, and PHE - degrading anaerobes in sediment-free cultures with nitrate and sulfate as terminal electron acceptors. In a separate study, the isolation and identification of pure cultures of NAP -degrading microbes was achieved under nitrate-reducing conditions (Rockne *et al.*, 2000). A few very recent studies have addressed the anaerobic degradation of PAHs under sulfate-reducing conditions. Langenhoff *et al*. illustrated NAP

disappearance in anaerobic sediment columns in the presence of sulfate (Langenhoff *et al.,* 1996). Further, Coates *et al.* (1996) demonstrated the degradation of PAHs under sulfate-reducing conditions by monitoring the oxidation of [^{14}C] NAP and PHE to $^{14}CO_2$ with sediments from San Diego Bay, California. Evidence was also presented in this report which conclusively linked sulfate reduction to PAH degradation. Further investigations with the San Diego Bay sediments revealed that methyl NAP, fluorene, and fluoranthene were also anaerobically transformed to carbon dioxide, while pyrene and benzo[a]pyrene were not (Coates *et al.*, 1997). Sediments from less-contaminated sites also showed degradative potential but only after a relatively long adaptation. Zhang *et al*. (1997) described the development of two highly enriched cultures capable of completely mineralizing NAP and PHE after 150 days of incubation. A later study by Meckenstock *et al.* (2000) reported that NAP could be degraded under sulfate-reducing conditions in a contaminated aquifer by adding NAP as the sole carbon and energy source. Most recently, Chang *et al.* (2001) reported the establishment of NAP- and PHE-degrading enrichment cultures utilizing Baltimore Harbor (Baltimore, MD) sediment under methanogenic conditions. This was the first report to demonstrate the degradation of PAHs under methanogenic conditions.

2.2 Isolation of PAH-degrading microorganisms

Although it has been shown that PAHs are degraded anaerobically, little is known about the microorganisms responsible for this activity. Thus far, there are two reports (McNally *et al.*, 1998; Rockne *et al.*, 2000) describing pure cultures with the ability to degrade PAHs under nitrate-reducing conditions, and one report demonstrating the

isolation of a NAP-degrading sulfate-reducing bacterium (Galushko *et al*., 1999); however, attempts to isolate PAH-degrading organisms under anaerobic conditions have failed for a long time (Heider *et al.*, 1999). Since anaerobic microorganisms, especially those known to degrade PAHs have generally very slow growth rates, conventional serial dilution or plating techniques can result in selection for bacteria growing on nutrient content in the media rather than the provided PAHs (Phelps *et al.*, 1998; McNally *et al.*, 1998; Rockne *et al.*, 2000).

Under aerobic conditions, however, numerous successful isolations of PAH-degrading microorganisms have been reported with the technique developed by Kiyohara *et al.* (1982). Briefly, the bacterial cultures were transferred to an agar plate with the appropriate growth medium. The surface of the agar plate was then sprayed with PAH solution in ether, and ether was allowed to evaporate from the surface. Following ether vaporization, a visible coating of the PAH on the surface of the agar plate remains. After the plates are incubated, colonies exhibiting PAH degradation can be detected by the appearance of clear zones surrounding the colony due to PAH uptake and utilization. However, there are limitations for the use of this method. First of all, direct contact of highly volatile solvent with inoculated bacteria on the plates may harmful to the bacteria. Because PAHs are highly toxic, great care must be taken not to contaminate a large area by spraying. In addition, it is difficult to spray plates while maintaining strict sterile conditions. Another alternative isolation method was described by Bogardt and Hemmingsen (1992). In this method, an ethanol:PHE solution was added to molten, cooled agarose and mixed by vortexing, resulting in a cloudy agarose solution because of the evenly dispersed fine PHE particles. After serial

dilutions of culture were added to this agarose solution, it was poured onto the surface of already solidified agar plates. Ethanol was utilized to dissolve PHE and disperse PHE, which is nearly water-insoluble, evenly within the agarose. PHE-degrading organisms were recognized by the clear zones resulting from the disappearance of PHE particles around colonies. However, this method involves the contact of incorporated solvent with bacteria within the agarose solution. Since the PHE particles are imbedded in the agarose overlayer, the clear zones would be less distinct than those by the spray-plate method. Recently, Alley and Brown developed a sublimation method to generate the visible white layer of water-insoluble compounds on to the agar surface without the use of solvents (Alley and Brown, 2000). In this method, an inoculated petri plate was inverted and placed in the heated aluminum dish containing the compound to be sublimed. While the inverted petri dish was cooled by the second aluminum dish containing ice on the petri dish, the compounds were deposited by sublimation. This method may solve some of the problems existing with other two isolation methods stated above; however, it involves a relatively complicated experimental apparatus. Furthermore, it takes considerable time and care to generate an even, visible layer of PAHs because the appropriate temperature should be maintained to sublime compounds.

2.3 Metabolism of PAH biodegradation

The genes encoding enzymes in the aerobic degradative pathway of several aromatic hydrocarbons such as toluene, NAP and PHE have been isolated, and research has shown that these genes are usually plasmid-borne (Burlage *et al*., 1989; Foght and Westlake, 1991; Menn *et al*., 1993, Sanseverino *et al*., 1993). For example, one of the

most extensively studied catabolic plasmid families, the TOL plasmids, bears the genes for toluene degradation (Burlage *et al.,* 1989). Similarly, genes for the aerobic degradation of NAP in pseudomonads were found to be located in relatively large plasmids such as the NAH7 plasmid (Eaton, 1994; Sanseverino et *al*., 1993; Yen and Serdar, 1988).

As opposed to PAH degradation under aerobic conditions, very little information is currently available on the genetic and regulatory mechanisms involved in the anaerobic microbial catabolism of PAHs. Very recently, Zhang and Young (1997) presented the first evidence for the identification of catabolic intermediates in the anaerobic degradation of NAP and PHE. In this report, carboxylation was proposed as the initial step in NAP and PHE degradation under sulfate reducing conditions. Intermediates were identified as 2-naphthoic acid and phenanthroic acid, which are structurally similar to benzoates. In another recent study, anaerobic degradation of NAP by a sulfate-reducing enrichment culture was confirmed by substrate utilization test and identification of metabolites by gas chromatography-mass spectroscopy (GC/MS) (Meckenstock *et al.*, 2000). In this study, 2-naphthoic acid was identified as a prominent metabolite in NAP-degrading cultures, which supports the results obtained by Zhang and Young (1997). Meckenstock *et al.* (2000) also observed incorporation of [^{13}C] bicarbonate into the carboxylic group of 2-naphthoic acid, indicating that NAP was activated through addition of a C1 compound. In another study, naphthalenol was detected as an intermediate consistently by GC/MS analysis in anaerobic NAP degradation by a sulfidogenic culture (Bedessem *et al.*, 1997). This study proposed hydroxylation as the initial step in NAP degradation under sulfate-reducing conditions.

However, hydroxylated intermediates were not identified by GC/MS in the study by Meckenstock *et al*. (2000). Further understanding of the mechanisms of anaerobic PAH degradation under sulfate-reducing conditions, however, remains unknown due to the lack of isolates. Besides, no information is available on the metabolism of PAH biodegradation under nitrate-reducing and methanogenic conditions.

Chapter 3 Characterization of anaerobic PAH-degrading enrichment cultures

3.1 Abstract

Previous research demonstrated that methanogenic cultures enriched from Baltimore Harbor (Baltimore, MD) sediments were able to degrade NAP and PHE. This research focuses on the characterization of anaerobic polycyclic aromatic hydrocarbons (PAHs)-degrading cultures under methanogenic conditions. The microbial consortia of PAHs-degrading cultures were monitored via comparative sequence analysis of genes coding for 16s rDNA. By using this analysis with "universal primers", the predominant RFLP types exhibited the high sequence similarities to *Clostridia* and to the δ-subgroup of sulfate-reducers. Several RFLP types had high sequence similarity to clones identified in a sulfate-reducing consortium, which mineralizes benzene. To further identify the PAH-degrading consortia, methanogen-specific primers have been designed and utilized to characterize the methanogenic population. Based on the acquired information, targeted isolation strategies have been designed.

3.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing two or more fused aromatic rings. They are produced in a variety of processes such as the incomplete combustion of fossil fuels and as a byproduct of coke production and petroleum refining (McNally *et al*., 1998). The environment, especially aquatic sediments, has suffered from the contamination of PAHs due to fuel spills, runoff,

sewage treatment plants, petrochemical industrial effluents, etc. (Cerniglia, 1992). PAHs can be classified into two classes based on the number of aromatic rings; low molecular weight PAHs with 2 or 3 aromatic rings and high molecular weight PAHs with 4 or more aromatic rings.

To remediate PAH-contaminated sites, aerobic PAH degradation has been studied extensively (Barnsley, 1983; Bauer and Capone, 1985; Heitkamp *et al*., 1988; Cerniglia, 1992; Kim *et al*., 1996; Moody *et al*., 2001); however, the hydrophobicity and the low water solubility of PAHs cause their accumulation in subsurface sediments where anaerobic processes become important. Only recently have several studies been conducted to investigate PAH degradation under various anaerobic conditions such as nitrate-reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 1998; Rockne *et al.*, 2000), sulfate-reducing conditions (Coates *et al.*, 1997; Langenhoff *et al.*, 1996; Zhang and Young, 1997) and methanogenic conditions (Chang *et al.,* 2001).

In a previous study in our research group, highly enriched anaerobic cultures capable of degrading both naphthalene (NAP) and phenanthrene (PHE) under methanogenic conditions were established (Chang, 2003). Briefly, enrichment cultures were initiated by transferring Baltimore Harbor sediments (10% [wet wt/vol]) into sterile estuarine medium anaerobically and incubating in a serum bottle under N_2:CO_2:H_2 (15:4:1). NAP and PHE (from Sigma Chemical Co., St. Louis, MO) were solubilized in acetone and supplemented to the cultures to provide initial PAH concentrations of 200μM. PAHs in the slurry cultures were extracted with hexane by shaking overnight and quantified using gas chromatography (GC) with flame ionization detection (FID). As a result, concentrations of NAP and PHE dropped below detection

limits (3 μM) after 286 and 168 days of incubation, respectively (Figure 3.1). After several refeeding of respective PAH, enrichment cultures were transferred to fresh medium (10% [vol/vol]). The transferred cultures showed immediate and continuous degradation of NAP and PHE over 160 days (Figure 3.2), demonstrating sustained PAH degrading activity without the addition of sediment.

In this study, characterization of anaerobic PAH-degrading enrichment cultures was performed with the transferred cultures because more enriched PAH-degrading cultures and reduced community diversity are expected with less sediment in which undefined nutrients might be present. Microbial consortia enriched for PAH degradation were analyzed by comparative sequence analysis of genes coding 16S rDNA amplified from total community DNAs (Holoman *et al.*, 1998). Briefly, genomic DNA is extracted from the enrichment cultures, the 16S rDNA is amplified by polymerase chain reaction (PCR) using universal and archaeal primers, a partial 16S rDNA PCR fragments clone library is constructed in *E. coli*, the clones are screened by restriction fragment length polymorphism analysis (RFLP), and unique clones are sequenced for comparative sequence analysis. Information obtained via phylogenetic analysis will be utilized to design targeted isolation strategies for anaerobic PAH-degrading microorganisms.

3.3 Materials and Methods

Extraction of Genomic DNA The methods described previously by Holoman *et al.* (1998) were employed here for the phylogenetic analysis of the enrichment cultures. Two or three ml of the cultures were withdrawn anaerobically in the Coy Anaerobic

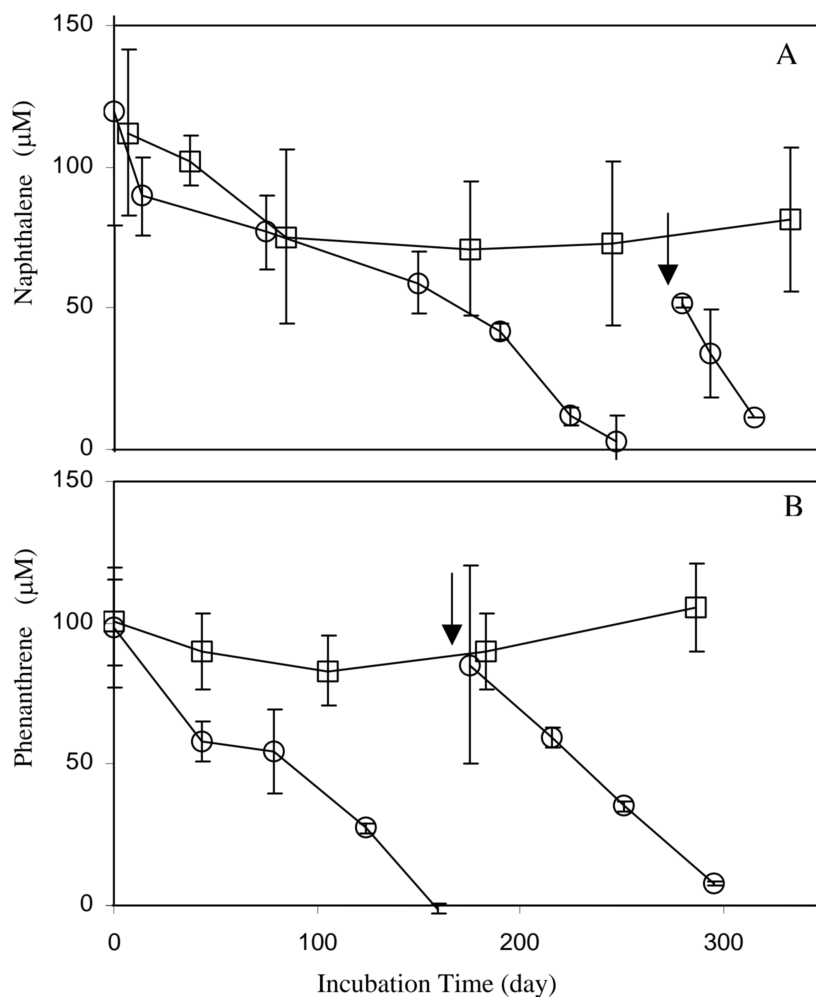

Figure 3.1 Initial degradation of naphthalene (A), phenanthrene (B) in Baltimore Harbor sediment- inoculated, methanogenic enrichment cultures with refeeding (→) of naphthalene (A) and phenanthrene (B) on day 286 and 168, respectively. The results are the means of triplicates for active cultures (O) and the means of duplicates for sterile controls (□).

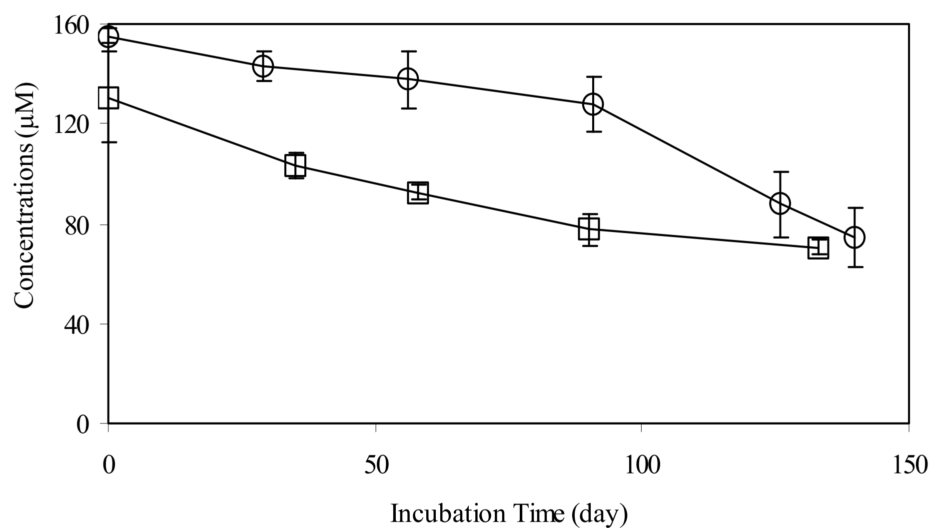

Figure 3.2 Initial degradation of naphthalene (□) and phenanthrene (O) in methanogenic enrichment cultures after the first transfer (10% [vol/vol]). The results are the means of triplicates.

Chamber (Coy Manufacturing Co., Ann Arbor, MI) and centrifuged at 14,000 rpm. The resulting pellet was resuspended in 250 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]), and placed in a screw-cap conical tube that contained 2.5g of autoclaved zirconia-silica beads (0.1mm) and 250 µl each of sodium phosphate buffer (0.1M, pH 8.0) and TS-SDS buffer (0.1M NaCl, 0.5M Tris [pH 8.0], 10% [wt/vol] sodium dodecyl sulfate). The sample was cooled on ice for 10 minutes and then placed in a Minibeater bead mill homonizer (Biospec Products) to lyse the cells for 5 minutes at 5,000 rpm at 4 ^{o}C. The cell debris and beads were removed by centrifugation at 14,000 rpm. Crude DNA in the supernatant was purified twice with equal volumes of tris-saturated phenol and chloroform-isoamyl alcohol (24:1), followed by extraction with an equal volume of chloroform. Phase-Lock gel (5-Prime-3 Prime, Inc., Boulder, CO.) was used to promote the separation of the phases. The aqueous phase was diluted to 1 ml with sterile distilled water and 0.15 g of insoluble polyvinylpolypyrrolidone (PVPP)(Sigma, St. Louis, Mo) was added to extract humic acids, which inhibit PCR (Tebbe and Vahjen, 1993). After PVPP was removed by centrifugation, the genomic DNA was precipitated with an equal volume of isopropanol at -20°C. The sample was centrifuged, and then the pellet was washed with 70% ethanol and centrifuged again. The supernatant was discarded and the DNA pellet was dried, then resuspended in 20µl of sterile distilled water. The resuspended DNA extract was loaded on a 1.3% low-melt agarose gel containing 2% polyvinypyrolidone with loading dye to further bind any residual humic acids by electrophoresis. The genomic DNA was recovered from the gel with a Promega Wizard PCR Prep Kit (Promega, Madison, Wis.).

16S rDNA amplification Bacterial and archaeal 16S rDNAs from the genomic DNA extracted from the enrichment cultures were amplified utilizing PCR. For the amplification of bacterial 16S rDNAs, universal primers 519F (5'-CAG CA/CG CCG CGG TAA TA/TC-3') and 1406R (5'-ACG GGC GGT GTG TA/GC-3') were utilized (Holoman *et al.*, 1998). Specific archaeal primers, 21F (5'-TTC CGG TGG ATC CYG CCG GA-3') and 958R (5'-TCC GGC GTT GAM TCC AAT T-3'), were utilized to amplify methanogen 16S rDNAs (Holoman *et al.*, 1998). GeneAmp PCR kits with *Taq* polymerase (Perkin Elmer, Inc.) were used for PCR. Conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94 ^{o}C; 30 amplification cycles of denaturation (30 s at 94 ^{o}C), annealing (30 s at 55 ^{o}C), and elongation (30 s at 72 ^{o}C); and a final extension step of 5 min at 72 ^{o}C. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) prior to ligation.

Cloning and restriction fragment length polymerphism (RFLP) analysis Purified PCR fragments were ligated into the *pCR*II vector with the Invitrogen TOPO TA cloning kit. (Invitrogen, Carlsbad, CA) to generate plasmid libraries for bacterial and archaeal 16S rDNA. The ligated plasmids were transformed into *Escherichia coli* competent cells supplied with the Invitrogen TOPO TA cloning kit. Eighty-four and forty-two clones were randomly chosen from bacterial and archaeal 16S rDNA libraries, respectively, and grown in Luria Broth (LB) with kanamycin (100μg/ml). These LB cultures were directly used for the partial 16S rDNA fragment amplification with the following PCR conditions: 1 cycle of 3min at 95 ^{o}C; 40 cycles of 95 ^{o}C for 30s; 55 ^{o}C for 30s, and 72 ^{o}C for 1min; and a final extension step of 72 ^{o}C for 5min. Two restriction endonucleases, *Hae III* and *Hha I* (New England Biolabs, Inc., Beverly, MA),

were used separately to digest the PCR products. The restriction digests were separated on a 3% Trevi-gel (TreviGen, Gaithersburg, MD) and visualized with ethidium bromide gel stain. Clones bearing unique restriction banding patterns were categorized.

Comparative 16S rDNA sequence analysis The unique RFLP patterns obtained for at least two clones were analyzed further so that the population of predominant microorganisms can be obtained via comparative 16S rDNA sequence analysis. Plasmid DNA was purified with the Qiagen Plasmid Mini kit (Qiagen, Inc.) and sequenced via the DNA sequencing facility at the University of Maryland Biotechnology Institute. The sequences were entered into the GenBank BLAST search program at the National Center for Biotechnology Information (NCBI) to obtain the closest phylogenetic relatives (Altschul *et al*., 1990).

3.4 Results and Discussion

Characterization of NAP-degrading enrichment cultures Microbial community profiles of NAP-degrading enrichment cultures were monitored at forty days after the second sequential transfer. Eighty-four randomly chosen clones from a bacterial library were screened by RFLP analysis. These clones exhibited thirteen predominant RFLP patterns representing at least two clones. 16S rDNA fragments for each predominant RFLP pattern were sequenced, and these sequences were analyzed with the GenBank BLAST search program to get the closest phylogenetic relatives. Table 3.1 shows the closest phylogenetic relatives to each predominant RFLP type in both the bacterial and the archaeal domain. In Table 3.1, frequency is based on the total number of clones (84 for bacterial domain and 42 for archaeal domain). The sequence similarity (%) is

Table 3.1 Phylogenetic affiliations of predominant RFLP types from NAP-degrading cultures

RFLP type	Frequency* (%)	Closest phylogenetic relative	Sequence Similarity (%)	Putative division
With universal primers				
U1	7.1	Methanococcus vannielii	98	*Methanococcales*
U2	2.4	Uncultured bacterium BURTON-35	97	green sulfur bacteria
U5	8.3	Unidentified eubacterium RFLP1	98	*Thermotogales*
U6	4.8	Uncultured bacterium SJA-117	91	green non sulfur bacteria
U7	4.8	Bacterium 2BP-6	99	*Proteobacteria* (delta)
U8	4.8	Bacterium Phenol-1	91	*Proteobacteria* (gamma)
U10	6.0	*Acidaminobacter hydrogenoformans*	99	low G+C gram positive
U11	10.7	Uncultured bacterium BB48	91	·
U17	2.4	Sulfate-reducing bacterium R-PropA1	95	*Proteobacteria* (delta)
U19	7.1	Uncultured bacterium SJA-68 16S rRNA	91	green non sulfur bacteria
U20	6.0	Uncultured bacterium SHA-21 16S	91	green non sulfur abcteria
U37	2.4	*Pseudomonas cf. Pseudoalcaligenes*	99	*Proteobacteria* (gamma)
U38	2.4	Uncultured *actinomycete* clone SUBT-5	98	high G+C gram positive
With archaeal primers				
A1	52.0	Methanococcus vannielii	98	*Methanococclaes*
A2	20.0	Methanococcus maripaludis	97	*Methanococclaes*

*Based on the total number of randomly chosen colonies (84 for bacterial and 42 for archaeal domain)

determined by the BLAST search program. In phylogenetic analysis, it is generally accepted that species with homology over 95% can be defined as specific strains and that values between 85 and 95% are used to group organisms belonging to genera (Ficker *et al*., 1999; Weisburg *et al*., 1991). As shown in Table 3.1, the most predominant RFLP type in the bacterial domain, U11, accounting for 10.7% of the total universal clones, has the highest sequence similarity to an uncultured bacterium BB48 which was found in an anaerobic digester (Godon *et al.*, 1997). No further information on phylogeny was available for this bacterium. The other RFLP types exhibited broad diversity within the domain *Bacteria* with distributions, as follows: 17.9% to green non-sulfur bacteria, 14.4% to the *Proteobacteria* (7.2% to the delta and 7.2% to the gamma subgroup), 8.3% to the *Thermotogales*, 6.0% to low G+C gram positive bacteria, 2.4% to the high G+C gram positive bacteria, and 2.4% to the green sulfur bacteria. Among the RFLP patterns with universal primers, only one RFLP type U1 was identified to be most closely related to the *Methanococcus* genus which belongs to the *Archaeabacteria* group. Three RFLP types (U6, U19, U20) previously detected in anaerobic dechlorinating microbial consortia fall within the green non-sulfur bacteria. Specifically, RFLP type U6 and U19 were found in an anaerobic trichlorobenzene-transforming microbial consortium (von Wintzinggerode *et al.*, 1999), and RFLP type U20 was detected in an anaerobic 1,2-dicholropropane-dechlorinating mixed culture (Schlotelburg *et al.,* 2000). Additionally, the closest phylogenetic relatives to RFLP types U5, U7, U8, and U37 were reported to belong to the following contaminant-degrading microbial communities from previous studies: U5, 2,3,5,6-tetrachlorobiphenyl-ortho-dechlorination microbial consortia (Holoman *et al*., 1998):

U7 and U8, bromophenol-dehalogenating and phenol-degrading consortia (Knight *et al*., 1999): U37, diclofop-methyl-degrading biofilm consortia (Laramee *et al*., 2000). These various hydrocarbon-degrading consortia may be involved in the degradation of NAP in our enrichment cultures.

From forty-two randomly chosen clones in the archaeal library, two predominant RFLP types, A1 and A2, were identified to be most closely related to the *Methanococcus* genus (Table 3.1). *Methanococcus* genus is known to produce CH_4 in the presence of H_2 and CO_2 (Holt *et al*., 1994). No information is available on whether the closest phylogenetic relatives to RFLP type A1 and A2 are capable of degrading contaminants

As mentioned before, RFLP types revealed broad diversity within the domain *Bacteria* because a certain amount of sediment was still present in cultures and may provide unidentified nutrients and electron acceptors, resulting in the growth of non-NAP degrading organisms. Accordingly, it is hard to elucidate the role of each species on anaerobic NAP degradation under methanogenic conditions. However, it is interesting to point out that most RFLP types except U5 were not detected at all in NAP-free cultures (Chang, 2003), suggesting that the microbial profiles were changed dramatically in the presence of NAP. Interestingly, no A*rchaeal* 16S rDNA amplification could be detected with NAP-free cultures in spite of extensive PCR analysis by optimizing protocols such as annealing temperature, the concentration of template DNA, etc. (Chang, 2003), implying the presence of NAP might stimulate the growth of methanogens utilizing the metabolic intermediates directly or indirectly during the NAP degradation.

To isolate NAP-degrading microbes, appropriate cultivation conditions should be chosen based on the information obtained from the microbial population profiles. In our enrichment cultures, NAP served as a carbon source in the minimal mineral medium containing NH_4Cl and $Na_2S \cdot 9H_2O$ as a nitrogen and sulfur source, respectively. Among thirteen RFLP types from universal primers, U7 and U17 have the highest sequence similarity to the sulfate-reducing bacteria in the delta subgroup of the *Proteobacteria*. Other RFLP types do not require specific electron acceptors such as nitrate and sulfate. Since methanogens were also detected with bacterial and archaeal primers, desirable isolation strategies for NAP-degrading microbes will be sulfate-reducing conditions and methanogenic conditions.

Characterization of PHE-degrading enrichment cultures Community profiles analyzed at 54 days after the first sequential transfer of PHE-degrading enrichment cultures are shown in Table 3.2. From the bacterial library, eleven predominant RFLP patterns, which had at least two representative clones, were analyzed to determine the closest phylogenetic relative. All RFLP types fell within three divisions of the *Bacteria* domain including low G+C gram positive, the delta subgroup of the *Proteobacteria*, and green non-sulfur bacteria, accounting for 73.9%, 7.2%, and 4.8% of the randomly chosen 84 universal clones, respectively. While the most predominant RFLP type in NAP-degrading microbial profiles (U11) represented 10.7% of the total population, two RFLP types in PHE-degrading cultures, U27 and U44, accounted for 22.6% and 21.4%respectively. RFLP type U27 showed the highest sequence similarity to the unidentified eubacterium RFLP 15 detected in the tetrachlorobiphenyl-dechlorinating microbial community (Holoman *et al*., 1998). This unidentified eubacterium RFLP 15

Table 3.2 Phylogenetic affiliations of predominant RFLP types from PHE-degrading cultures

RFLP type	Frequency* (%)	Closest phylogenetic relative	Sequence Similarity (%)	Putative division
With universal primers				
U10	4.8	Acidaminobacter hydrogenoformans	99	low G+C gram positive
U27	22.6	Unidentified eubacterium RFLP15	96	low G+C gram positive
U43	10.7	*Clostridium ultunal*	92	low G+C gram positive
U44	21.4	*Desulfosporosinus* sp.	88	low G+C gram positive
U46	6.0	*Paenibacillus* sp.	89	low G+C gram positive
U48	6.0	Uncultured bacterium SJA-118	97	low G+C gram positive
U49	3.6	Benzene mineralizing consortium clone SB-1	98	*Proteobacteria* (delta)
U51	2.4	Uncultured eubaterium WCHBI-54	95	low G+C gram positive
U55	2.4	Uncultured eubacterium WCHA1-69	93	green non-sulfur bacteria
U57	3.6	Uncultured eubacterium	88	*Proteobacteria* (delta)
U65	2.4	Uncultured bacterium PENDANT-36	90	green non-sulfur bacteria
With archaeal primers				
A3	47.4	*Methanosarcina* sp. FR	98	Methanosarcinales
A4	31.6	Methanosarcina mazei	99	Methanosarcinales

*Based on the total number of randomly chosen colonies (84 for bacterial and 42 for archaeal domain)

was analyzed to be most closely related to the *Clostridium* genus as reported by Holoman *et al*. (1998). RFLP type U44 falls into the *Desulfosporosinus* genus, which was identified in an aquifer contaminated with gasoline (Roberston *et al*., 2000). In addition to RFLP type U27 and U44, the known various hydrocarbon-degrading consortia were found to be the closest phylogenetic relatives to RFLP 48, 49, 51, 55 and 57 as follows: U48, trichlorobenzene-transforming microbial consortium (von Wintzingerode *et al*., 1999): U49, benzene mineralizing sulfate-reducing consortium (Phelps *et al*., 1998): U51and U55, hydrocarbon- and chlorinated-solvent-degrading consortia (Dojka *et al*.,1998): U57, hydrocarbon-degrading sulfate-reducing bacteria (Rueter *et al*.,1994). Interestingly, even though sulfate was not added to the cultures, three RFLP types (U44, U49, U57) accounting for 28.6% of the total clones belonged to the sulfate-reducing bacteria, which is a relatively higher detection frequency than that detected in NAP-degrading cultures. Indeed, recent studies have demonstrated coexistence of sulfate-reducing bacteria and methanogens in sulfate-depleted environments (Raskin *et al*, 1996; Tasaki *et al*., 1993). Some species of the *Desulfosporosinus* (previously *Desulfotomaculum*) are known as sulfate-reducing bacteria, which can grow by utilizing fatty acids in the absence of sulfate (Tasaki *et al*., 1993). This could explain the predominance of the *Desulfosporosinus* genus representing 21.4% of the total population in PHE-degrading cultures. The predominant microbial populations of the NAP-degrading cultures and PHE-degrading cultures do not share common RFLP types except RFLP type U10. This result suggests that different microbial consortia may be responsible for the degradation of NAP and PHE due to the different physical and chemical properties such as water solubility,

hydrophobicity, and bioavailability (Piatt *et al*., 1996). No shared RFLP type between PHE-degrading cultures and PHE-free cultures was detected (Chang, 2003), demonstrating that the presence of PHE shifted the microbial population to optimize PHE degradation.

With the archaeal primers, two predominant RFLP types were identified. RFLP type A3 was most similar to *Methanosarcina* sp. FR found in a perchloroethylene-degrading consortium (Cabirol *et al*., 1998). The other RFLP type, A4, exhibited the closest sequence similarity to *Methanosarcina mazei* from an anaerobic trichlorobenzene transforming microbial consortium (von Wintzingrode and Goebel, directly submitted to GenBank). Interestingly, *Archaeal* communities with NAP cultures were totally different from those of PHE cultures. While the *Methanococcus* species, which are hydrogenotrophic methanogens, were detected in NAP cultures, the *Methanosarcina* species, which are acetate-utilizing methanogens, were found in PHE cultures. This phenomenon might result from the significantly different bacterial communities between NAP- and PHE-degrading cultures, accordingly affecting archaeal communities which usually utilize the bacterial metabolic intermediates. In fact, RFLP type 10, closely related to hydrogen-producing bacteria, was detected in NAP cultures, which might support the growth of hydrogen-utilizing *Archaea*l species such as the *Methanococcus* species. In PHE cultures, on the contrary, RFLP types U27 and U44, related to *Clostridium* sp. and *Desulfosporosinu*s sp., respectively, were found which are known to produce acetate as a major product (Fendrich *et al*., 1990; Robertson *et al*., 2000). Results gained from the microbial population of PHE-

degrading cultures will be used to design targeted isolation strategies again for methanogens and a sulfate-reducing consortium.

In conclusion, the microbial community structures of PAH-degrading methanogenic cultures were monitored by employing molecular characterization via comparative 16S rDNA sequence analysis. It was revealed that the microbial community in NAP-degrading cultures was significantly different from that of PHE-degrading cultures, suggesting different microbial consortia may be required for NAP and PHE degradation. The results obtained here will be utilized to design isolation strategies for the possible microbial species responsible for PAH-degradation.

Chapter 4 Modified isolation method for bacteria degrading solid PAHs on the agar-overlay plate and its use under anaerobic conditions

4.1 Abstract

A modified plating method was developed for detecting microorganisms degrading solid PAHs on an agar-overlay plate under aerobic conditions as well as anaerobic conditions. In this technique, a molten agar solution containing PAH-degrading microorganisms was spread on an agar plate which had a visible white layer of PAHs generated by spreading and evaporating NAP:methanol or PHE:acetone solutions. After incubation, colonies exhibiting hydrocarbon degradation were detected by the appearance of clear zones surrounding the colony due to hydrocarbon uptake and utilization. By preparing agar plates and solvents anaerobically, this method was successfully applied under anaerobic conditions. To our knowledge, this is the first report to utilize clear zones on the agar plate for the isolation of anaerobic PAH-degrading microorganisms.

4.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are among the most widely distributed organic contaminants in aquatic sediments because of their presence in coal and petroleum. Their toxicity, due to carcinogenic and mutagenic properties (Keith and Telliard, 1979), has led to considerable research efforts to understand the microbial degradation of PAHs in contaminated sites. Until recently, research on the

biodegradation of PAHs has focused primarily on aerobic biological processes (Barnsley, 1983; Bauer and Capone, 1985; Heitkamp *et al*., 1988; Cerniglia, 1992; Moody *et al*., 2001). Although it had been thought that PAHs were recalcitrant to biodegradation without oxygen (Bauer and Capone, 1985; Evans and Fuchs, 1988), recent studies have demonstrated PAH degradation under anaerobic conditions (Coates *et al*., 1997; Langenhoff *et al.*, 1996; Zhang and Young, 1997). Isolation of PAH-degrading microorganisms has been attempted in the last few decades to gain a detailed understanding of the metabolism of PAH biological degradation with pure cultures. Among the isolation techniques, the methods using zones of clearing on agar plates, developed by Kiyohara *et al*. (1982), Bogardt and Hemmingsen (1992), and Alley and Brown (2000) have been widely used to screen PAH-degrading microorganisms under aerobic conditions.

The spray-plate method developed by Kiyohara *et al*. (1982) has been utilized and cited by numerous researchers for the isolation of the microorganisms degrading water-insoluble hydrocarbons. This method involved an agar plate that was inoculated with bacteria and sprayed with PAH solution in ether. Ether was allowed to evaporate from the surface, resulting in a visible coating of the PAH on the surface of the agar plate. After the plates were incubated, colonies exhibiting PAH degradation could be detected by the appearance of clear zones around the colony due to PAH uptake. Though this method has been widely used to isolate PAH-degrading microbes, it has several disadvantages. The direct contact of a highly volatile solvent with inoculated bacteria on the plates may possibly kill the bacteria. Because PAHs are highly toxic, great care must be taken not to contaminate a large area by spraying, and personal protection

should be involved. In addition, it is difficult to spray plates while maintaining strict sterile conditions.

Another method, the overlayer technique developed by Bogardt and Hemmingsen (1992), could be utilized for the isolation of PHE-degrading bacteria in an aseptic manner. In this method, an agarose-overlayer containing bacteria and ethanol solutions of PHE was poured onto the surface of a prepared agar underlayer. Since the agar overlayer was opaque due to the evenly dispersed fine PHE particles, PHE-degrading colonies could be screened by detecting clear zones around colonies. However, this method also involves the contact of incorporated solvent with bacteria when they are mixed in the agarose-overlayer. Since the PHE particles are imbedded in the agarose overlayer, the clear zones would be less distinguishable than those by the spray-plate method, especially with small colonies. Recently, Alley and Brown developed a sublimation method to generate the visible white layer of water-insoluble compounds on to the agar surface without the use of solvents (Alley and Brown, 2000). With this method, an inoculated petri plate was inverted and placed in the heated aluminum dish containing the compound to be sublimed. While the inverted petri dish was cooled by the second aluminum dish containing ice on the petri dish, the compounds were deposited by sublimation. This method potentially avoids some of the problems discussed above; however, it involves a relatively complicated experimental apparatus. Additionally, it takes considerable time and care to generate an even, visible layer of PAHs by maintaining the appropriate temperature necessary to sublime compounds. The residual compounds remaining in the heated aluminum dish after the sublimation would also be a problem.

Despite several problems with previously developed isolation methods mentioned above, numerous successful isolations of PAH-degrading microorganisms have been reported by utilizing those methods under aerobic conditions (Churchill *et al*., 1999; Geiselbrecht *et al.*, 1996; Heitkamp *et al*., 1988; Daane *et al*., 2001). As opposed to aerobic conditions, however, there is no published report on the isolation of PAH-degrading microbes under anaerobic conditions by utilizing those methods. In anaerobic conditions, the conventional serial dilution and plating methods have been generally utilized to obtain pure cultures. However, the slow growth of PAH-degrading microorganisms could cause the outgrowth of bacteria growing on nutrient content in the media rather than the intended PAHs (Phelps *et al.*, 1998; McNally *et al.*, 1998; Rockne *et al.*, 2000). As a result, the isolation of PAH-degrading microbes under anaerobic conditions has not been successful (Heider *et al.*, 1999). Until recently, there are only three reports describing pure cultures with the ability to degrade PAHs, including two reports under nitrate-reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 2000) and one report demonstrating the isolation of a NAP-degrading sulfate-reducing bacterium (Galushko *et al*., 1999). Consequently, there has been a need for effective isolation methods not only applicable under anaerobic conditions but also solving some of the problems with previously developed isolation methods.

In this chapter, we describe a modified isolation method for PAH-degrading microorganisms that could be used under aerobic conditions and, with additional modification, are applicable under anaerobic conditions as well. This technique involved an agar plate (bottom agar) which has a visible white layer of PAHs on the surface created by spreading and evaporating NAP: methanol or PHE: acetone solutions.

The resulting plate was then spread with molten agar (top agar) containing bacteria without disturbing hydrocarbons layer. This simple, but significant modification allows us to avoid the problems such as the direct contact of a volatile solvent with bacteria and contamination of the working area by spraying toxic PAH solutions while zones of clearing can still be easily detected with a visible PAH layer. We also evaluated the applicability of this technique under anaerobic conditions by preparing agar plates and PAH solutions anaerobically.

4.3 Materials and Methods

Organisms Three species of aerobic PAH-degrading microorganisms were used to evaluate the modified isolation method. The NAP-degrading microorganism, *Pseudomonas fluorescens* Uper-1 (Brian *et al*., 1998), was provided by Dr. Donald R. Lueking in the Department of Biological Sciences, Michigan Technological University. *Pseudomonas putida* CRE7, a PHE-degrading aerobic microorganism (Zhang *et al*., 1997), was obtained from Dr. Eric A. Seagren in the Department of Civil and Environmental Engineering, University of Maryland, College Park. Dr. Seagren obtained *P. putida* CRE7 originally from Dr. Raina M. Maier in the Department of Soil, Water and Environmental Science at University of Arizona. *Mycobacterium sp*. strain PYR-1 (NRRL B-24157), capable of degrading NAP, PHE and PYR (Heitkamp and Cerniglia, 1989), was obtained from Dr. David P. Labeda, Agricultural Research Service Culture Collection, Illinois. Under anaerobic conditions, NAP- and PHE-degrading anaerobic enrichment cultures that have been studied in our lab (Chang *et al*., 2001) were employed to examine the isolation method. Also, NAP-degrading sulfate-

reducing bacterium, NaphS2 (Galushko *et al.*, 1999), was obtained from Dr. Alexander Galushko, University Konstanz, Germany.

All microorganisms were maintained on either the mineral salt agar recommended in each reference with the corresponding PAH as sole carbon source (Brian *et al*., 1998; Zhang *et al*., 1997; Heitkamp and Cerniglia, 1989; Galushko *et al.*, 1999) or R2A medium for aerobic bacteria (Difco Inc., Detroit, MI).

Anaerobic agar plate preparation The medium was prepared anaerobically in an atmosphere that contained N_2:CO_2 (4:1) (Sowers and Noll, 1995). The estuarine medium for PAH-degrading methanogenic cultures contained (per liter of distilled water) 8.4g of NaCl, 3.95g of $MgCl_2 \cdot 6H_2O$, 0.27g of KCl, 3.0g of $CaCl_2 \cdot 2H_2O$, 0.5g of NH_4Cl, 3.0g of Na_2CO_3, 1.12g of Na_2HPO_4, 0.25g of cysteine-HCl·H_2O, 10ml of vitamin and trace metal solutions (Wolin *et al*., 1963), and 1ml of resazurin solution. Resazurin was added as a redox indicator that is initially colorless but changes pink in the presence of oxygen (Sowers and Noll, 1995). For the sulfate-reducing conditions, 12.32g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water was added to the estuarine medium. For NapS2 cultures, defined bicarbonate-buffered, sulfide-reduced mineral agar medium was used (Galushko *et al.,* 1999). After the pH of the media was adjusted to 6.8, media (100ml) was transferred to 160 ml serum vials that contained 1.25% or 0.5% (w/v) agar for bottom or top agar, respectively (Apolinario and Sowers, 1996). The vials were sealed with Teflon-coated rubber stoppers and aluminum crimp seals under a N_2:CO_2 (4:1) headspace. Media containing agar was autoclaved at 121 ^{o}C for 20min and cooled to about 60 ^{o}C. To make bottom agar, cooled molten agar medium was transferred to the Coy Anaerobic Chamber and poured into sterile polystyrene petri

plates (60×15mm). Plates were dried in the Coy Anaerobic Chamber for 2 days before use as recommended by Apolinario and Sowers (1996). The anaerobic chamber contained an atmosphere of N_2: CO_2: H_2 (15:4:1).

Modified plating method The surface of bottom agar was spread with either 0.3ml of 5% w/v NAP:methanol solution or 2% w/v PHE:acetone solution. Methanol and acetone were chosen as solvents for NAP and PHE, respectively after several solvents were tested to generate uniform white layers of PAHs. The solvents were evaporated immediately and a visible thin white layer of PAHs remained. To inoculate bottom agar, 0.1ml serially diluted PAH-degrading cultures was mixed with 0.9ml of 37 ^{o}C 0.5% molten agar solutions (top agar) and poured on bottom agar, followed by swirling the plates to cover the surface. As this point, due to the weak adherence of PAHs to the bottom agar, the white layers of PAHs moved to the surface of top agar although top agar was poured onto the white coatings of PAHs. In this procedure, the plates should be swirled carefully to avoid disturbing the white layer of PAHs. Inoculated plates were then incubated at room temperature and examined daily to detect clear zones.

For anaerobic conditions, acetone was purged with ultra pure N_2 gas for an hour to remove dissolved oxygen prior to use. After acetone was transferred into the Coy chamber, 0.1% (w/v) cysteine-HCl·H_2O was added to lower the redox potential (Sowers and Noll, 1995). All other procedures were the same as those stated in aerobic conditions, but performed in the Coy Anaerobic Chamber. Inoculated plates were placed in wide-mouth glass canning jars (Ball Corporation, El Paso, TX) with a 5ml serum vial containing 1 ml of 2.5% $Na_2S \cdot 9H_2O$ solution that serves as a reducing agent

(Sowers and Noll, 1995). The plates were incubated at room temperature under N_2: CO_2: H_2 (15:4:1) in the jars and examined monthly to detect clear zones.

4.4 Results

Detection of clear zones by the modified isolation method under aerobic conditions A modified isolation method was examined with known PAH-degrading microorganisms under aerobic conditions. As shown in Figure 4.1, colonies of *P. fluorescens* Uper-1, NAP-degrading bacterium, were distinguished with clear zones on the white layer of NAP after 3 days of incubation. After *P. putida* CRE7 was incubated for 1 to 2 days on the agar plates with white PHE layer, clear zones started to appear even when colonies were too small to detect. After incubating for 5 days, agar plates revealed distinct clear zones as *P. putida* CRE7 was growing by the uptake of PHE on the top agar (Figure 4.1). Another PHE-degrading bacteria, *Mycobacterium sp*. strain PYR-1, was also incubated on the prepared agar plates. The colonies were observable after one week, but clear zones were not distinguished on the PHE layer until 3 weeks of incubation (Figure 4.1). As shown in Figure 4.1, the surface of top agar was evenly covered with a white PAH layer even after molten top agar solution was poured on the PAH layer. Pouring the top agar solution over the PAH layer, however, should be done carefully because impacting molten agar solution could disrupt the PAH layer. Flowing the molten agar solution along the wall of petri dish may minimize damage to the PAH layer.

Applications of the modified isolation method under anaerobic conditions NAP- and PHE-degrading enrichment cultures under methanogenic conditions (Chang *et al*.,

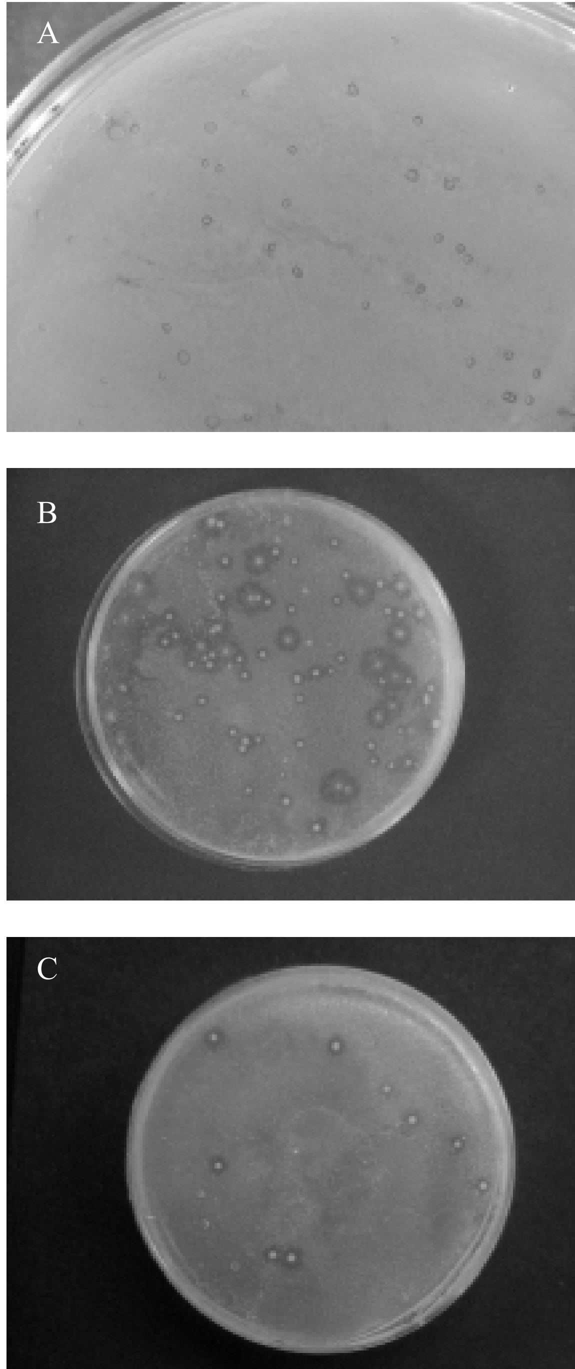

Figure 4.1 Clear zones on the white coating of NAP with *Pseudomonas fluorescens* Uper-1 (A) and on the PHE layer with *Pseudomonas putida* CRE7 (B) and *Mycobacterium sp*. strain PYR-1 (C).

2001) were utilized to evaluate the modified isolation method. Since sulfate-reducing bacteria (SRB) have been monitored in those enrichment cultures based on 16S rDNA analysis (Chang, 2003), both methanogenic and sulfate reducing conditions were employed. With the NAP-degrading enrichment cultures, numerous colonies could be detected on the agar plate as early as 30 days after incubation under both methanogenic and sulfate-reducing conditions, but no clear zones were found and the NAP-layer was less distinguishable. As the incubation time was extended up to 3 months, the colonies became larger, but the NAP layer was no longer visible because of the vaporization of NAP during incubation. With the NapS2 cultures, numerous colonies without detectable clear zones were also observed.

Unlike NAP, however, a white visible layer of PHE remained on the surface of the top agar after long periods of incubation because of the lower volatility of PHE compared to that of NAP. With PHE-degrading enrichment cultures, clear zones were detected both under methanogenic and sulfate-reducing conditions after 7 months of incubation (Figure 4.2). PHE-degrading colonies with clear zones were obviously distinguished from other colonies which did not generate clear zones. The clear zones under anaerobic conditions were less pronounced than those detected under aerobic conditions, implying slow PHE degradation under anaerobic conditions. Further study on anaerobic PHE-degrading colonies isolated by this method will be discussed in Chapter 5.

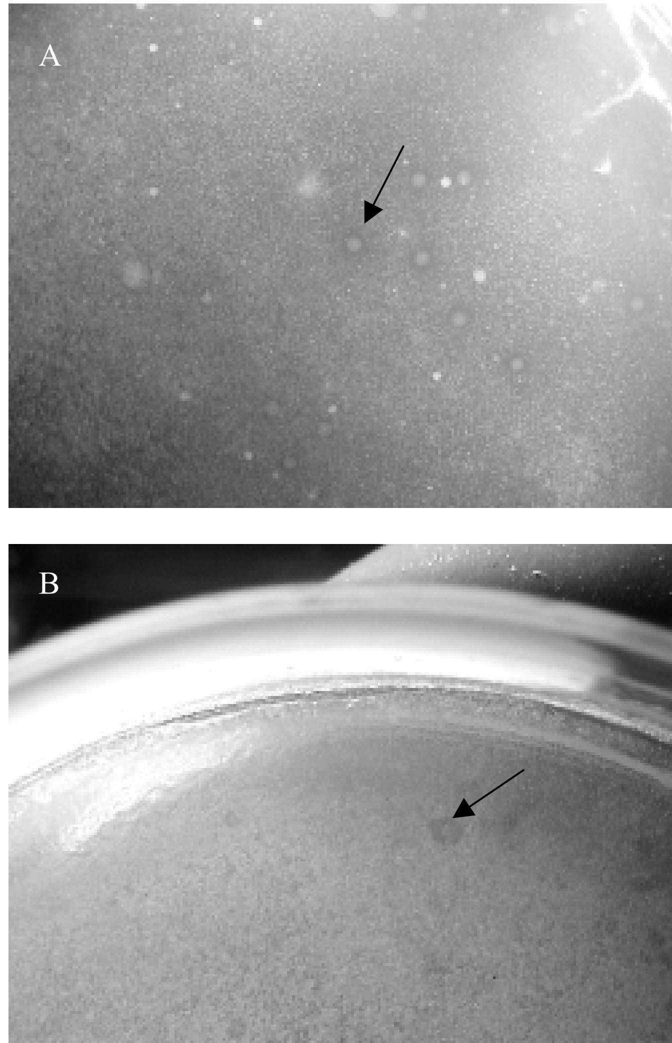

Figure 4.2 Clear zones on the white coating of PHE with PHE-degrading enrichment cultures under methanogenic conditions (A) and sulfate reducing conditions (B)

4.5 Discussion

Several isolation methods for PAH-degrading microorganisms utilizing zones of clearing on the agar plates have been utilized (Kiyohara *et al.,* 1982; Bogardt and Hemmingsen, 1992; Alley and Brown, 2000); however, those methods have some problems as stated before and cannot be employed under anaerobic conditions without modification. The modified isolation method presented in this study was designed to solve those problems and limitations.

In the modified isolation method, direct contact of solvent with bacteria could be avoided by creating the PAH layer first and then pouring molten agar solutions containing bacteria. As the PAH layer migrated to the top of molten agar solution, clear zones could be detected on the evenly coated PAH layer. More care was required to spread the molten agar solution over the NAP layer because the NAP layer was strictly adhered on the bottom agar possibly due to the less hydrophobicity than that of PHE and consequently it was not wetted well by molten agar solution like water droplets on a Teflon-coated pan. To solve this problem, after acetone was evaporated, the agar plate with NAP layer was covered with a lid and placed upside-down for 30 min. This procedure made the NAP layer's adhesion to the bottom agar weaker probably because of the moisture between the NAP layer and the bottom agar. Consequently,the NAP layer was easily separated from the bottom agar, allowing molten agar solution to be spread evenly without damaging the NAP layer. Alternatively, the PAH solution could be spread after the molten agar solution was solidified. However, this modification resulted in fewer colonies on the agar plate possibly due to the toxicity of the solvent on the bacteria at the top of the agar overlayer (data not shown). Distinct clear zones were

detectable even with small colonies after *P. putida* CRE7 was incubated for 1 day, whereas large colonies of *Mycobacterium sp*. strain PYR-1 did not reveal clear zones until incubated for 3 weeks. This result may indicate that the time for the formation of detectable clear zones is not affected by the size of colony, but influenced by the degradation ability of the microorganisms.

To apply the modified isolation method under anaerobic conditions, acetone was first purged with ultra pure N_2 gas for an hour. The purged acetone, however, made the solidified agar media turn pink in color when spread on the agar plates, indicating that the removal of dissolved oxygen was not sufficient to create the proper reduced conditions necessary for the growth anaerobic organisms. To lower the redox potential further, 0.1% (w/v) cysteine-HCl·H_2O, a reducing agent, was added to acetone. Accordingly, the further reduced acetone did not change the color of agar plate media, thus enabling us to utilize the modified plating method under anaerobic conditions. Under anaerobic conditions, clear zones on the NAP layer could not be observed with NAP-degrading methanogenic cultures and NapS2 because the NAP layer disappeared due to the volatilization of NAP during incubation. However, it would be possible to detect clear zones on NAP layer if there are anaerobic microorganisms that can degrade NAP quickly enough to generate clear zones while the NAP layer remains visible.

Under anaerobic conditions, the agar plate should contain enough electron acceptors, such as sulfate and nitrate, for the bacteria to be able to degrade a certain amount of PAH and thus form clear zones. For this isolation method, 50 mM of sulfate and nitrate could be used both for bottom (9ml) and for top agar (1ml), which resulted in approximately 0.5mmol of sulfate and nitrate. Since 0.3ml of 5% NAP:methanol and

0.3ml of 2% PHE:acetone solutions were spread, based on the stoichiometry ratio stated in Rocken and Strand (1998), 0.50mmol and 0.28mmol of sulfate would be required for the degradation of NAP and PHE, respectively, and 0.81mmol and 0.44mmol of nitrate for NAP and PHE degradation, respectively. According to this estimation, more nitrate would be required to degrade the entire NAP layer. However, because the amount of NAP on the agar decreased significantly due to NAP evaporation during incubation and the degradation of NAP occurred only around colonies, the actual required amount of electron acceptors would be less than the calculated amount for the degradation of the entire NAP layer, thus suggesting that supplied electron acceptor would be enough.

In recent years, PAH degradation under anaerobic conditions has drawn remarkable attention, resulting in a growing number of reports on NAP- and PHE-degradation with mixed cultures (Coates *et al.,* 1997; Chang *et al*., 2001; Rockne and Strand, 1998; Meckenstock *et al*., 2000; Zhang and Young, 1997) ; however, there is little information on anaerobic microorganisms in pure cultures responsible for the degradation due to the lack of effective isolation methods applicable under anaerobic conditions, (Phelps *et al*., 1998). The modified isolation method presented in this study was developed to isolate both aerobic and anaerobic PAH-degrading microorganisms as well as to solve some problems existing with other previous isolation methods utilizing clear zones. To our knowledge, this is the first report to utilize clear zones on agar plates for the isolation of anaerobic PAH-degrading microorganisms. Since the lack of isolates has limited the understanding of anaerobic metabolism of PAHs, the modified isolation method presented here will likely stimulate the research on anaerobic PAH

degradation with isolated pure cultures, making it more feasible to gain a comprehensive understanding of PAH-degradation.

Chapter 5 Identification and isolation of a syntrophic Phenanthrene (PHE)-degrading bacterium under methanogenic conditions

5.1 Abstract

PHE-degrading microorganisms under methanogenic conditions were isolated from enrichment cultures utilizing Baltimore Harbor (Baltimore, MD) sediment using a modified plating method. Based on 16S rDNA analysis, the isolated colony was a mixed community, consisting of 3 species of bacteria and 2 species of methanogens. The degradation of PHE was partially inhibited by 2-bromoethanesulfonic acid; however, no $^{14}CH_4$ was detected when [9-^{14}C] PHE was employed, indicating partial mineralization of PHE. One species of bacterium was isolated and identified as an initial microbial catalyst for PHE-degradation. This is the first report, to our knowledge, that demonstrates the identification and isolation of a fermentative PHE-degrading bacterium under strict anaerobic conditions.

5.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds containing two or more fused aromatic hydrocarbon rings. Numerous PAHs have been listed as priority pollutants by United States Environmental Protection Agency due to their carcinogenic and mutagenic properties (Keith and Telliard, 1979). To remediate PAH-contaminated sites, aerobic PAH degradation has been studied extensively (Barnsley, 1983; Bauer and Capone, 1985; Heitkamp *et al*., 1988; Cerniglia, 1992; Kim *et al*., 1996; Moody *et al*., 2001); however, the hydrophobicity and low

water solubility of PAHs cause their accumulation in subsurface sediments where anaerobic processes become important.

Only recently have several studies been conducted to investigate PAH degradation under various anaerobic conditions such as nitrate-reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 1998; Rockne *et al.*, 2000), sulfate-reducing conditions (Coates *et al.*, 1997; Langenhoff *et al.*, 1996; Zhang and Young, 1997) and methanogenic conditions (Chang *et al.,* 2001). While it has been demonstrated that PAHs are degraded under anaerobic conditions, little is known about the microorganisms responsible for PAH degradation. Thus far, there are only two reports describing pure cultures with the ability to degrade PAHs under nitrate-reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 2000) and one report demonstrating the isolation of a NAP-degrading sulfate-reducing bacterium (Galushko *et al*., 1999). To date, however, there is no published report presenting the isolation of PAH-degrading microbes under methanogenic conditions. In particular, under methanogenic conditions, it is difficult to isolate pure cultures of the fermentative bacteria responsible for the initial transformation of complex hydrocarbons because of the syntrophic relationship between the fermentative bacteria and hydrogen-consuming organisms such as methanogens (Craig *et al*., 1998; Grbic-Galic, 1991; Gibson and Harwood, 2002). So far, there have been a few reports demonstrating the isolation of fermentative bacteria utilizing simple substituted aromatic compounds such as benzoate (Auburger and Winter, 1995; Wallrabenstein *et al*., 1995), but no report on the fermentative bacteria degrading unsubstituted aromatic hydrocarbons.

In this report, we describe the isolation of a consortium of PHE-degrading microorganisms under methanogenic conditions by utilizing the modified plating method with highly enriched PHE-degrading cultures from Baltimore Harbor (Baltimore, MD) sediment. The identification and isolation of the fermentative bacterium initiating the transformation of PHE was demonstrated. Also, the inhibitory effect of 2-bromoethanesulfonic acid, a selective inhibitor of methanogens, on PHE degradation was examined to investigate the syntrophic association between microorganisms.

5.3 Materials and Methods

Isolation of PHE-degrading microorganisms The PHE-degrading methanogenic cultures enriched from Baltimore Harbor sediments (Baltimore, MD) (Chang *et al.*, 2001) were used as the inoculum source for the isolation experiments. The isolation of PHE-degrading microbes was performed with the modified plating method described in Chapter 4. Briefly, PHE:acetone solution was spread on a minimal salt agar plate and acetone was allowed to evaporate, thereby resulting in a visible white coating of PHE on the surface of agar plates. The plates were then inoculated with 0.5% molten agar containing enrichment cultures onto a white coating of PHE. The plates were incubated in glass jars under N_2: CO_2: H_2 (15:4:1) at room temperature. Colonies generating clear zones were transferred to agar plates with a PHE white layer and to the estuarine liquid medium (5ml) in serum bottles containing PHE. Before the addition of liquid medium, a PHE solution in acetone was added to the empty serum bottles and acetone was allowed to evaporate, which resulted in a coating of PHE on the serum bottle walls.

After medium was added in serum bottles, the resulting PHE concentration was 10 ppm. All procedures were performed under strict anaerobic conditions.

The medium was prepared anaerobically in an atmosphere that contained N_2:CO_2 (4:1) (Sowers and Noll, 1995). The mineral estuarine medium for methanogens contained (per liter of distilled water) 8.4g of NaCl, 3.95g of $MgCl_2 \cdot 6H_2O$, 0.27g of KCl, 3.0g of $CaCl_2 \cdot 2H_2O$, 0.5g of NH_4Cl, 3.0g of Na_2CO_3, 1.12g of Na_2HPO_4, 0.25g of cysteine-$HCl \cdot H_2O$, 10ml of vitamin and trace metal solutions (Wolin *et al*., 1963), and 1ml of resazurin solution. For sulfate-reducing conditions, 12.32g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water was added. After the pH of the media was adjusted to 6.8, the medium (100ml) was transferred to 160 ml serum vials. For the agar plates, 1.25% or 0.5% (w/v) agar for bottom or top agar were added, respectively (Apolinario and Sowers, 1996). The vials were sealed under a N_2:CO_2 (4:1) headspace with a butyl rubber stopper and an aluminum crimp seal, and autoclaved at 121 ^oC for 20min. The Coy anaerobic chamber contained an atmosphere of N_2: CO_2: H_2 (15:4:1).

Characterization of isolated colonies via 16S rDNA sequence analysis To investigate whether the isolated colonies were pure cultures, different carbon sources other than PHE were used to stimulate the growth of microorganisms in the liquid cultures. After the isolated colonies were transferred to the media containing PHE as the sole carbon source and incubated for 30 days, they were transferred again to fresh media containing 0.1 % yeast extract and each of the following substrates (all at 0.1% w/v): starch, glucose, sodium acetate, sodium formate, trimethylamine, methanol, ethanol, 2-propanol and 2-buthanol. When turbidity was observed, each liquid culture was examined under the microscope to determine the morphology of the

microorganisms, and the headspace was sampled with a gas tight syringe (Hamilton, Reno, NV) and analyzed by gas chromatography (GC) (Hewlett Packard 7673A) with flame ionization detector (FID) to measure CH_4 production (Sowers and Schreier, 1995).

Microorganisms growing on various carbon sources were investigated further with 16S rDNA gene sequence analysis. For the amplification of bacterial 16S rDNAs, universal primers 519F (5'-CAG CA/CG CCG CGG TAA TA/TC-3') and 1406R (5'-ACG GGC GGT GTG TA/GC-3') were utilized (Holoman *et al.*, 1998). Specific archaeal primers, 340F (5'-CCT ACG GGG CGC AC/GC AGG CC/GG C-3') and 915R (5'-GTG CTC CCC CGC CAA TTC CT-3') were utilized to amplify methanogen 16S rDNAs (Loffler *et al.*, 1999). GeneAmp PCR kits with *Taq* polymerase (Perkin Elmer, Inc.) were used for PCR and conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94 ^{o}C; 30 amplification cycles of denaturation (30 s at 94 ^{o}C), annealing (30 s at 55 ^{o}C), and elongation (30 s at 72 ^{o}C); and a final extension step of 5 min at 72 ^{o}C. Since it was found that the isolated colonies were not pure cultures, the PCR products were ligated into the *pCR*II vector with the Invitrogen TOPO TA cloning kit. (Invitrogen, Carlsbad, CA) to generate plasmid libraries for bacterial and archaeal 16S rDNA. The ligated plasmids were transformed into *Escherichia coli* INVαF' competent cells supplied with the Invitrogen TOPO TA cloning kit. Thirty clones were randomly chosen from bacterial and archaeal 16S rDNA libraries, respectively, and grown in Luria Broth (LB) with kanamycin (100μg/ml). These LB cultures were directly used for the partial 16S rDNA fragment amplification with the following PCR conditions: 1 cycle of 3min at 95 ^{o}C; 40 cycles of 95 ^{o}C for 30s; 55 ^{o}C for 30s, and 72 ^{o}C for 1min; and a final extension step of 72 ^{o}C for 5min. Two

restriction endonucleases, *Hae III* and *Hha I* (New England Biolabs, Inc., Beverly, MA), were used separately to digest the PCR products. The restriction digests were separated on a 3% Trevi-gel (TreviGen, Gaithersburg, MD) and visualized with ethidium bromide gel stain. Clones bearing unique restriction banding patterns were categorized and the corresponding plasmid DNA containing the 16S rDNA gene inserts was purified with the Qiagen Plasmid Mini kit (Qiagen, Inc., CA). 16S rDNA fragments were sequenced via the DNA sequencing facility at the University of Maryland Biotechnology Institute and the sequences were entered into the GenBank BLAST search program at the National Center for Biotechnology Information (NCBI) to obtain the closest phylogenetic relatives (Altschul *et al*., 1990). For construction of a phylogenetic tree, more than 600-bp segments of sequenced bacterial and archaeal 16s rDNAs were aligned using the Ribosomal Database Project (RDP) alignment function (Maidak *et al*., 1997) with selected sequences of the closely related microbial species searched in RDP and GenBank databases. The distance matrix was generated with unambiguously aligned sequences (582 bases of the bacterial alignment, 518 bases of the archaeal alignment) by using the DNADIST program with a maximum likelihood method included in Phylip, version 3.5c (Felsenstein, 1993). The phylogenetic tree was constructed using a neighbor-joining method of the NEIGHBOR program included in Phylip, version 3.5c (Felsenstein, 1993).

Screening for biosurfactant activity The clear zone-generating colonies were examined for the ability to produce surfactant by cultivation in the liquid cultures containing PHE and various carbon sources. The drop-collapse method was employed for the determination of surfactant production (Bodour and Miller-Maier, 1998). Briefly,

the polystyrene lid of a 96-microwell plate (VWR, CA, USA) was coated with a thin layer of mineral oil, and 5 μl of liquid culture or distilled water as a control were dropped into the well. The shape of the drop on the surface of oil was observed after 1 min. According to Bodour and Miller-Maier (1998), the droplet will spread out or collapsed compared to the droplet of distilled water in the presence of surfactant.

PHE-degradation experiments Isolated clear-zone-forming colonies were tested for the ability to degrade PHE in liquid media. Isolated colonies were cultivated in liquid media with PHE (10ppm), yeast extract and each of carbon sources stated above. Cells were harvested by centrifugation at 6000 rpm for 5 minutes at the end of the exponential phase or early stationary growth phase, and then inoculated to 3 ml mineral liquid media in 10 ml of serum bottles containing dissolved PHE at the concentration of 0.3~0.4 ppm. To investigate if methanogenic activity was involved for PHE degradation, 3 mM of 2-bromoethanesulfonic acid (BES) was added. Control cultures were prepared without biomass and all liquid cultures were made in duplicate. After 40 days of incubation in the Coy anaerobic chamber, 3 ml of hexane was added to the cultures for the extraction of PHE. The serum bottles were shaken for 1 hour thoroughly and PHE concentrations were measured quantitatively by GC/FID as described before (Chang *et al*., 2001).

To test the ability of the isolated colonies to mineralize PHE, radiolabeled [9-^{14}C] PHE (Sigma Chemical Co., St. Louis, MO) was employed to monitor the evolution of $^{14}CO_2$ and $^{14}CH_4$. Cells and cultures were prepared in the same manner as described before for the study with non-radiolabeled PHE. Added radioactivity was 0.5μCi per each culture and 0.2 ppm non-radiolabeled PHE was also added as the dissolved phase

in fresh media, which resulted in 0.5ppm of total PHE concentration. Cultures without cells were examined as controls, and all samples, including controls, were made in triplicate. The production of $^{14}CO_2$ and $^{14}CH_4$ was measured monthly over the 5 months as described by Falz *et al*. (1999). Briefly, 200 µl of the headspace was sampled with a 1000 µl gas tight syringe in which there was 200 µl of 2N NaOH to trap $^{14}CO_2$. The syringe was shaken vigorously to absorb $^{14}CO_2$. To measure $^{14}CH_4$ production, the remaining gas phase in the syringe was injected to scintillation vials containing 20ml of scintillation grade toluene containing 4 g/l of Omnifluor (PerkinElmer, MA). For this purpose, scintillation vials were modified with septum placed between the scintillation vial and the screw cap to retain gas phase samples (Zehnder *et al*., 1979). Also, the screw cap of the scintillation vial was modified by making a small hole for the injection of gas phase samples (Zehnder *et al*., 1979). Toluene-based cocktail was employed to detect $^{14}CH_4$ production due to the high solubility of methane in toluene (Zehnder *et al*., 1979). Liquid phase in the syringe was transferred to scintillation vials containing 20 ml of cocktail (Scintisafe™ Econol, Fisher Scientific, NJ) to analyze $^{14}CO_2$. After the scintillation vials containing either $^{14}CO_2$ or $^{14}CH_4$ were vigorously shaken to mix cocktails and samples, the radioactivities were determined with LKB/Wallac 1219 RackBeta Spectral Liquid Scintillation Counter (Finland). The radioactivities of headspace in control cultures were analyzed in the same manner and compared with those in active cultures to ensure the evolution of $^{14}CO_2$ and $^{14}CH_4$.

5.4 Results

Isolation of colonies creating clear zones The modified plating method was utilized to screen PHE-degrading colonies on the agar plates. Since sulfate-reducing bacteria (SRB) have been monitored in PHE-degrading enrichment cultures based on 16S rDNA analysis (Chang, 2003), both methanogenic and sulfate-reducing conditions were employed. Under methanogenic conditions, one colony with a clear zone (M-PCZ) was detected as early as 4 months after incubation and distinguished more clearly 2 months later. As shown in Figure 5.1A, the clear zone around M-PCZ could be easily detected against the white coating of PHE. As the incubation time was extended up to 12 months, several more colonies were distinguished with clear zones. Under sulfate-reducing conditions, one colony (S-PCZ) showed a clear zone on the white layer of PHE after 8 months of incubation (Figure 5.2A), and more clear zones were developed approximately 3 to 4 months later. As revealed in Figure 5.2A, the zone around the colony was transparent since PHE was utilized by the colony.

To confirm the degradation activity, M-PCZ and S-PCZ were transferred to the white PHE layers on the agar plates. After 6 months of incubation, under methanogenic conditions, the agar plate with transferred M-PCZ revealed numerous small clear zones and those were more distinct after an additional 2 months of incubation (Figure 5.1B). After those transferred colonies generating clear zones were retransferred to the white layer of PHE, more than 13 months of incubation was required for colonies to develop detectable clear zones. After the second transfer, however, no clear zones around colonies were observed during 16 months of incubation and it was not feasible to detect clear zones after 16 months as PHE layer disappeared because of evaporation. With

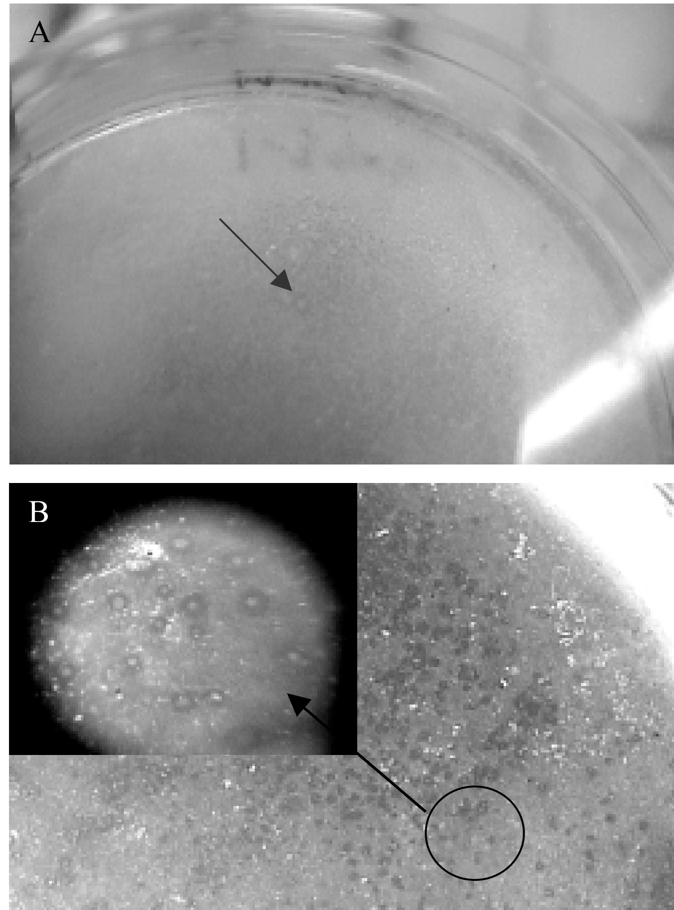

Figure 5.1 Photograph of the PHE-degrading colony, M-PCZ, surrounded by a clear zone on the white coating of PHE (A) and the transferred colonies revealing clear zones (B)

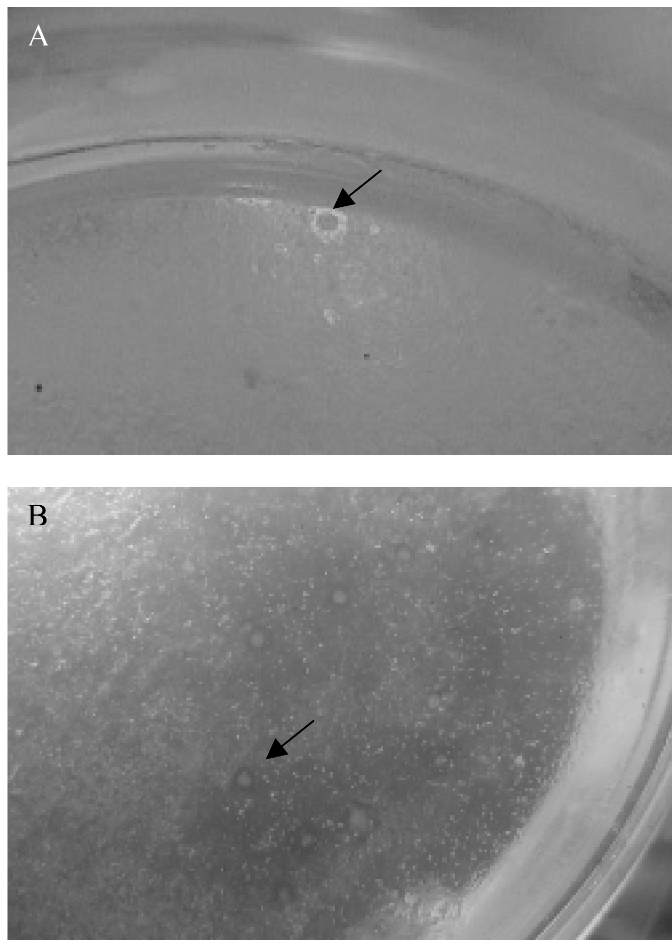

Figure 5.2 Photogtaph of the PHE-degrading colony, S-PCZ, surrounded by a clear zone on the white coating of PHE under sulfate-reducing conditions (A) and the transferred colonies with clear zones (B)

transferred S-PCZ, clear zones surrounding colonies began to appear on the PHE layer after 10 months of incubation.

Identification of microbial species in isolated colonies Since it is believed that several species of microorganisms interact cooperatively to utilize complex organic compounds under methanogenic conditions, the transferred liquid cultures of M-PCZ and S-PCZ were subcultured and cultivated with various carbons sources to encourage the growth of diverse anaerobic microbes, facilitating characterization of the microbial composition in M-PCZ and S-PCZ. With M-PCZ, the visual turbidity was observed with starch, glucose, trimethylamine and methanol after 5 days of incubation. CH_4 production was detected with the cultures growing on trimethylamine and methanol, but no CH_4 production with starch and glucose. Further identification of microorganisms in the liquid cultures was performed via 16S rDNA analysis. Based on RFLP analysis, there were 2 distinct restriction patterns from bacterial clones and 1 pattern from archaeal clones, and a representative clone of each unique RFLP pattern was further identified by 16S rDNA sequence analysis. From the cultures containing starch or glucose, only one bacterial clone (M-B1) was detected. As shown in Figure 5.3, clone M-B1 revealed the highest sequence similarity to the members of *Cytophagal* (*Cytophagal/Flavobacterium/ Bacteroides*). According to microscopic examination, the morphology of clone M-B1 was changed from a rod-shape at the exponential growth phase to a coccus-shape at the stationary phase. In addition to the bacterial clone M-B1, another bacterial clone (M-B2) and one archaeal clone (M-A1) were identified with the cultures containing trimethylamine and methanol, which were closely related to the *Acholeplasma* genus (Figure 5.3) and the *Methanosacinales* Genus (Figure 5.4),

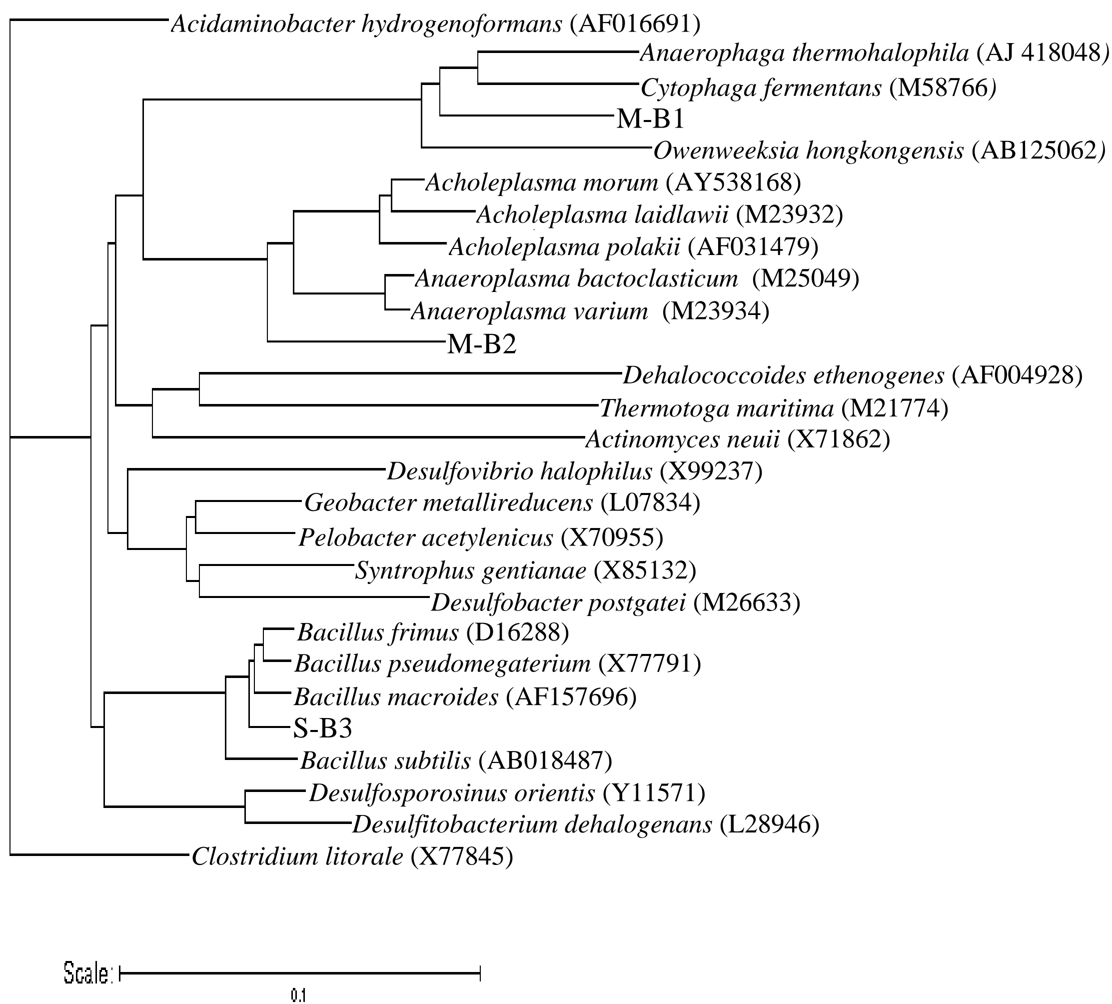

Figure 5.3 Phylogenetic tree of bacterial 16S rDNA sequences retrieved from M-PCZ and S-PCZ. The GeneBank accession number for each reference sequence is shown in parenthesis. The sequence of *Clostridium litorale* was used as an out-group. The scale bar represents 10 nucleotide substitutions per 100 bases.

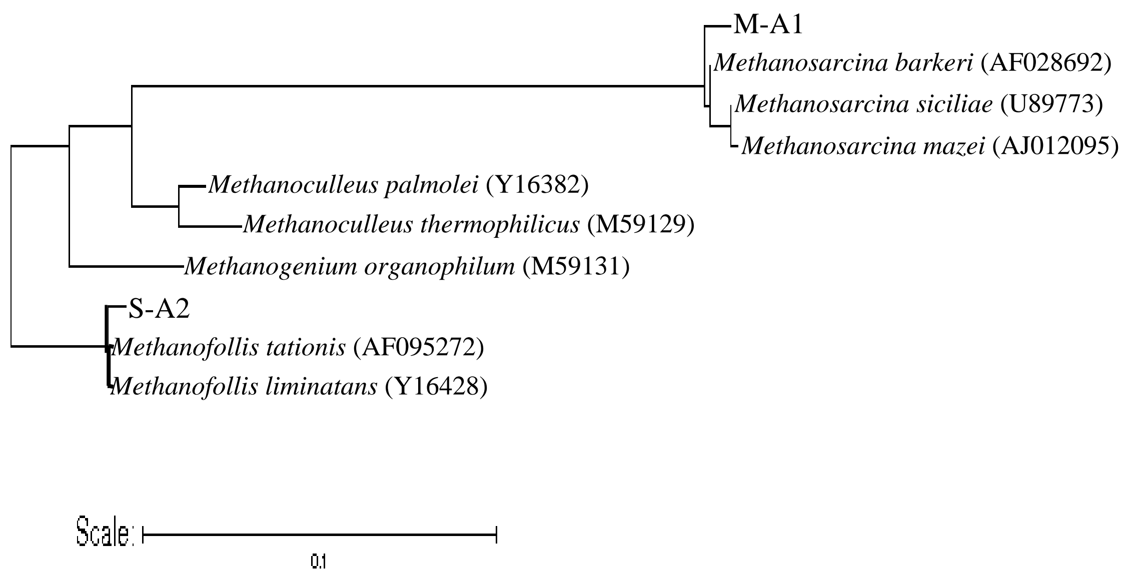

Figure 5.4 Phylogenetic tree of archaeal 16S rDNA sequences retrieved from M-PCZ and S-PCZ. The GeneBank accession number for each reference sequence is shown in parenthesis. The scale bar represents 10 nucleotide substitutions per 100 bases.

respectively. Clone M-B2 exhibited the highest sequence similarity to an unidentified eubacterium RFLP 24 detected in the tetrachlorobiphenyl-dechlorinating microbial community (Table 5.1) (Holoman *et al*., 1998).

With S-PCZ, turbidity and CH_4 production were observed with all cultivated liquid cultures containing each of the carbon sources stated before. Interestingly, similar growth behavior (*i.e*., OD vs. time) was observed even in the absence of sulfate. RFLP analysis with amplified 16S rDNA revealed 3 different bacterial clones and 2 archaeal clones (Table 5.1). Among them, 2 bacterial clones (M-B1 and M-B2) and 1 archaeal clone (M-A1) were previously identified in theM-PCZ cultures. The third bacterial clone (S-B3) showed the highest 16S rDNA sequence similarity to the species of the *Bacillus* (Figure 5.3), and the other archaeal clone (S-A2) to the *Methanofollis* genus (Figure 5.4). The bacterium corresponding to clone M-B1 detected from S-PCZ was confirmed to be the same organism represented by clone M-B1 from M-PCZ based on the 16S rDNA sequence analysis. Clone M-B1 and S-B3 accounted for more than 85 % of the total universal clones. The microbial species and their populations cultivated without sulfate were found to be similar to those in the liquid cultures with sulfate.

Identification and isolation of an organism responsible for creating clear zones

To investigate which microorganism was responsible for generating clear zones as the result of the initial attack on PHE, 16S rDNA analysis was performed with clear-zone-forming colonies developed on the transferred M-PCZ and S-PCZ agar plates. The colonies were picked using sterile toothpicks and suspended in 50 μl distilled water. The suspended cells were frozen and thawed to lyse the cells, then utilized as a template for 16S rDNA amplification. According to RFLP analysis with both the transferred M-

Table 5.1 Phylogenetic affiliations of the consortia from M-PCZ and S-PCZ based on bacterial and archaeal 16S rDNA sequences

Clone	Source of colony[†]	Closest phylogenetic relative	% similarity to closest relative
		Bacterial domain	
M-B1	M, S	Uncultured benzene mineralizing clone SB-1[a]	98
M-B2	M, S	Unidentified eubacterium RFLP24[b]	96
S-B3	S	Unidentified bacterium IrT-RS2[c]	99
		Archaeal domain	
M-A1	M, S	Uncultured archaeon clone RFLP 308A[d]	98
S-A2	S	*Methanofollis tationis*[e]	98

[†] M, M-PCZ; S, S-PCZ

[a] Phelps *et al*. (1998)

[b] Holoman *et al*. (1998)

[c] Selenska-Pobell (2002)

[d] Cho *et al*., direct submission (2000)

[e] Zellner *et al*. (1999)

PCZ and S-PCZ, only one RFLP pattern corresponding to clone M-B1 was observed, implying that a bacterium corresponding to M-B1 was the actual clear zone-generating microorganism. The microorganism represented by clone M-B1, designated bacterium M-Phe-1, was isolated as a pure culture by repeated streaking on the agar plate. Bacterium M-Phe-1 revealed the same growth curves with and without sulfate, and the glucose cultures showed the longer lag phase compared to the starch cultures (data not shown). Since bacterium M-Phe-1 was also detected on the agar plates with the transferred S-PCZ under sulfate reducing conditions, 20mM sodium molybdate (SM), the selective inhibitor of sulfate-reducing bacteria (SRB), was added in the liquid culture with starch to examine whether isolated M-Phe-1 was a SRB. It appeared that the growth of M-Phe-1 was inhibited in the liquid cultures when colonies of pure culture M-Phe-1 were picked and inoculated. But, when 2% (v/v) of the liquid cultures at the exponential phase cultivated without SM was inoculated along with 20 mM SM, the growth behavior was the same of that without SM except that a longer lag period was required to acclimate, indicating that M-Phe-1 was not SRB, but the molybdate, a heavy metal, would be toxic, consequently inhibiting the growth of M-Phe-1 apparently when the very small number of active microorganisms were inoculated. The production of surfactant by M-Phe-1 was not detected with the drop-collapse method (Bodour and Miller-Maier, 1998), confirming that the clear zones were formed due to the uptake of PHE, not the dissolution of PHE by surfactant. Since the distinct clear zones were developed obviously around the colonies in which only clone M-B1 was detected, this result confirmed that M-Phe-1 is a fermentative bacterium responsible for initiating PHE degradation under methanogenic conditions.

PHE degradation in the liquid media with isolated clear-zone-forming colonies

The PHE degradation activities of the isolated colonies were examined in the liquid media. Since the growth of isolated colonies on PHE were too slow to detect PHE degradation (data not shown), cells previously growing on other carbon sources were used to provide more biomass for the degradation of PHE. For the experiment with M-PCZ, cells in the liquid cultures containing PHE and each of carbon sources such as starch, glucose and methanol were utilized after centrifugation. After 40 days of incubation, however, no reproducible degradation data were obtained based on the GC results. In opposition to the result with M-PCZ, PHE degradation was detected with the microbial consortium of S-PCZ by utilizing biomass in the liquid cultures growing on PHE and separately added starch, glucose, ethanol, 2-propanol, 2-butanol and trimethylamine (Figure 5.5). Furthermore, as shown in Figure 5.5, the addition of BES brought about the partial inhibition of PHE degradation. Since the growth of bacterial species was not inhibited by BES in the liquid cultures (data not shown), it may be concluded that the inhibitory effect of BES on methanogens influenced on PHE degradation, indicating that methanogenic activity was possibly linked to PHE degradation. Radiolabeled [9-^{14}C] PHE was added to investigate whether the consortium of S-PCZ mineralized PHE to CO_2 and CH_4. Over 5 months, however, neither $^{14}CO_2$ nor $^{14}CH_4$ was detected, implying that the consortium did not mineralize PHE completely.

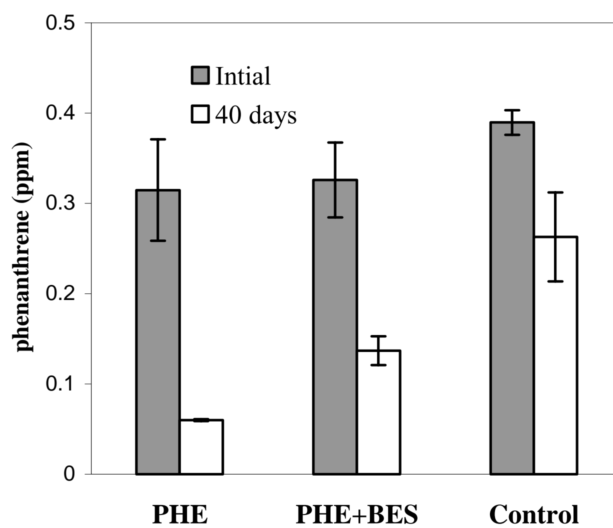

Figure 5.5 Phenanthrene degradation with the microbial consortium of S-PCZ

5.5 Discussion

Very recently, methanogenic conditions have drawn remarkable attention on the degradation of aromatic hydrocarbons and hexadecane, which had previously been considered non-degradable under methanogenic conditions (Kazumi *et al*., 1997; Weiner and Lovley, 1998; Beller and Edwards, 2000; Chang *et al*., 2001; Zengler *et al.*, 1999; Parkes, 1999; Anderson and Lovley, 2000). The identification and isolation of microorganisms initiating degradation, however, has not been successful because of the syntrophic interactions of multiple species (Gibson and Harwood, 2002).

In this study, we have identified and isolated a fermentative bacterium responsible for the initial attack on PHE under methanogenic conditions by utilizing the modified plating method. M-Phe-1 generated clear zones on the white layer of PHE under methanogenic conditions as well as sulfate-reducing conditions, implying that sulfate may not be necessary for M-Phe-1 to initiate PHE degradation. Furthermore, based on the 16S rDNA sequence analysis and the inhibition study with SM, it is clear that bacterium M-Phe-1 does not belong to the SRB. A RFLP pattern corresponding to bacterium M-Phe-1 has been detected in PHE-degrading enrichment methanogenic cultures (Chang, 2003). 16S rDNA sequence analysis of bacterium M-Phe-1 revealed the highest sequence similarity to the uncultured benzene mineralizing clone SB-1 from a benzene-degrading sulfate-reducing consortium (Phelps *et al*., 1998), falling within the subgroup of *Cytophagales* in the CFB (*Cytophagal/Flavobacterium/ Bacteroides*) group (Figure 5.3). The species of the subgroup of *Cytophagales* have been found in the subsurface microbial cultures including hydrocarbon-degrading anaerobic communities (Balkwill *et al*., 1997; Phelps *et al*., 1998; Dojka *et.al*., 1998). Recently, there have

been several reports illustrating anaerobic *Cytophaga* species (Denger *et al*., 2002; Denger and Schink, 1995; Haack and Breznak, 1993). Among them, *Anaerophaga thermohalophila* is similar to bacterium M-Phe-1 in terms of its comparably fast growth on starch and glucose by fermentation without H_2-consuming microorganisms (Denger *et al*., 2002). However, *Anaerophaga thermohalophila* grows neither on the surface of agar nor at 30°C in liquid medium, whereas bacterium M-Phe-1 grew well both on the agar plate and in the liquid medium at 30°C.

With liquid cultures of the consortium of S-PCZ, the concentration of PHE was successfully decreased by biological transformation, but there was no observable PHE degradation with the consortium of M-PCZ. However, it should be noted that the colonies transferred directly from M-PCZ exhibited the clear zones, indicating that the PHE degradation activity of transferred M-PCZ was sustained on the agar plate. The probable explanation for these conflicting results could be the fact that the different fermentative patterns in the presence of carbon sources other than PHE may bring about the selective outgrowth of specific microorganisms as well as possibly the disappearance of certain species which were necessary for the degradation of PHE (Schink, 2002). As stated before, the use of various carbon sources to stimulate growth of sufficient biomass for the study of PHE degradation possibly caused a shift in the microbial composition of M-PCZ, thus resulting in no observable PHE degradation. On the contrary, the colonies on the agar plate were incubated on PHE as the sole carbon source, consequently revealing the sustained degradation activity.

Although the transferred M-PCZ on the agar plate showed sustained PHE degradation activity, the required incubation time for the colonies to create the

detectable clear zones increased from 4 months to 6 months after the first transfer and even no clear zones were detectable after second transfer during 16 months of incubation, suggesting that there may be the lack of necessary nutrient to support the growth of microorganisms or the change of microbial population through transfer as a result of the selective cultivation on mineral minimal medium. Also, since it is known to be difficult to cultivate naturally occurring environmental microorganisms in the lab, the microorganisms necessary for the degradation of PHE could be lost through transfer (Schink, 2002).

In contrast to M-PCZ, S-PCZ revealed PHE degradation activity both in the liquid cultures and on the agar plate. Since clones S-B3 and S-A2 were detected only with S-PCZ, the microorganisms represented by those clones may play important roles in the syntrophic degradation process by consuming metabolic intermediate such as H_2 and organic compounds, making the degradation of hydrocarbons thermodynamically favorable (Gibson and Harwood, 2002; Widdel and Rabus, 2001). Based on the inhibitory effect of BES on PHE degradation as shown Figure 5.5, it could be suggested that methanogenic metabolism was involved to some extent in anaerobic PHE degradation by the consortium of S-PCZ. Especially, *Methanofollis* species represented by clone S-A2, which is known to utilize H_2, was likely associated with the interspecies transfer of H_2 with other microorganisms, influencing PHE degradation positively. The proposed role of *Bacillus* species corresponding to clone S-B3 could be the production of simple organic acids such as acetate, butyrate and pyruvate by fermentation of intermediates. The other two species of microorganisms, *Methanosacinales* species represented by clone M-A1 utilizing methanol and trimethylamine and *Acholeplasma*

species by clone M-B2, might be involved in the syntrophic degradation of PHE. However, it is not clear yet whether all species of the microorganisms corresponding to the detected clones were necessary for the degradation of PHE or if some of them persist with the metabolic byproducts. There was no inhibitory effect of SM on the growth of cultures, indicating that sulfate may not be the terminal electron acceptor. Thus, it could be suggested that sulfate was not required for PHE degradation by S-PCZ even though S-PCZ was discovered coincidently on the agar plate containing sulfate.

Of interest is the fact that bacterium M-Phe-1 could grow on starch or glucose by fermentation without syntrophic interactions while methanogenic activity was required for the degradation of PHE based on the study with BES. There has been an increasing number of reports describing pure cultures of bacteria known to grow only by syntrophic interactions with hydrogen-utilizing organisms (Auburger and Winter, 1995; Beaty and McInerney, 1987; Jackson *et al*., 1999). It has been feasible to obtain the pure cultures of obligate syntrophs since some bacteria exhibiting syntrophic degradation of specific hydrocarbons are able to grow as pure cultures with other carbon sources (Gibson and Harwood, 2002). Similarly, bacterium M-Phe-1 could be considered as a syntrophic PHE-degrading microorganism, capable of growing on starch and glucose as a pure culture without syntrophic interactions. Based on the partial inhibition of PHE degradation in the presence of BES, however, it appeared that bacterium M-Phe-1 did not strictly interact with methanogens. When the consortium of each of M-PCZ and S-PCZ was tested for the ability of PHE mineralization with [9-^{14}C] in the liquid media, no production of $^{14}CH_4$ and $^{14}CO_2$ was detected, suggesting the incomplete mineralization of PHE with the consortia of M-PCZ and S-PCZ. Zhang and

Young (1997) proposed carboxylation as the initial step in NAP and PHE degradation under sulfate reducing conditions. Also, it was demonstrated that the carboxylation of PHE did not occur at the carbon-9 position as an initial degradation step but likely on the one of the outer ring carbon. It might be the reason for the phenomena that $^{14}CH_4$ or $^{14}CO_2$ was not detected with [9-^{14}C] PHE despite the transformation of PHE was observed as shown in Figure 5.5. However, it should not be excluded that our cultivation methods may omit unculturable microorganisms necessary for the complete mineralization of PHE, possibly causing no observable $^{14}CH_4$ or $^{14}CO_2$. The fate of the radiolabeled PHE could be elucidated by monitoring the ^{14}C activity incorporated into biomass and the activity in the liquid media.

In summary, this report describes the identification and isolation of a fermentative bacterium that is responsible for the initial catalyst activity for the transformation of PHE under methanogenic condition. To our knowledge, this is the first report to isolate the pure cultures of a syntrophic unsubstituted aromatic hydrocarbon-degrading bacterium under anaerobic conditions. Further study on the mechanisms and the factors affecting anaerobic PAH degradation with pure cultures of microorganisms will facilitate comprehensive understanding on anaerobic PAH-degradation.

Chapter 6 Isolation and characterization of Naphthalene (NAP)-degrading microorganisms under methanogenic conditions

6.1 Abstract

NAP-degrading microorganisms under methanogenic conditions were isolated by utilizing an agar-overlay containing evenly dispersed fine particles of NAP. A NAP-degrading colony was detected by the disappearance of NAP particles around the colony. According to 16S rDNA analysis with the transferred liquid cultures grown on various carbon sources and NAP, the isolated colony was a mixed culture with 2 species of bacteria and one species of hydrogenotrophic methanogen. One species of the bacteria was the same microorganism as an initial microbial catalyst of PHE-degradation identified before, and the other one showed a syntrophic relationship with methanogen species. To our knowledge, this is the first report to isolate NAP-degrading microorganisms under methanogenic conditions by utilizing clear zones on the agar plate.

6.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing two or more fused aromatic rings. They are produced in a variety of processes such as the incomplete combustion of fossil fuels and as a byproduct of coke production and petroleum refining (McNally *et al*., 1998). The environment, especially aquatic sediments, has suffered from the contamination of PAHs due to fuel spills, runoff, sewage treatment plants, petrochemical industrial effluents, etc. (Cerniglia, 1992).

PAHs can be classified into two classes based on the number of aromatic rings; low molecular weight PAHs with 2 or 3 aromatic rings and high molecular weight PAHs with 4 or more aromatic rings. Research has demonstrated that low molecular weight PAHs are acutely toxic to aquatic organisms due to the relatively high aqueous solubility (Neff, 1979).

Naphthalene (NAP) is the lowest molecular weight PAH consisting of two fused aromatic rings. The biodegradation of NAP with oxygen has been extensively studied with mixed cultures and pure cultures (Herbes and Schwall, 1979; Heikamp and Cerniglia, 1988; Bouchea *et al*., 1995; Brian *et al*., 1998). Only very recently did a number of studies begin to demonstrate the anaerobic biodegradation of NAP under nitrate reducing conditions (McNally *et al*., 1998; Mihelcic and Luthy, 1988; Rockne and Strand, 1998), under sulfate reducing conditions (Coates *et al*., 1996; Langenhoff *et al*., 1996; Meckenstock *et al*., 2000; Zhang and Young, 1997), and methanogenic conditions (Chang *et al*., 2001). Thus far, two reports have described pure cultures of NAP degrading microorganisms under nitrate reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 2000) and one report demonstrating the isolation of a NAP-degrading sulfate-reducing bacterium (Galushko *et al*., 1999); however, there has been no information on pure cultures of NAP-degrading organisms under methanogenic conditions.

It has been generally believed that three species of anaerobes are required to mineralize complex hydrocarbons other than simple fatty acids under methanogenic conditions; the hydrolytic fermentative bacteria, the syntrophic acetogenic organisms transforming intermediates to methanogenic precursors such as acetate and hydrogen,

and the methanogenic bacteria converting them to CH_4 and CO_2 (Grbic-Galic and Vogel, 1987; Phelps *et al*., 1998). Due to the syntrophic associations of various microbial species in methanogenic hydrocarbon-degrading cultures, it has been difficult to isolate pure cultures of fermentative bacteria responsible for the initial attack on hydrocarbons (Ficker *et al*., 1999).

Previous research in our lab demonstrated the degradation of NAP under methanogenic conditions by utilizing Baltimore Harbor (Baltimore, MD) sediments to initiate anaerobic enrichment cultures (Chang *et al*., 2001). In this study, we describe the isolation of NAP degrading microorganisms with highly enriched NAP-degrading methanogenic cultures by utilizing the opaque overlayer technique developed by Bogardt and Hemmingsen (1992). The microbial consortium of isolated clear-zone-forming colony was analyzed by comparative sequence analysis of 16S rDNA, and the degradation activity was examined with liquid cultures.

6.3 Materials and Methods

Medium and Agar plate preparation The medium was prepared anaerobically in an atmosphere that contained N_2:CO_2 (4:1) (Sowers and Noll, 1995). The estuarine medium for methanogens contained (per liter of distilled water) 8.4g of NaCl, 3.95g of $MgCl_2 \cdot 6H_2O$, 0.27g of KCl, 3.0g of $CaCl_2 \cdot 2H_2O$, 0.5g of NH_4Cl, 3.0g of Na_2CO_3, 1.12g of Na_2HPO_4, 0.25g of cysteine-HCl·H_2O, 10ml of vitamin and trace metal solutions (Wolin *et al*., 1963), and 1ml of resazurin solution. Resazurin was added as a redox indicator that is initially colorless but changes pink in the presence of oxygen (Sowers and Noll, 1995). After the pH of the media was adjusted to 6.8, medium was transferred

to serum vials. For the agar plates, 1.25% or 0.5% (w/v) agar for bottom or top agar (overlayer) were added, respectively (Apolinario and Sowers, 1996). The vials were sealed under a N_2:CO_2 (4:1) headspace with a butyl rubber stopper and an aluminum crimp seal, and autoclaved at 121 ^{o}C for 20min. Media containing agar was cooled to about 60 ^{o}C. To make bottom agar, cooled molten agar medium was transferred to the Coy Anaerobic Chamber and poured into sterile polystyrene petri plates (60×15mm). Plates were dried in the Coy Anaerobic Chamber for 2 days before use as recommended by Apolinario and Sowers (1996). The anaerobic chamber contained an atmosphere of N_2: CO_2: H_2 (15:4:1).

Isolation of NAP degrading colony with the overlayer technique Due to the high solubility of NAP in acetone, acetone was used as a solvent to make NAP solution instead of ethanol that was originally employed by Bogardt and Hemmingsen (1992), resulting in more NAP particles in the overlayer and thus facilitating the detection of clear zones after a long period of incubation. Prior to use, acetone was purged with ultra pure N_2 gas for an hour to remove dissolved oxygen. After purged acetone was transferred into the Coy chamber, 0.1% (w/v) cysteine-HCl·H_2O was added to lower redox potential (Sowers and Noll, 1995). Top agar solution (0.9ml) was mixed with 0.05ml of NAP solution in acetone (2% [w/v]) by vortex, resulting in cloudy top agar solution due to the evenly dispersed NAP particles. After the top agar solutions were mixed with 0.1ml of serially diluted enrichment cultures, top agar solutions were poured on bottom agar and dried for more than 1 hour, creating opaque overlayer. Inoculated plates were then placed in wide-mouth glass canning jars (Ball Corporation, El Paso, TX) with a 5ml serum vial containing 1 ml of 2.5% $Na_2S \cdot 9H_2O$ solution that

serves as a reducing agent (Sowers and Noll, 1995). The plates were incubated at room temperature under N_2: CO_2: H_2 (15:4:1) in the jars. Colony generating a clear zone was transferred to agar plates with opaque top agar solutions and to the estuarine liquid medium (5ml) in serum bottles containing NAP as the sole carbon source. Before the colony was transferred to liquid medium, NAP solution in acetone was added to the empty serum bottles, and acetone was allowed to evaporate, leaving a coating of NAP on the serum bottle walls. After medium was added in serum bottles, the resulting concentration of NAP was 5 ppm. All procedures were performed under strict anaerobic conditions.

Characterization of isolated colony via 16S rDNA sequence analysis After the isolated colony was transferred to the liquid medium containing NAP as the sole carbon source, and incubated for 30 days, it was subcultured in fresh media containing 0.1 % yeast extract, 5 ppm NAP and each of the following substrates (all at 0.1% w/v): starch, glucose, sodium acetate, sodium formate, trimethylamine, methanol, ethanol, 2-propanol and 2-buthanol. Once turbidity was observed, each liquid culture was examined under the microscope, and the headspace was sampled with a gas tight syringe (Hamilton, Reno, NV) and analyzed by gas chromatography (GC) (Hewlett Packard 7673A) with flame ionization detector (FID) to measure CH_4 production (Sowers and Schreier, 1995)

Microorganisms growing on various carbon sources were investigated further with 16S rDNA gene sequence analysis. For the amplification of bacterial 16S rDNAs, universal primers 519F (5'-CAG CA/CG CCG CGG TAA TA/TC-3') and 1406R (5'-ACG GGC GGT GTG TA/GC-3') were utilized (Holoman *et al.*, 1998). Specific

archaeal primers, 340F (5'-CCT ACG GGG CGC AC/GC AGG CC/GG C-3') and 915R (5'-GTG CTC CCC CGC CAA TTC CT-3') were utilized to amplify methanogen 16S rDNAs (Loffler *et al*., 1999). GeneAmp PCR kits with *Taq* polymerase (Perkin Elmer, Inc.) were used for PCR and conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94 ^{o}C; 30 amplification cycles of denaturation (30 s at 94 ^{o}C), annealing (30 s at 55 ^{o}C), and elongation (30 s at 72 ^{o}C); and a final extension step of 5 min at 72 ^{o}C. Since it was found that the isolated colony was not a pure culture, the PCR products were ligated into the *pCR*II vector with the Invitrogen TOPO TA cloning kit. (Invitrogen, Carlsbad, CA) to generate plasmid libraries for bacterial and archaeal 16S rDNAs. The ligated plasmids were transformed into *Escherichia coli* INVαF' competent cells supplied with the Invitrogen TOPO TA cloning kit. Thirty clones were randomly chosen from bacterial and archaeal 16S rDNA libraries, respectively, and grown in Luria Broth (LB) with kanamycin (100μg/ml). These LB cultures were directly used for the partial 16S rDNA fragment amplification with the following PCR conditions: 1 cycle of 3min at 95 ^{o}C; 40 cycles of 95 ^{o}C for 30s; 55 ^{o}C for 30s, and 72 ^{o}C for 1min; and a final extension step of 72 ^{o}C for 5min. Two restriction endonucleases, *Hae III* and *Hha I* (New England Biolabs, Inc., Beverly, MA), were used separately to digest the PCR products. The restriction digests were separated on a 3% Trevi-gel (TreviGen, Gaithersburg, MD) and visualized with ethidium bromide gel stain. Clones bearing unique restriction banding patterns were categorized and the corresponding plasmid DNA containing the 16S rDNA gene inserts was purified with the Qiagen Plasmid Mini kit (Qiagen, Inc., CA). 16S rDNA fragments were sequenced via the DNA sequencing facility at the University of Maryland Biotechnology Institute and the

sequences were entered into the GenBank BLAST search program at the National Center for Biotechnology Information (NCBI) to obtain the closest phylogenetic relatives (Altschul *et al*., 1990).). For construction of a phylogenetic tree, more than 600-bp segments of sequenced bacterial 16s rDNAs were aligned using the Ribosomal Database Project (RDP) alignment function (Bonnie *et al*., 1997) with selected sequences of the closely related microbial species searched in RDP and GenBank databases. The distance matrix was generated with unambiguously aligned sequences (581 bases of the bacterial alignment) by using the DNADIST program with a maximum likelihood method included in Phylip, version 3.5c (Felsenstein, 1993). The phylogenetic tree was constructed using a neighbor-joining method of the NEIGHBOR program included in Phylip, version 3.5c (Felsenstein, 1993).

Screening for biosurfactant activity A clear zone-generating colony was examined for the ability to produce surfactant by cultivation in the liquid cultures containing NAP and various carbon sources. The drop-collapse method was employed for determination of surfactant production (Bodour and Miller-Maier, 1998). Briefly, the polystyrene lid of a 96-microwell plate (VWR, CA, USA) was coated with a thin layer of mineral oil, and 5 μl of liquid culture or distilled water as a control were dropped into the well. The shape of the drop on the surface of oil was observed after 1 min. According to Bodour and Miller-Maier (1998), the droplet will spread out or collapsed compared to the droplet of distilled water in the presence of surfactant.

NAP-degradation experiments Isolated colonies were cultivated in the liquid media with NAP (5 ppm), yeast extract and each of carbon sources stated above. Cells were harvested by centrifugation at 6000 rpm for 5 minutes at the end of the

exponential phase or early stationary growth phase, and then inoculated to 3 ml mineral liquid media in 10 ml of serum bottles containing dissolved NAP (~1 ppm). Control cultures were prepared in the same way without biomass and all liquid cultures were made in duplicate. After 40 days of incubation, 3 ml of hexane was added to the cultures for the extraction of NAP. The serum bottles were shaken for 1 hour thoroughly and NAP concentrations were measured quantitatively by GC/FID as described before (Chang *et al*., 2001).

6.4 Results and Discussion

Isolation of NAP-degrading colony generating clear zone The overlayer technique was utilized to isolate NAP-degrading microbes under anaerobic conditions. The plating procedure of Bogardt and Hemminsen (1992) was modified to include the anaerobic preparation of acetone by purging it with ultra pure N_2 gas for an hour in order to remove dissolved oxygen and adding 0.1% (w/v) cysteine-$HCl{\cdot}H_2O$ to lower the redox potential further. Accordingly, the reduced solvent did not change the color of agar solution, thus enabling the utilization of the overlayer technique under anaerobic conditions. Bogardt and Hemminsen (1992) originally utilized an ethanolic solution of PHE (8.5mg/ml) to enumerate PHE-degrading bacteria. However, it was our experience that loss of NAP particles by evaporation was considerable because of the long incubation times required under anaerobic conditions even though the fine NAP particles were imbedded in the opaque top agar layer, making it difficult to detect clear zones. In order to supply more NAP particles, instead of ethanol, acetone was utilized as a solvent because the solubility of NAP in acetone is higher than in ethanol, and

acetone is miscible with water. By using 2% (w/v) NAP in acetone, visible fine NAP particles remained even after long periods of incubation, and thus the clear zones could be detected.

After 8 months of incubation, one colony with a clear zone (M-NCZ) was distinguished from other colonies showing no clear zones under methanogenic conditions (Figure 6.1). The clear zone was detected a few months after colonies appeared on the agar plates. The similar circular shape of a clear zone has been shown from other studies under aerobic conditions (Kiyohara *et al*., 1982; Shiaris and Cooney, 1983; Alley and Brown, 2000). Interestingly, except this one NAP-degrading colony (M-NCZ), no additional colonies with clear zones were observed although numerous colonies existed on other plates. One possible reason for this result is that a consortium of microorganisms is necessary for the degradation of NAP under methanogenic conditions. Because it may occur rarely for multiple microbial species required for NAP-degradation to grow together in a single colony, the possibility to detect the colonies with clear zones may also decrease. Another possible reason is that the lack of nutrients in the minimal mineral agar medium may make it difficult for NAP-degrading microbes to form adequately sized colonies, thus resulting in indistinguishable clear zones. Several reports advised the utilization of nutrient agar plates to obtain distinct clear zones with proper size colonies (Bogardt and Hemmingsen, 1992; Rockne *et al*., 2000; Heitkamp and Cerniglia, 1988). However, it should be considered that the vapor phase of NAP could be utilized by microorganisms (Kastner *et al*., 1994), suggesting that there might exist more colonies capable of NAP degradation without exhibiting clear zones.

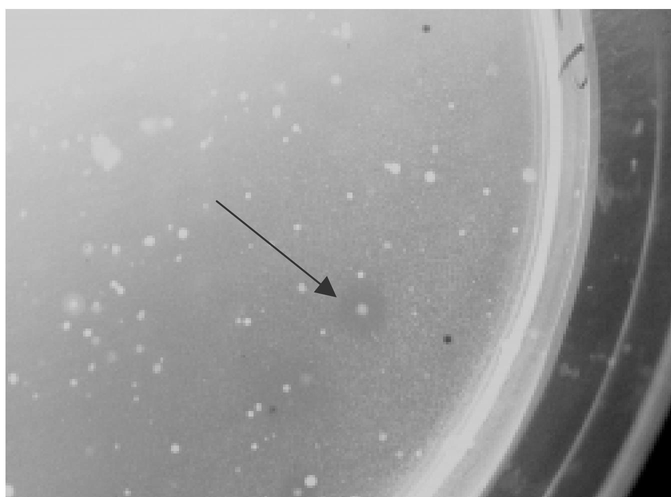

Figure 6.1 Photograph of the NAP-degrading colony, M-NCZ (→), surrounded by a clear zone on the opaque overlayer under methanogenic conditions

Characterization of the clear-zone-forming colony To support the growth of possible microorganisms existing in M-NCZ, the transferred M-NCZ was subcultured with various carbon sources in the presence of NAP. Turbidity was detected after 5 days of incubation in the starch culture and 7 days in the glucose and 2-propanol cultures. In the glucose culture, flock-like aggregates were observed with vigorous CH_4 production. Other cultures exhibiting turbidities also revealed CH_4 production, but much less than that of glucose culture. Microscopical observation of the glucose culture showed filamentous organisms which formed flock-like aggregates and irregular coccus-shape organisms which were likely methanogens. Further identification of the microorganisms was performed via RFLP analysis followed by 16S rDNA sequence analysis. According to RFLP pattern analysis, one bacterial and one archaeal clone were found in the starch and 2-propanol cultures. They were identified as clone M-B1 and S-A2 that had been detected in PHE-degrading colonies (Table 6.1). With the glucose culture, bacterial clone (M-B4) corresponding to the filamentous organisms as well as archaeal clone S-A2 were observed. Based on 16S rDNA sequence analysis, clone M-B4 was affiliated with the green non-sulfur group with the highest sequence similarity (95%) to anaerobic trichlorobenzene-degrading consortium clone SJA-170 (Figure 6.2) (von Wintzingerode *et al*., 1999). Since CH_4 production in the glucose culture was much greater than those of other cultures, the existence of a syntrophic relationship was examined with 2-bromoethanesulfonic acid (BES), a selective inhibitor of methanogens. Interestingly, little cotton-like flocs were observed in the presence of BES without detectable CH_4 production, but the quantity of these aggregates was far less than with the BES-free culture, indicating that the growth of the filamentous microorganisms was

Table 6.1 Phylogenetic affiliations of the consortia from M-NCZ based on bacterial and archaeal 16S rDNA sequences

Clone	Closest phylogenetic relative	% similarity to closest relative
	Bacterial domain	
M-B1	Uncultured benzene mineralizing clone SB-1[a]	98
M-B4	Uncultured bacterium SJA-170[b]	95
	Archaeal domain	
S-A2	*Methanofollis tationis*[c]	98

[a] Phelps *et al*. (1998)

[b] von Wintzingerode *et al*. (1999)

[c] Zellner *et al*. (1999)

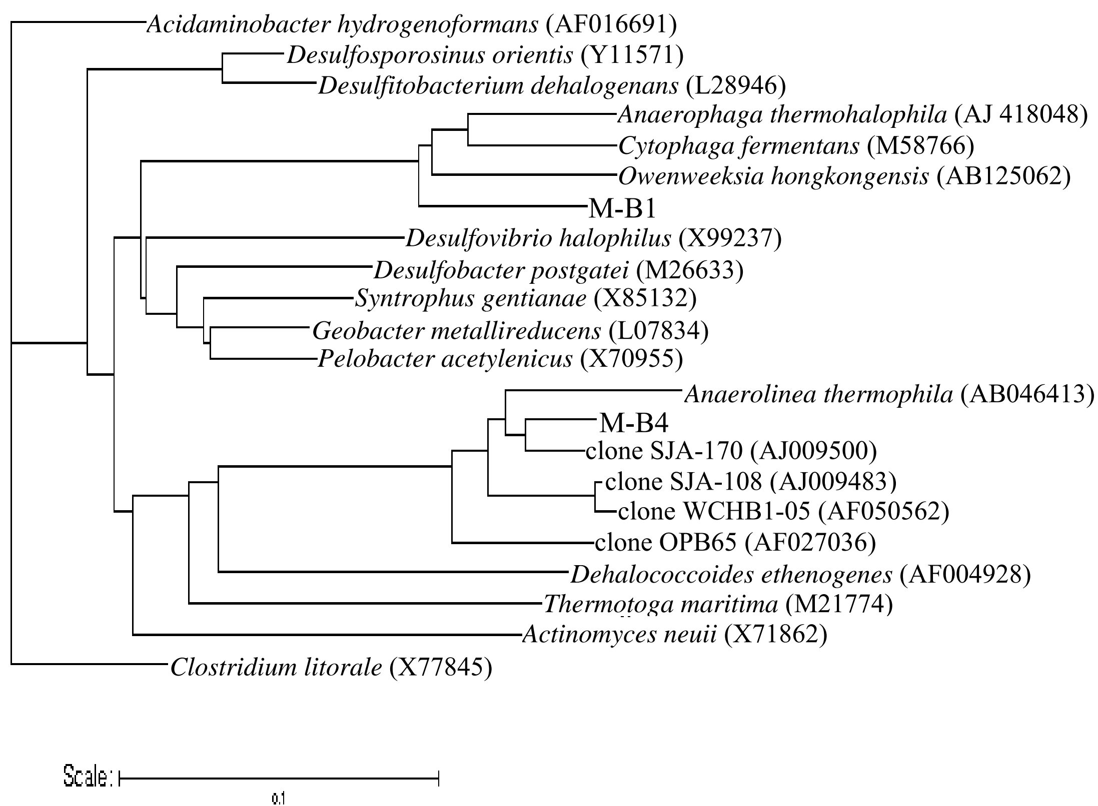

Figure 6.2 Phylogenetic tree of bacterial 16S rDNA sequences retrieved from M-NCZ. The GeneBank accession number for each reference sequence is shown in parenthesis. The sequence of *Clostridium litorale* was used as an out-group. The scale bar represents 10 nucleotide substitutions per 100 bases.

stimulated significantly in the presence of hydrogen-consuming methanogens, and accordingly suggesting that there was a syntrophic cooperative interaction to some extent between the filamentous bacterium and a hydrogen-consuming archaeal organism represented by clone S-A2. Also, it appeared that filamentous microorganisms corresponding to clone M-B4 did not grow without complex nitrogen sources such as yeast extract. Sekiguchi *et al*. (2001) reported one species of green non-sulfur bacteria (strain UNI-1) revealing similar aspects of this filamentous organism. Strain UNI-1 was described as a filamentous floc-forming bacterium exhibiting some extent of sensitivity to H_2 and growing on carbohydrate such as starch, sucrose and glucose only in the presence of complex nitrogen sources. However, strain UNI-1 did not grow below 45°C while the filamentous bacterium represented by clone M-B4 showed growth at 30°C. When the glucose culture was transferred to the liquid medium containing starch, the slow growth of filamentous bacterium was observed. It is an interesting result because clone M-B4 corresponding to the filamentous bacteria was not monitored when colony M-NCZ was initially cultivated in starch culture. The plausible explannation for this result is that the bacterium (M-Phe-1) represented by M-B1 grew fast, and thus outcompeted the filamentous bacterium in the starch culture. In the glucose culture, however, since M-Phe-1 revealed the long lag phase as demonstrated in chapter 5, the filamentous organism could grow and become a dominant organism in spite of the slow growth rate. No production of surfactant in the liquid cultures growing on starch, glucose and 2-propanol was detected with the drop-collapse method (Bodour and Miller-Maier, 1998), implying that the clear zone was not generated due to the dissolution of NAP by surfactant.

NAP degradation study with agar plate and liquid medium In order to investigate if NAP-degrading activity was retained through transfer, the clear-zone-forming colony M-NCZ was transferred to an agar plate with an opaque overlayer. As the incubation time was extended to 10 months, however, detecting clear zones was not possible because of the sublimation of NAP. Furthermore, although there were numerous tiny colonies with the size of 0.1 to 0.2 mm in diameter on the agar plates, they were identified as a species of the *Methanofollis* genus corresponding to clone S-A2. Since methanogens have been known to utilize simple carbon sources containing 1 or 2 carbons such as acetate, formate or methanol, the colonies on the agar plate may have utilized H_2 in the jar rather than the supplied NAP. The possible reason for the absence of bacterial colonies would be the lack of nutrients necessary for the growth of bacterial species participating in syntrophic NAP degradation. As demonstrated before, the filamentous bacterium could grow only in the presence of complex nitrogen nutrients. Since complex nitrogen sources or other necessary nutrient components could be supplied along with enrichment cultures, which were used as inoculum sources, containing undefined nutrients, it may be feasible to support the growth of microorganisms required for NAP degradation and consequently to generate the clear zone at the first attempt of isolation.

The degradation of NAP was also examined with the M-NCZ subcultures growing on starch, glucose and 2-propanol followed by centrifugation of the cultures. However, there was no significant disappearance of NAP compared to control cultures during 90 days of incubation. This result could suggest that microorganisms corresponding to clone M-B1, M-B4 and S-A2 did not represent a NAP-degrading consortium. We used

various carbon sources to stimulate the growth of different microorganisms; however, specific carbon sources can cause the outgrowth of certain species of microbes, resulting in the exclusion of slow-growing microorganisms required for NAP degradation, like the filamentous bacterium in the starch culture as demonstrated above. In addition, since it has been known that over 95% of microorganisms detectable by molecular phylogenetic studies with 16S rDNA analysis are not cultivable with conventional techniques (Amann *et al*., 1995), our cultivation methods may omit unculturable microorganisms necessary for the degradation of NAP, thus limiting the culture's NAP degradation capability.

Based on the results obtained so far, it is not clear yet which microorganism is responsible for the initial attack on NAP. Nevertheless, it could be presumably suggested that bacterium M-Phe-1 initiated the degradation of NAP since M-Phe-1 has been identified as an initial attacker on PHE as described in chapter 5. It would be a reasonable assumption based on previous studies demonstrating that microorganisms degrading aromatic hydrocarbons can generally utilize lower molecular weight aromatic compounds as well (String and Aitken, 1995; Heitkamp et al., 1988).

In conclusion, the isolation of NAP-degrading microorganisms was successfully performed by utilizing the opaque overlayer technique. Though the cultivated microbial consortium did not reveal NAP degradation activity on the agar plate and in the liquid media, it should not be excluded that the naturally existing microbial consortium which cannot be recovered in the lab with standard cultivation technique may degrade NAP as well as other recalcitrant compounds.

Chapter 7 Conclusions and Recommendations

This study describes not only the first isolations of NAP- and PHE-degrading microorganisms under methanogenic conditions by employing modified plating methods but also, more remarkably, the first identification and isolation of a fermentative bacterium responsible for initiating a syntrophic PHE-degradation.

To isolate PAH-degrading microbes under anaerobic conditions, a modified plating method was first developed for detecting the microorganisms degrading solid PAHs on the agar-overlay plate. The modified plating method presented in this study was successfully utilized to isolate both aerobic and anaerobic PAH-degrading microorganisms. In addition, it was verified that this developed method addressed the problems existing with other isolation methods such as the toxicity of the volatile solvents on the bacteria, contamination of the working area with PAH solutions, complicated experimental apparatus and so on. Since the lack of isolates has limited the understanding of anaerobic metabolism of PAHs, the modified plating method will likely stimulate more research on anaerobic PAH degradation with isolated pure cultures.

Molecular characterization of PAH-degrading methanogenic cultures via comparative 16S rDNA sequence analysis was utilized to monitor the microbial community structure and consequently to design isolation strategies for the possible microbial species responsible for PAH-degradation. By employing the modified plating method, PHE-degrading microorganisms under methanogenic conditions were successfully isolated from the enrichment cultures utilizing Baltimore Harbor

(Baltimore, MD) sediments. With the defined microbial consortium of PHE-degrading colonies grown on various carbon sources, it was observed that the degradation of PHE was partially inhibited by 2-bromoethanesulfonic acid and no $^{14}CH_4$ was detected when [9-^{14}C] PHE was added, indicating incomplete mineralization of PHE. However, it should not be excluded that our cultivation methods may omit unculturable microorganisms necessary for the complete mineralization of PHE, possibly causing no observable $^{14}CH_4$ or $^{14}CO_2$. One species of bacterium falling within the subgroup of *Cytophagales* in the CFB group was isolated and identified as an initial microbial catalyst for the transformation of PHE. This is the first report, to our knowledge, that demonstrates the isolation of a syntrophic unsubstituted aromatic hydrocarbon-degrading microorganism under methanogenic conditions.

NAP-degrading microorganisms under methanogenic conditions were also isolated by utilizing an agar-overlay containing evenly dispersed fine particles of NAP. The microbial consortium of the isolated NAP-degrading colony grown on various carbon sources, however, did not reveal NAP-degradation activity in the liquid media possibly due to the loss of specific microbial species that were required for the syntrophic NAP-degradation. In NAP-degrading colony, one species of the bacteria was identified to be the same microorganism as a fermentative bacterium presumably responsible for the initial catalyst activity for the transformation of PHE, suggesting that this bacterium may initiate NAP-transformation as well.

The depletion of electron acceptors such as nitrate, sulfate and iron results in methanogenic conditions in soil and sediment, especially, closest to the source of contamination (Lovely, 1997). Very recently, methanogenic conditions have drawn

remarkable attention on the degradation of recalcitrant compounds such as aromatic hydrocarbons and hexadecane, which were considered to be non-degradable compounds under methanogenic conditions (Kazumi *et al*., 1997; Weiner and Lovley, 1998; Beller and Edwards, 2000; Chang *et al*., 2001; Zengler *et al.*, 1999; Parkes, 1999; Anderson and Lovley, 2000). The identification and isolation of microorganisms initiating degradation, however, has not been successful because of the syntrophic relationship between multiple species under methanogenic conditions (Gibson and Harwood, 2002). The presented studies here will likely contribute to the isolation of anaerobic PAH-degrading microbes with developed modified plating method as well as the identification of the microbial consortia for the biodegradation of recalcitrant compounds under anaerobic conditions. Since it has been known that over 95% of microorganisms detectable by molecular phylogenetic studies are not cultivable with conventional techniques (Amann *et al*., 1995), it should be considered that our cultivation methods may omit unculturable microorganisms necessary for the degradation of complex compounds, bringing about no observable degradation in the laboratory while leaving a possibility of the degradation in natural environments.

In addition to the presented studies, future studies are necessary to understand PAH-degradation under methanogenic conditions with the isolated microorganisms. Further characterization of the isolates could be conducted based on morphology, temperature range, substrate range, and oxygen sensitivity. Also, the key enzymatic reactions can be elucidated and the enzymes purified and characterized. Enzymes involved in PAH degradation can be screened by employing two-dimensional (2D) gel

electrophoresis to detect proteins that are expressed or increased after PAH exposure. Comparison of proteins from PAH-containing and PAH-free cultures on 2D gels will make it possible to monitor expressed proteins in the presence of PAHs, which might be enzymes involved in PAH degradation. By utilizing the GenBank BLAST search program at the National Center for Biotechnology Information, the amino acids sequences can be conducted to find the homology to other known catabolic enzymes, enabling us to identify the role of enzymes in PAH degradation. Genomic study can be also utilized to identify genes in PAH catabolic pathways. Under sulfate reducing conditions, carboxylation has been proposed as an initial step in NAP and PHE degradation (Zhang and Young, 1997) resulting in the transformation of PAH to benzoate-like structures. The first step in the benzoate anaerobic degradative pathway is catalyzed by a well-characterized enzyme, benzoate-CoA ligase (Harwood and Gibson, 1997). Using the gene sequences for benzoate-CoA ligase, molecular probe/PCR primers can be developed to determine if this enzyme or similar enzymes are present in the PAH-degrading microorganisms. Also, the metabolic intermediates can be investigated with gas chromatography/mass spectrometry. Radiolabeled PAHs could be utilized to verify the intermediates and metabolic mechanisms. Identifying a defined microbial consortium for the complete mineralization of PAH and determining the role of each species in the syntrophic degradation process would be challenging since standard cultivation methods employed in the laboratory might cause the dramatic change of microbial environment, accordingly resulting in different microbial community from the endogenous one. Nevertheless, the further study on the mechanisms and the factors affecting anaerobic PAH degradation with pure cultures of

microorganisms will make it more feasible to gain a detailed understanding on PAH-degradation.

REFERENCES

Alley, J.F., and Brown, L.R. (2000) Use of sublimation to prepare solid microbial media with water- insoluble substrates. *Appl Environ Microbiol* 66: 439.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., and Lipman, D.J. (1990) Basic local alighnment search tool. *J. Mol. Biol.* 215: 403.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic Identification and in-Situ Detection of Individual Microbial-Cells without Cultivation. *Microbiological Reviews* 59: 143.

Anderson, R.T., and Lovley, D.R. (2000) Hexadecane decay by methanogenesis. *Nature* 404: 722.

Apolinario, E.A., and Sowers, K.R. (1996) Plate colonization of *Methanococcus maripaludis* and *Methanosarcina thermophila* in a amodified canning jar. *FEMS Microbiology ecology* 145: 131.

Auburger, G., and Winter, J. (1995) Isolation and physiological characterization of *Syntrophus buswellii* strain GA from a syntrophic benzoate degrading strictly anaerobic coculture. *Applied Microbiology and Biotechnology* 44: 241.

Balkwill, D.L., Reeves, R.H., Drake, G.R., Reeves, J.Y., Crocker, F.H., King, M.B., and Boone, D.R. (1997) Phylogenetic characterization of bacteria in the subsurface microbial culture collection. *FEMS Microbiol Rev* 20: 201.

Barnsley, E.A. (1983) Bacterial oxidation of naphthalene and phenanthrene. *J Bacteriol* 153: 1069.

Bauer, J.E., and Capone, D.G. (1985) Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and maphthalene in intertidal marine sedmints. *Appl Environ Microbiol* 50: 81.

Beaty, P.S., and McInerney, M.J. (1987) Growth of *Syntrophomonas-Wolfei* in Pure Culture on Crotonate. *Archives of Microbiology* 147: 389.

Bedessem, M.E., SwobodaColberg, N.G., and Colberg, P.J.S. (1997) Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiology Letters* 152: 213.

Beller, H.R., and Edwards, E.A. (2000) Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl Environ Microbiol* 66: 5503.

Bodour, A.A., and Miller-Maier, R.M. (1998) Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *Journal of Microbiological Methods* 32: 273.

Bogardt, A.H., and Hemmingsen, B.B. (1992) Enumeration of phenanthrene-degrading bacteria by an overlayer technique and its use in evaluation of petroleum-contaminated sites. *Appl Environ Microbiol* 58: 2579.

Bouchez, M., Blanchet, D., and Vandecasteele, J.P. (1995) Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. *Appl Microbiol Biotechnol* 43: 156.

Burlage, R.S., Hooper, S.W., and Sayler, G.S. (1989) The TOL (pWW0) catabolic plasmid. *Appl Environ Microbiol* 55: 1323.

Cabirol, N., Villemur, R., Perrier, J., Jacob, F., Fouillet, B., and Chambon, P. (1998) Isolation of a methanogenic bacterium, *Methanosarcina* sp. strain FR, for its ability to degrade high concentration of perchloroethylene. *Can J Microbiol* 44: 1142.

Cerniglia, C.E. (1992) Biodegradation of polycyclic aromatic hydrocarbons. *biodegradation* 3: 351.

Chang, W. (2003) Anaerobic biodegradation of Polycyclic Aromatic Hydrocarbons. Ph.D. disseration. Department of Chemical engineering. College Park: University of Maryland.

Chang, W., T. Jones, and T. R. Pulliam Holoman (2001) Anaerobic polycyclic aromatic hydrocarbon (PAH)-degrading enrichment cultures under methanogenic conditions. In *Bioremediation of Energetics, Phenolics, and Polynuclear Aromatic Hydrocarbons, Proceedings of the Sixth International In Situ and On-Site Bioremediation Symposium*. V. Magar, G.J., S. Ong, and A. Leeson (ed). Battelle, Columbus, OH, pp. 205.

Churchill, S.A., Harper, J.P., and Churchill, P.F. (1999) Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Appl Environ Microbiol* 65: 549.

Coates, J.D., Anderson, R.T., and Lovely, D.R. (1996) Oxidation of Polycyclic aromatic hydrocarbons under sulfate-reducing conditions. *Appl Environ Microbiol* 62: 1099.

Coates, J.D., Woodward, J., Allen, J., Philp, P., and Lovley, D.R. (1997) Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl Environ Microbiol* 63: 3589.

Daane, L.L., Harjono, I., Zylstra, G.J., and Haggblom, M.M. (2001) Isolation and characterization of polycyclic aromatic hydrocarbon- degrading bacteria associated with the rhizosphere of salt marsh plants. *Appl Environ Microbiol* 67: 2683.

Denger, K., and Schink, B. (1995) New halo and thermotolerant fermenting bacteria producing surface-active compounds. *Applied Microbiology and Biotechnology* 44: 161.

Denger, K., Warthmann, R., Ludwig, W., and Schink, B. (2002) *Anaerophaga thermohalophila* gen. nov., sp nov., a moderately thermohalophilic, strictly anaerobic fermentative bacterium. *International Journal of Systematic and Evolutionary Microbiology* 52: 173.

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R. (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent- contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* 64: 3869.

Eaton, R.W. (1994) Organization and evolution of naphthalene catabolic pathways: sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and trans- o-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. *J Bacteriol* 176: 7757.

Evans, W.C., and Fuchs, G. (1988) Anaerobic degradation of aromatic compounds. *Annu Rev Microbiol* 42: 289.

Falz, K.Z., Holliger, C., Grosskopf, R., Liesack, W., Nozhevnikova, A.N., Muller, B. (1999) Vertical distribution of methanogens in the anoxic sediment of Rotsee (Switzerland). *Applied and Environmental Microbiology* 65: 2402.

Felsenstein, J. 1993. in *http://evolution.genetics.washington.edu/phylip.html* PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.

Fendrich, C., Hippe, H., and Gottschalk, G. (1990) *Clostridium-Halophilium* Sp-Nov and *Clostridium-Litorale* Sp-Nov, an Obligate Halophilic and a Marine Species Degrading Betaine in the Stickland Reaction. *Archives of Microbiology* 154: 127.

Ficker, M., Krastel, K., Orlicky, S., and Edwards, E. (1999) Molecular charaterization of a Toluene-degrading methanogenic consortium. *Appl. Environ. Microbiol.* 65: 5576.

Foght, J.M., and Westlake, D.W. (1991) Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. *Can J Microbiol* 37: 924.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* 1: 415.

Geiselbrecht, A.D., Herwig, R.P., Deming, J.W., and Staley, J.T. (1996) Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget sound sediments. *Appl Environ Microbiol* 62: 3344.

Gibson, J., and Harwood, C.S. (2002) Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Annual Review of Microbiology* 56: 345.

Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol* 63: 2802.

Grbic-Galic, D. (1991) Anaerobic transformation of aromatic hydrocarbon pollution under fernentative/methanogenic conditions. *Periodicum Biologorum* 93: 533.

Grbic-Galic, D., and Vogel, T.M. (1987) Transformation of toluene and benzene by mixed methanogenic cultures. *Appl Environ Microbiol* 53: 254.

Haack, S.K., and Breznak, J.A. (1993) *Cytophaga-Xylanolytica* Sp-Nov, a Xylan-Degrading, Anaerobic Gliding Bacterium. *Archives of Microbiology* 159: 6.

Harwood, C.S., and Gibson, J. (1997) Shedding light on anaerobic benzene ring degradation: a process unique to prokaryotes? *J Bacteriol* 179: 301.

Heider, J., Spormann, A.M., Beller, H.R., and Widdel, F. (1998) Anaerobic bacterial metabolism of hydrocarbons. *Fems Microbiology Reviews* 22: 459.

Heitkamp, M.A., and Cerniglia, C.E. (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. *Appl Environ Microbiol* 54: 1612.

Heitkamp, M.A., and Cerniglia, C.E. (1989) Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. *Appl Environ Microbiol* 55: 1968.

Heitkamp, M.A., Franklin, W., and Cerniglia, C.E. (1988) Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. *Appl Environ Microbiol* 54: 2549.

Herbes, S.E., and Schwall, L.R. (1978) Microbial Transformation of Polycyclic Aromatic-Hydrocarbons in Pristine and Petroleum-Contaminated Sediments. *Applied and Environmental Microbiology* 35: 306.

Holoman, T.R., Elberson, M.A., Cutter, L.A., May, H.D., and Sowers, K.R. (1998) Characterization of a defined 2,3,5, 6-tetrachlorobiphenyl-ortho- dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. *Appl Environ Microbiol* 64: 3359.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, A.T., and Williams, S.T. (1994) *Bergey's Manual of determinative bacteriology*. Baltimore, MD: Willams & Wilkins.

Jackson, B.E., Bhupathiraju, V.K., Tanner, R.S., Woese, C.R., and McInerney, M.J. (1999) *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Archives of Microbiology* 171: 107.

Johnson, A.C., Larsen, P.F., Gadbois, D.F., and Humason, A.W. (1985) The Distribution of Polycyclic Aromatic-Hydrocarbons in the Surficial Sediments of Penobscot Bay (Maine, USA) in Relation to Possible Sources and to Other Sites Worldwide. *Marine Environmental Research* 15: 1.

Kastner, M., Breuer-Jammali, M., and Mahro, B. (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). *Appl Microbiol Biotechnol* 41: 267.

Kazumi, J., Caldwell, M.E., Suflita, J.M., Lovley, D.R., and Young, L.Y. (1997) Anaerobic degradation of benzene in diverse anoxic environments. *Environmental Science & Technology* 31: 813-818.

Keith, L.H., and Telliard, W.A. (1979) Priority pollutants, I-a perspective view. *Environ. Sci. Technol.* 13: 418.

Kim, E., Aversano, P.J., Romine, R.P., Schneider, R.P., and Zylstra, G.J. (1996) Homology between genes for aromatic hydrocarbon degradation in surface and deep-subsurface *Sphingomonas* strains. *Appl. Environ. Microbiol.* 62: 1467.

Kiyohara, H., Nagao, K., Kouno, K., and Yano, K. (1982) Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on grar plates. *Appl Environ Microbiol* 43: 454.

Knight, V.K., Kerkof, L.j., and Haggblom, M.M. (1999) Community analyses of sulfidogenic 2-bromophenol-dehalogenating and phenol-degrading microbial consortis. *FEMS Microbiology ecology* 29: 137.

Langenhoff, A.A.M., Zehnder, A.J.B., and Schraa, G. (1996) Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* 7: 267.

Laramee, L., Lawrence, J.R., and Greer, C.W. (2000) Molecular analysis and development of 16S rRNA oligonucleotide probes to characterize a diclofop-methyl-degrading biofilm consortium. *Can J Microbiol* 46: 133.

Loffler, F.E., Ritalahti, K.M., and Tiedje, J.M. (1997) Dechlorination of chloroethenes is inhibited by 2-bromoethanesulfonate in the absence of methanogens. *Applied and Environmental Microbiology* 63: 4982-4985.

Lovley, D.R. (1997) Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. *Journal of Industrial Microbiology & Biotechnology* 18: 75.

Maidak, B., Olsen, G., Larsen, N., Overbeek, R., McCaughey, M., and Woese, C. (1997) The RDP (Ribosomal Database Project). *Nucl. Acids. Res.* 25: 109.

McNally, D.l., Michelcic, J.R., and Lueking, d.R. (1998) Biodegradation of three and four ring polycylic aromatic hydrocarbons under aerobic and denitrifying conditions. *Environ. Sci. Technol.* 32: 2633.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000) Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* 66: 2743.

Menn, F.M., Applegate, B.M., and Sayler, G.S. (1993) NAH plasmid-mediated catabolism of anthracene and phenanthrene to naphthoic acids. *Appl Environ Microbiol* 59: 1938.

Michelcic, J.R., and Luthy, R.G. (1988) Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl Environ Microbiol* 54: 1182.

Mihelcic, J.R., and Luthy, R.G. (1988) Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. *Appl Environ Microbiol* 54: 1188.

Moody, J.D., Freeman, J.P., Doerge, D.R., and Cerniglia, C.E. (2001) Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* 67: 1476.

Neff, J.M. (1979) *Polycyclic aromatic hydrocarbons in th aquatic environment: Sources, fates and biological effects*. London, England: Applied Sciecnce Publishers, Ltd.

Parkes, J. (1999) Cracking anaerobic bacteria. *Nature* 401: 217.

Phelps, C.D., Kerkhof, L.J., and Young, L.Y. (1998) Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. *FEMS Microbiology ecology* 27: 269.

Piatt, J.J., Backhus, D.A., Capel, P.D., and Eisenreich, S.J. (1996) Temperature-dependent sorption on naphthalene, phenanthrene and pyrene to low organic carbon aquifer sediments. *Environ. Sci. Technol* 30: 751.

Raskin, L., Rittmann, B.E., and Stahl, D.A. (1996) Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Appl Environ Microbiol* 62: 3847.

Riser-Roberts, E. (1998) *Biodegradation of petroleum-contaminated soils: Biological, physical and chemical proesses*. Boca Raton, FL: Lewis publishers.

Robertson, W.J., Franzmann, P.D., and Mee, B.J. (2000) Spore-forming, Desulfosporosinus-like sulphate-reducing bacteria from a shallow aquifer contaminated with gasoline. *J Appl Microbiol* 88: 248.

Rockne, K.J., and Strand, S.E. (1998) Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environ. Sci. Technol.* 32: 3962.

Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000) Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl Environ Microbiol* 66: 1595.

Rueter, P., Rabus, R., Wilkes, H., Aeckersberg, F., Rainey, F.A., Jannasch, H.W., and Widdel, F. (1994) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature* 372: 455.

Sanseverino, J., Applegate, B.M., King, J.M., and Sayler, G.S. (1993) Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. *Appl Environ Microbiol* 59: 1931.

Schink, B. (2002) Synergistic interactions in the microbial world. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 81: 257.

Schlotelburg, C., von Wintzingerode, F., Hauck, R., Hegemann, W., and Gobel, U.B. (2000) Bacteria of an anaerobic 1,2-dichloropropane-dechlorinating mixed culture are phylogenetically related to those of other anaerobic dechlorinating consortia. *Int J Syst Evol Microbiol* 50 Pt 4: 1505.

Sekiguchi, Y., Takahashi, H., Kamagata, Y., Ohashi, A., and Harada, H. (2001) In situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: a novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. *Appl Environ Microbiol* 67: 5740.

Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H., and Kamagata, Y. (2003) *Anaerolinea thermophila* gen. nov., sp nov and *Caldilinea aerophila* gen. nov., sp nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. *International Journal of Systematic and Evolutionary Microbiology* 53: 1843.

Selenska-Pobell,S. (2002) Diversity and activity of bacteria in uranium mining waste piles in *Radioactivity in the environment vol.22-Interactions of microorganisms with radionuclides*, Keith-Roach,M.J. and Lievens,F.R. (Eds.), Elsevier, pp 225.

Shiaris, M.P., and Cooney, j.J. (1983) Replica plating method for estimating phenanthrene-utilizing and phenanthrene-cometanolizing microorganisms. *Appl Environ Microbiol* 45: 706.

Sowers, K.R. (1995) Techniques for monitoring methanogen cell growth. In *Archaea: A Laboratory Manual*. Robb, F.T., Place, A.R., Sowers, K.R., Schreier, H.J., DasSarma, S., and Fleischmann, E.M. (eds). Plainview, NY: Cold Spring Harbor Laboratory Press.

Sowers, K.R., and Noll, K.M. (1995) Techniques for anaerobic growth. In *Archaea: A Laboratory Manual*. Robb, F.T., Place, A.R., Sowers, K.S., Schreier, H.J., DasSarma, S., and Fleischmann, E.M. (eds). Plainview, NY: Cold Spring Harbor Laboratory Press, pp. 15.

Stringfellow, W.T., and Aitken, M.D. (1995) Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. *Appl Environ Microbiol* 61: 357.

Tasaki, M., Kamagata, Y., Nakamura, K., Okamura, K., and Minami, K. (1993) Acetogenesis from Pyruvate by *Desulfotomaculum-Thermobenzoicum* and Differences in Pyruvate Metabolism among 3 Sulfate-Reducing Bacteria in the Absence of Sulfate. *Fems Microbiology Letters* 106: 259.

Tebbe, C.C., and Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl Environ Microbiol* 59: 2657.

von Wintzingerode, F., Selent, B., Hegemann, W., and Gobel, U.B. (1999) Phylogenetic analysis of an anaerobic, trichlorobenzene-transforming microbial consortium. *Appl Environ Microbiol* 65: 283.

Wallrabenstein, C., Gorny, N., Springer, N., Ludwig, W., and Schink, B. (1995) Pure Culture of Syntrophus-Buswellii, Definition of Its Phylogenetic Status, and Description of Syntrophus-Gentianae Sp-Nov. *Systematic and Applied Microbiology* 18: 62.

Weiner, J.M., and Lovley, D.R. (1998) Rapid Benzene Degradation in Methanogenic Sediments from a Petroleum-Contaminated Aquifer. *Appl Environ Microbiol* 64: 1937.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173: 697.

Whitman, B.E., Lueking, D.R., and Mihelcic, J.R. (1998) Naphthalene uptake by a Pseudomonas fluorescens isolate. *Canadian Journal of Microbiology* 44: 1086.

Widdel, F., and Rabus, R. (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Current Opinion in Biotechnology* 12: 259.

Wolin, E.A., Wolin, M.J., and Wolfe, R.S. (1963) Formation of Methane by Bacterial Extracts. *Journal of Biological Chemistry* 238: 2882.

Yen, K.M., and Serdar, C.M. (1988) Genetics of naphthalene catabolism in pseudomonads. *Crit Rev Microbiol* 15: 247.

Zehnder, A.J.B., Huser, B., and Brock, T.D. (1979) Measuring Radioactive Methane with the Liquid Scintillation-Counter. *Applied and Environmental Microbiology* 37: 897.

Zellner, G., Boone, D.R., Keswani, J., Whitman, W.B., Woese, C.R., Hagelstein, A. (1999) Reclassification of *Methanogenium tationis* and *Methanogenium liminatans* as *Methanofollis tationis* gen. nov., comb. nov. and *Methanofollis liminatans* comb. nov. and description of a new strain of *Methanofollis liminatans*. *International Journal of Systematic Bacteriology* 49: 247.

Zengler, K., Richnow, H.H., Rossello-Mora, R., Michaelis, W., and Widdel, F. (1999) Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* 401: 266.

Zhang, X., and Young, L.Y. (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* 63: 4759.

Zhang, Y., Maier, W.J., and Miller, R.M. (1997) Effect of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. *Environ. Sci. Technol.* 31: 2211.