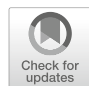

Anaerobic Biodegradation of Hydrocarbons: Metagenomics and Metabolomics

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Lisa M. Gieg and Courtney R. A. Toth

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Abstract

The biodegradation of hydrocarbons under anaerobic conditions is a significant process that is now known to occur in diverse environments. Understanding this process has important implications for the bioremediation of hydrocarbon-contaminated terrestrial and marine environments, for enhanced energy recovery from deep subsurface fossil energy reservoirs, and for climate change effects related to the release of methane and other hydrocarbons from natural seeps and hydrothermal vents. While much understanding of anaerobic hydrocarbon

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metabolism has been gleaned from cultivation-based studies, cultivation-independent meta-omics approaches such as metagenomics can offer new insights into the process in more complex, natural hydrocarbon-containing environments. Further, a metabolomic approach that seeks specific metabolites diagnostic of anaerobic hydrocarbon biodegradation can provide the “ultimate proof” that this process is occurring in situ. This chapter highlights the key pathways of anaerobic hydrocarbon metabolism and summarizes metagenomic information garnered to date from sequencing hydrocarbon degraders, enrichment cultures, and diverse hydrocarbon-containing environmental samples. Further, a brief overview of hydrocarbon metabolomics is presented, along with research needs on this topic.

1 Introduction

Hydrocarbons are ubiquitous across the globe. Comprised of carbon and hydrogen atoms, hydrocarbons are considered natural products; they are synthesized by some algal, plant, and animal species to serve a variety of functions (Harms et al. 1999) and are generated from forest fires and other anthropogenic sources (Vergnoux et al. 2011). By far though, the Earth’s terrestrial and marine subsurface environments contain the largest inventory of hydrocarbons (including the smallest hydrocarbon, methane). These are found beneath the ocean floor where they can be naturally emitted via cold seeps or generated within hydrothermal vent systems (Boetius and Wenzhöfer 2013; Teske et al. 2014) or are entrained in crude oil, coal, or shale reservoirs, where hydrocarbons were generated from ancient buried biomass subject to high temperatures and pressures over geologic time. Due to humankind’s predominant use of petroleum-based energy, the recovery of gaseous and liquid hydrocarbons from such deposits and its subsequent distribution, refining, storage, and combustion have led to widespread water and land contamination with hydrocarbons. While hydrocarbons are considered chemically inert (Widdel and Musat 2010), it has now been definitively demonstrated that naturally occurring microbial communities contain a multitude of enzymes that can readily biotransform hydrocarbons (including methane) both in the presence and absence of oxygen (Widdel and Musat 2010). Microbial hydrocarbon degradation is now known to occur in surface soils and sediments, shallow groundwater environments, subsurface fossil energy reservoirs, cold seeps, marine waters and sediments, and hydrothermal vent systems. This fact has many important implications for the remediation of hydrocarbon-contaminated areas (Techtmann and Hazen 2016), for crude oil quality in reservoirs or for enhanced energy recovery (Head et al. 2014), and for minimizing greenhouse gas (methane) emissions from natural terrestrial and marine systems (Boetius and Wenzhöfer 2013). This chapter will focus on hydrocarbon biodegradation by anaerobic microorganisms; for information on aerobic hydrocarbon biodegradation, the reader is referred to recent reviews (Rojo 2009; Ghosal et al. 2016).

Knowledge regarding anaerobic hydrocarbon biodegradation has been gained mainly from research conducted in the last 30 years. Such research has led to the isolation of dozens of anaerobic hydrocarbon-utilizing isolates (nitrate-reducing, metal-reducing, sulfate-reducing bacteria and archaea) and enriched methanogenic consortia (e.g., Tables 1 and 2; Widdel et al. 2010; Jiménez et al. 2016) along with the description of numerous anaerobic hydrocarbon-degrading enrichment cultures or processes (e.g., a search on PubMed for the term "anaerobic hydrocarbon biodegradation" yielded >7000 references, accessed October 2016). Thus, as the topic of anaerobic hydrocarbon biodegradation is vast, this chapter will provide a general overview as to how recent cultivation-independent "omics" approaches are starting to be applied to our understanding of this process. Many omics/meta-omics technologies have emerged in recent years that examine biological molecules at various levels of information flow: from DNA ((meta)genomics, genetic potential for activity) to RNA ((meta)transcriptomics, identifying expressed genes) to proteins ((meta)proteomics, detecting enzymes or other proteins actually made to catalyze biochemical reactions) and finally to metabolites (metabolomics, identifying biochemical products of expressed genes and enzymes). Here, we highlight how metagenomic (and metatranscriptomics or metaproteomics in some cases) approaches have been used to understand anaerobic hydrocarbon biodegradation in general and to gain insight into the ecological principles governing hydrocarbon-containing environments including in hydrocarbon resource environments (oil reservoirs, coal seams, and shale formations), in natural hydrocarbon-releasing cold seeps and hydrothermal vents, and in hydrocarbon-contaminated marine and terrestrial environments. A discussion on the use of metabolomics to assess anaerobic in situ biodegradation in hydrocarbon-containing environments is included here as well, but the reader is referred to a more detailed consideration of this topic in this manual (Gieg and Toth 2016) and to other recent reviews (Agrawal and Gieg 2013; Callaghan 2013a).

2 Anaerobic Hydrocarbon Metabolism: A General Overview

Recent publications (including several chapters in this manual, e.g., *Biochemistry of Anaerobic Degradation of Hydrocarbons*) describe in detail the anaerobic biodegradation of various classes of hydrocarbons including alkanes (Callaghan 2013b; Musat 2015; Rabus et al. 2016), monoaromatic hydrocarbons (Foght 2008), and polycyclic aromatic hydrocarbons (PAHs) (Meckenstock et al. 2016), so here we only highlight some of the major hydrocarbon activation mechanisms. Notably, the principles governing anaerobic hydrocarbon metabolism have been mainly determined by studying either highly enriched cultures or pure cultures isolated from hydrocarbon-bearing environments. Compilations of such isolates are available (Widdel et al. 2010; Heider and Schühle 2013; Stagars et al. 2016) and include nitrate-reducing, iron-reducing, and sulfate-reducing bacteria (and one sulfate-reducing archaeum); some examples are provided in Tables 1 and 2. Highly enriched

Table 1 Summary of anaerobic hydrocarbon-degrading isolates for which a whole genome sequence is available

Isolate	Electron acceptor	Hydrocarbons utilized	Genome status and accession number	Relevance of study	References
Saturated hydrocarbon degraders					
Gamma proteobacterium HdN1	NO_3^-	Alkanes C_6–C_{30}	Complete, NC_014366	Failure to detect an *ass*- or *mas*-like gene offered evidence that fumarate-independent mechanisms of anaerobic alkane activation were indeed possible, as proposed by previous studies	Ehrenreich et al. (2000) and Zedelius et al. (2011)
Desulfosarcina sp. BuS5	SO_4^{2-}	Alkanes C_3–C_4	Whole genome shotgun sequence, NZ_AXAM00000000	Demonstrated fumarate addition to propane at the C_1 and C_2 position. Genome analysis identified a single putative *masD* gene, suggesting that the same gene is responsible for C_3 and C_4 activation	Kniemeyer et al. (2007), and Sievert et al. (unpublished – submitted 2013)
Desulfococcus oleovorans Hxd3	SO_4^{2-}	*n*-Alkanes C_{12}–C_{20}; *n*-1-alkanes C_{14}–C_{17}	Complete, NC_009943	Genome analysis did not detect homologs to *ass* genes, supporting the theory that an alternate mechanism of alkane activation occurs in this isolate	Aeckersberg et al. (1998), So et al. (2003), and Copeland et al. (unpublished – submitted 2007)
Desulfatibacillum alkenivorans AK-01	SO_4^{2-}	*n*-Alkanes C_{13}–C_{18}, 1-hexadecene and 1-pentadecene	Complete, NC_011768	Confirmed physiological evidence of metabolic processes involved in anaerobic alkane degradation, such as fumarate regeneration, and successfully predicted this strain's ability to grow syntrophically with methanogens	So and Young (1999) and Callaghan et al. (2012)
Archaeoglobus fulgidus VC-16	SO_4^{2-}, $S_2O_3^{2-}$	C_{10}–C_{21}, *n*-alk-1-enes C_{12}–C_{21}	Complete, AE000782	First sequenced genome of a sulfate-reducing archaeum. Predicted to metabolize a variety of hydrocarbons due to the presence of 57 β-oxidation genes. This was recently experimentally verified, and a fumarate addition gene was also detected	Klenk et al. (1997) and Khelifi et al. (2010, 2014)

Aromatic hydrocarbon degraders					
Dechloromonas aromatica RCB	NO_3^-	Benzene, toluene, xylene, chlorobenzoate	Complete, NC_007298	Homologs to known anaerobic monoaromatic hydrocarbon-degrading genes (e.g., *bss*, *pcr*, *bcr*) were notably absent, though aerobic genes were present, leading to the suggestion that benzene degradation was coupled to oxygen produced during nitrate reduction	Coates et al. (2001) and Salinero et al. (2009)
Azoarcus toluclasticus MF63	NO_3^-	Toluene	Whole genome shotgun sequence, NZ_ARJX00000000	Part of JGI's efforts to sequence 1000 genomes (proposal ID: 733)	Song et al. (1999) and Kyrpides et al. (unpublished – submitted 2013)
Azoarcus sp. CIB	NO_3^-	Toluene, xylene	Complete, CP011072	Metabolic flexibility suggests both aerobic and anaerobic monoaromatic hydrocarbon biodegradation is possible and that the strain can adapt to varying environmental stressors, making it a potential candidate for future remediation studies	Martín-Moldes et al. (2015)
Aromatoleum aromaticum EbN1	NO_3^-	Toluene, ethylbenzene	Complete, NC_006513 Two plasmids also sequenced; NC_006823, NC_006824	First sequenced genome of an anaerobic aromatic-degrading member of the Betaproteobacteria. Genes for 10 major peripheral pathways of aromatic metabolism (aerobic and anaerobic) were detected, including the *bss* operon and *ebdABC*	Rabus and Widdel (1995) and Rabus et al. (2005)
Geobacter metallireducens GS-15	Fe(III)	Toluene	Complete, NC_007517 One plasmid also sequenced; NC_007515	*G. metallireducens* was predicted to have greater metabolic diversity than *G. sulfurreducens*, including the ability to degrade toluene (experimentally verified). Physiology and gene regulation are also predicted to be substantially different from other *Geobacteraceae*	Aklujkar et al. (2009)

(*continued*)

Table 1 (continued)

Isolate	Electron acceptor	Hydrocarbons utilized	Genome status and accession number	Relevance of study	References
Geobacter sp. Ben	Fe(III)	Toluene, benzene	Draft genome, unpublished	Second sequenced genome of an anaerobic benzene-degrading isolate. Genomic insight confirmed aerobic benzene degradation is not possible	Zhang et al. (2012)
Ferroglobus placidus AEDII12DO	Fe(II)	Benzene	Complete, NC_013849	Sequenced genome of an anaerobic benzene-degrading thermophile	Holmes et al. (2011) and Anderson et al. (2011)
Desulfobacula toluolica Tol2	SO_4^{2-}	Toluene	Complete, NC_018645	First sequenced genome of an anaerobic aromatic-degrading marine sulfate reducer. Predicted the metabolism of toluene (experimental verified and metabolites detected) and carboxylated/hydroxylated derivatives. Another notable feature was the genome's unusually high plasticity, which seems to correlate with flexible substrate catabolism	Rabus et al. (1993) and Wöhlbrand et al. (2013)
Deltaproteobacterium NaphS2	SO_4^{2-}	2-Methyl-naphthalene	Whole genome shotgun sequence, NZ_ADZZ00000000	Predication of ring reduction genes (*ncr*) for naphthoyl-CoA in a manner analogous to *bcr* (for anaerobic benzoate ring reduction)	Galushko et al. (1999) and DiDonato et al. (2010)

Table 2 Summary of anaerobic hydrocarbon-degrading enrichment cultures for which a metagenomic sequence is available

Enrichment culture	Electron accepting process	Hydrocarbons utilized	Metagenome accession #	Relevance of study	References
Deltaproteobacterium N47	SO_4^{2-}	2-Methyl-naphthalene, naphthalene	Available across 17 contigs, FR695864–FR695880	Combined metagenomics and proteomics to predict/identify novel genes expressed during anaerobic growth on PAHs	Bergmann et al. (2011)
m-Xylene-degrading	SO_4^{2-}	*m*-Xylene	European Nucleotide Archive, PRJEB11632	Supported previous hypotheses that Epsilonproteobacteria do not initiate xylene degradation in this enrichment culture. Rather, this group was suggested to be mixotrophic and thought to scavenge acetate within the syntrophic consortium	Bozinovski et al. (2012, 2014) and Keller et al. (2015)
Benzene degrading	Methanogenesis	Benzene	Short Read Archive, PRJNA281117	Confirmed *Deltaproteobacterium* ORM2 as a key benzene degrader; metagenomic surveys identified sequences belonging Parcubacteria (candidate division OD1) not detected by qPCR. This organism was present in all benzene-degrading cultures evaluated (after suitable primers were designed) and may be a key player in benzene degradation	Luo et al. (2016)
SCADC	Methanogenesis	Short-chain alkanes (C_6–C_{10}, mixture also contains traces of 2-methylpentane and methylcyclopentane)	Short Read Archive, SRX831148 Draft genomes also available	Initial genomic surveys failed to detect sequence homologs for *assA* and *bssA* despite further targeted gene analysis confirming their presence (Tan et al. 2015b). A secondary genomic survey showed improved homology for *assABC* (Tan et al. 2015a), in addition to detecting other anaerobic hydrocarbon activation genes	Tan et al. (2013, 2015a, b)

(*continued*)

Table 2 (continued)

Enrichment culture	Electron accepting process	Hydrocarbons utilized	Metagenome accession #	Relevance of study	References
NAPDC	Methanogenesis	Naphtha, a mixture of monoaromatics and C_6–C_{10} alkanes	Short Read Archive, SRX831147	Much like SCADC, reported sequence homology for several anaerobic hydrocarbon activation genes across several hydrocarbon classes, albeit not all with high sequence homology	Tan et al. (2015a)
TOLDC	Methanogenesis	Toluene	Short Read Archive, SRX831099	Despite being enriched solely on toluene for >10 years, several anaerobic hydrocarbon activation genes were found to be present (including *bss*A and *ass*A)	Fowler et al. (2012) and Tan et al. (2014, 2015a)
n-C_{16} degrading	Methanogenesis	Hexadecane	DDBJ/EMBL/ Genbank, LNQE00000000	Used metagenomic binning, metatranscriptomic analysis, and metabolic modeling to deduce interspecies interactions between taxa driving syntrophy in methanogenic communities	Embree et al. (2014, 2015), Tan et al. (2014)
n-C_{28} degrading	Methanogenesis	Octacosane (C_{28}), $C_{10–18}$, C_{40}, C_{50}	Short Read Archive, PRJNA293354 Draft genomes also available	Revealed *Smithella* as key degrader by fumarate addition and genes associated with syntrophic interactions with methanogens	Wawrik et al. (2016)

methanogenic cultures, wherein syntrophic partnerships are needed to convert hydrocarbons to methane, have also been studied in order to understand the bio-conversions of specific hydrocarbons under these conditions (e.g., Gieg et al. 2014; Jiménez et al. 2016). The pathways for anaerobic hydrocarbon metabolism and the corresponding genes and enzymes were largely identified prior to the widespread use of "omics" approaches; typically, cultures were incubated with the substrate of interest, and processes were tracked using analytical chemistry (e.g., to identify pathway intermediates) and classical molecular biology/genetic approaches (e.g., Sanger sequencing, closing, gene selection, etc.). From these studies, hydrocarbon activation mechanisms including fumarate addition, carboxylation, and hydroxylation have been identified (along with a couple of studies suggesting activation by methylation of unsubstituted aromatics, Ulrich et al. 2005; Safinowski and Meckenstock 2006). Of these, the addition of hydrocarbons to the double bond of fumarate (simply deemed "fumarate addition") is the most widespread and understood mechanism catalyzed by a glycyl radical enzyme and occurs for the activation of alkylated aromatics and linear and cyclic alkanes (Fig. 1), with a couple of notable exceptions (ethylbenzene degradation under nitrate-reducing conditions occurs via hydroxylation, Rabus et al. 2005; *n*-alkane degradation may also occur via hydroxylation and/or carboxylation; So et al. 2003; Callaghan et al. 2009; Callaghan 2013b). Carboxylation and hydroxylation are the primary mechanisms proposed for the activation of non-substituted aromatics such as benzene, naphthalene, and phenanthrene (Meckenstock et al. 2016). Most evidence to date supports carboxylation as the most likely mechanism for the activation of non-substituted aromatics (Zhang and Young 1997; Holmes et al. 2011; Abu Laban et al. 2010; Mouttaki et al. 2012; Luo et al. 2014), with the exception of an iron-reducing species (*Geobacter metallireducens*) that appears to activate benzene by hydroxylation, yielding phenol (Zhang et al. 2013). Figure 1 summarizes the most widely reported mechanisms for the activation of different classes of hydrocarbons under anoxic conditions. Along with identifying the metabolites (intermediates) in hydrocarbon degradation pathways, studies have also revealed the key genes and characterized several of the enzymes associated with these pathways. For example, benzylsuccinate synthase (BSS) was identified as the enzyme catalyzing toluene activation over 20 years ago, encoded by the *bss*A gene; dozens of publications now describe this novel and uniquely anaerobic biochemical reaction (as reviewed in Foght 2008; Widdel et al. 2010; Heider and Schühle 2013). For alkane activation by fumarate addition, alkylsuccinate synthase (ASS, also called (1-methyl)alkylsuccinate synthase, MAS) is the key activation enzyme, encoded by the *ass*A/*mas*D gene (different designations for the same gene identified concurrently by two different research groups; Callaghan et al. 2008; Grundmann et al. 2008). Similarly, the *nms*A gene encodes the naphthyl-2-methylsuccinate synthase enzyme subunit that activates 2-methylnaphthalene by addition to fumarate (Meckenstock et al. 2016). Most recently, a thermophilic anaerobic consortium (enriched from the Guaymas Basin hydrothermal vent samples) dominated by an archaeal phylotype closely related to *Methanosarcinales* was proposed to activate *n*-butane (and *n*-propane) via an alkyl-coenzyme M mechanism in a manner analogous to that demonstrated for anaerobic

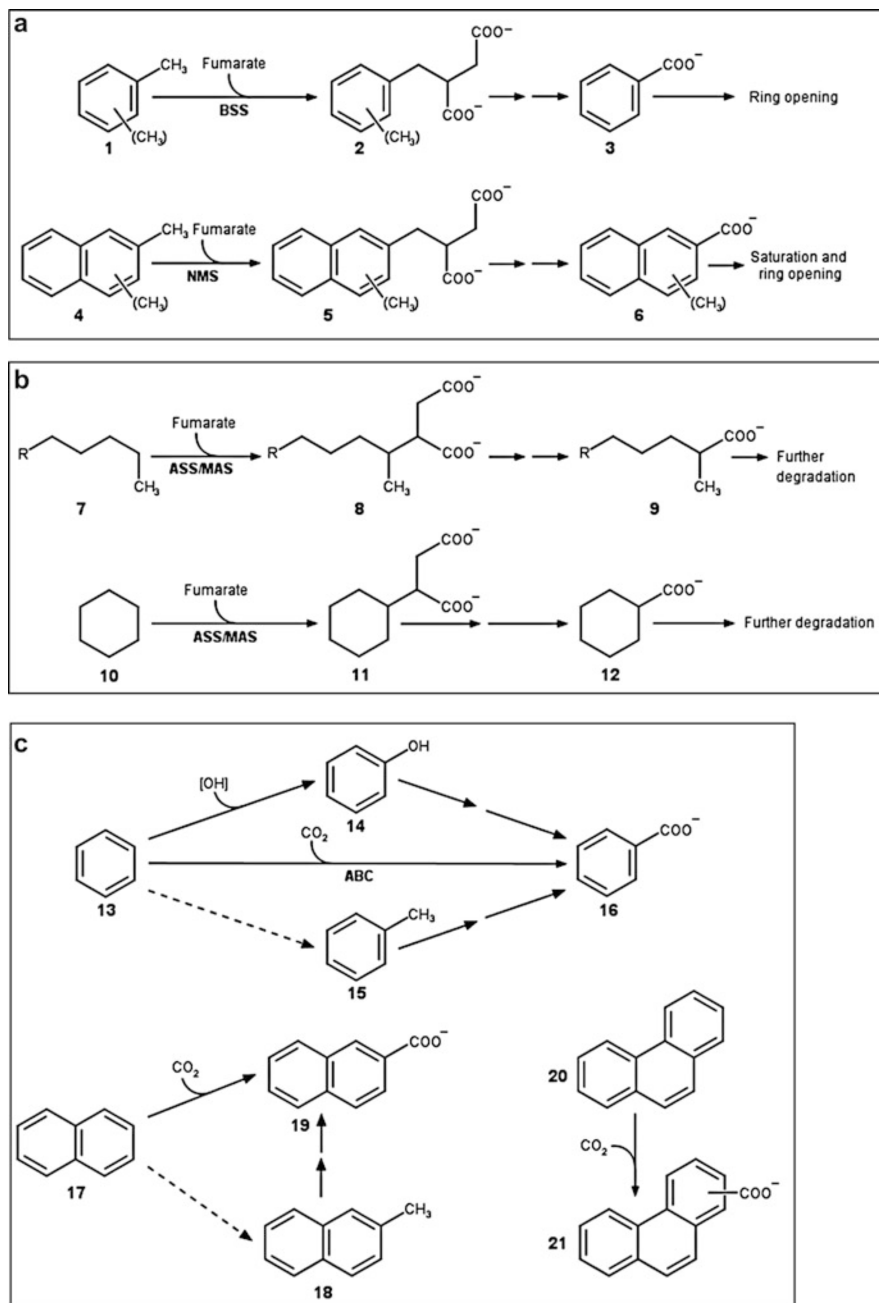

Fig. 1 Overview of the major hydrocarbon activation mechanisms under anaerobic conditions. Metabolites are shown as free acids. Multiple *arrows* represent more than one enzymatic step; *dashed arrows* represent an unknown reaction. *Structure nomenclature*: *1* toluene (or xylene);

methane oxidation operating via reverse methanogenesis (Laso-Pérez et al. 2016). Although further work is needed to understand and confirm this mechanism, this finding has revealed a potential novel mechanism of hydrocarbon activation and expands upon the substrate range of methanogen-like organisms. The key hydrocarbon activation genes and metabolites identified through these laboratory-based physiological studies have important applications in assessing in situ hydrocarbon biodegradation processes in various environments (see sections below).

3 Insights on Anaerobic Hydrocarbon Biodegradation Using Metagenomic Approaches

Even though several anaerobic hydrocarbon-utilizing isolates have been described within the last 30 years, obtaining pure isolates is usually difficult, tedious, and time-consuming. Further, it is unclear as to whether laboratory isolates truly represent the diversity of hydrocarbon degraders that may be found in various natural environments. Genomics has emerged as a field of science that examines the genetic material, or genomes, of organisms in a culture-independent manner and thus can greatly expand our knowledge of microbial processes occurring in diverse environments. The term "metagenome" refers to the genetic (genomic) compositions of *all* organisms in a given sample (e.g., of the total microbial community) without the need for cultivation; this field of science is referred to as metagenomics. Metagenomic approaches are typically conducted in either a targeted or nontargeted fashion. Targeted metagenomics is more commonly known as amplicon sequencing; here, a gene of interest is amplified by PCR using primer sets specific to that gene in order to capture multiple sequences of that gene in a given sample. For example, targeting a portion of the small subunit ribosomal RNA gene (the 16S rRNA gene for prokaryotes) is the most common way to obtain taxonomic information, or microbial community composition, of a given sample. Other genes diagnostic of particular microbial groups (e.g., *dsr*AB for sulfate reducers or *mcr*A for methanogens, Johnson et al. 2015; Gründger et al. 2015) or encoding functional genes (e.g., for anaerobic hydrocarbon biodegradation, *ass*A, *bss*A, *nms*A) are often utilized as well (von Netzer et al. 2013, 2016). In nontargeted metagenomics, or shotgun metagenomics, all genetic material is sequenced in order to capture the complete suite of genes within a given sample in order to gain an understanding of the overall

Fig. 1 (continued) 2 (methyl)benzylsuccinate; *3* benzoate; *4* 2-methylnaphthalane/dimethylnaphthalene; *5* naphthylmethylsuccinate; *6* (methyl)-2-naphthoate; *7* *n*-alkane; *8* (2-methyl)alkylsuccinate; *9* 2-alkylmalonate; *10* cyclohexane; *11* cyclohexyl succinate; *12* cyclohexanecarboxylate; *13* benzene; *14* phenol; *15* toluene; *16* benzoate; *17* naphthalene; *18* 2-methylnaphthalene; *19* 2-naphthoate; *20* phenanthrene; and *21* phenanthrene carboxylate. *Enzyme nomenclature*: **BSS** catalytic subunit of benzylsuccinate synthase, **NMS** catalytic subunit of naphthyl-2-methylsuccinate synthase; **ASS/MAS** catalytic subunit of alkylsuccinate synthase, **ABC** putative gene encoding anaerobic benzene carboxylase

functional potential of the microbial ecosystem. To conduct metagenomic analysis, DNA is extracted from the requisite culture(s) or environmental sample(s), and the sample is processed for sequencing using a parallel sequencing approach (e.g., Illumina MiSeq or HiSeq is currently the most widely employed sequencing approach although Ion Torrent and Pac Bio are also used; most analyses, e.g., prior to ~2014, used the pioneering Roche 454 pyrosequencing system which is no longer supported). Once sequences are obtained, they are subjected to quality control analyses and processed using a variety of software tools that not only allow for the identification of genes, but can also assign, or bin, genes to specific taxa (Segata et al. 2013; Pérez-Wohlfeil et al. 2016; Anantharaman et al. 2016). Reconstructing individual genomes within metagenomic datasets can provide enormously valuable information regarding the potential functionalities and interactions between community members within an ecosystem and can also lead to the discovery of new, previously unclassified taxa (Anantharaman et al. 2016).

4 Genomes of Anaerobic Hydrocarbon-Degrading Isolates and Enrichment Cultures as Benchmarks for Metagenomes

Although metagenomic approaches do not require cultivation, analyzing and interpreting metagenomic datasets benefit greatly by comparing sequences to reference genes and genomes for easier gene annotation and identification. Thus, publishing the genome sequences of anaerobic hydrocarbon-degrading isolates not only provide insights into the overall functional capabilities of these types of microorganisms but also serve as invaluable “blueprints” for interpreting metagenomic datasets from hydrocarbon-impacted environments. The genomes of several hydrocarbon-degrading nitrate reducers, iron reducers, and sulfate reducers have now been described (see Table 1 for examples) providing important information about the physiological characteristics of anaerobic hydrocarbon degraders. For example, genomic analysis of *Desulfatibacillum alkenivorans* AK-01 identified 2 loci harboring the alkylsuccinate synthase gene and putative genes for an alkyl-CoA ligase, mutase, and carboxyltransferase (presumably needed for further metabolic transformations of alkylsuccinates; Callaghan et al. 2012). Further, the AK-01 genome revealed several genes believed to be important for its growth on alkanes under syntrophic conditions an activity that was experimentally confirmed (Callaghan et al. 2012). Target gene amplification and genomic sequencing of the nitrate-reducing strain HdN1, in addition to hydrocarbon metabolite analysis, failed to detect evidence for an *ass*A/*mas*D-like gene which suggested an alternate mechanism of *n*-alkane activation (Zedelius et al. 2011). Physiological tests revealed that alkane metabolism may instead be coupled to the denitrification intermediates NO_2^- and NO wherein O_2 is generated to serve as a co-reactant for hydroxylation (Zedelius et al. 2011; Callaghan 2013b) similar to that shown during methane oxidation under nitrate-reducing conditions (Ettwig et al. 2010). *Desulfococcus oleovorans* Hxd3 is another sulfate-reducing alkane degrader that does not utilize fumarate addition for *n*-alkane activation (So et al. 2003). Instead, its genome sequence revealed the

presence of an ethylbenzene dehydrogenase-like complex that may allow for alkane hydroxylation (Callaghan 2013b). Thus, sequencing the genomes of isolates greatly helps to elucidate the biochemical reactions associated with anaerobic hydrocarbon metabolism (along with other physiological processes) and provides benchmarking information to help reconstruct individual genomes from metagenomic datasets.

The biodegradation of hydrocarbons under methanogenic communities is a syntrophic process wherein different microorganisms (e.g., fermentative bacteria, syntrophs, and methanogens) collectively carry out this reaction in a thermodynamically interdependent manner (Sieber et al. 2012; Gieg et al. 2014). Therefore, a shotgun metagenomic approach is essential to determine the genetic composition of these culture systems. To date, the metagenomes of six methanogenic hydrocarbon-degrading cultures have been sequenced and described in publications; one methanogenic benzene-degrading culture (Luo et al. 2016), two cultures enriched from oil sands tailings ponds that degrade naphtha (NAPDC; naphtha is refinery product consisting of monoaromatics and C_6-C_{10} alkanes and is present in many oil sands tailings ponds) or short-chain alkanes (SCADC; C_6-C_{10} *n*-alkanes with small proportions of 2-methylpentane and methylcyclopentane) (Tan et al. 2013, 2015a), one culture enriched from a gas condensate-contaminated aquifer on toluene (TOLDC) (Fowler et al. 2012; Tan et al. 2015a), one culture enriched on *n*-hexadecane (Zengler et al. 1999; Embree et al. 2014), and one culture enriched from San Diego Bay sediments on *n*-octacosane (Wawrik et al. 2016). A comparative metagenomic analysis of SCADC, NAPDC, and TOLDC revealed that all cultures harbored fumarate addition genes to degrade a variety of hydrocarbon types along with genes for H_2- and acetate-utilizing methanogenesis (Tan et al. 2015a). Further, a comparison of these metagenomes with 41 other environmental metagenomic datasets revealed an enrichment of genes associated with anaerobic hydrocarbon biodegradation, syntrophy, and methanogenesis, suggesting these are hallmark features of this process (Tan et al. 2015a). In metagenomic sequencing of one methanogenic benzene-degrading culture, combined with other molecular methods used to analyze 15 other cultures, Luo et al. (2016) unequivocally identified a *Deltaproteobacterium* designated ORM2 as the key benzene degrader under methanogenic conditions. Studies with the *n*-hexadecane-degrading culture helped to reveal the key taxa involved in hydrocarbon degradation (*Smithella*, *Desulfovibrio*, *Methanoculleus*, *Methanocalculus*, and *Methanosaeta*) and key hydrocarbon biodegradation genes (associated with *Smithella*, Tan et al. 2014) as well as potential interactions among members of the syntrophic community (Embree et al. 2014, 2015). Similar findings were discovered following the metagenomic sequencing of an *n*-octacosane-degrading culture; a *Smithella* phylotype harbored the *ass*A genes needed for *n*-alkane activation along with the genes required for syntrophic interactions and energy conservation (Wawrik et al. 2016). Although work with isolates has shown that nitrate or sulfate reducers can completely mineralize hydrocarbons (e.g., Table 1), there are also several examples wherein syntrophic consortia are required to utilize hydrocarbons under nitrate- or sulfate-reducing conditions (Gieg et al. 2014). For example, Luo et al. (2014) used a metatranscriptomic approach to reveal that benzene degradation by a

nitrate-reducing culture requires at least two partner organisms, one of which appears to contain the putative genes associated with syntrophic benzene activation.

5 Hydrocarbon-Impacted Environments: Targeted Metagenomics (Amplicon Sequencing)

By far, the most commonly used genomic approach in the field of environmental microbiology is taxonomic (phylogenetic) profiling of a given sample based on amplifying a portion of the 16S rRNA gene for prokaryotic identification (or the 18S rRNA gene for eukaryotes). Prior to ~10 years ago, assessing microbial diversity in a cultivation-independent manner was usually performed using clone libraries and Sanger sequencing or techniques such as denaturing gradient gel electrophoresis or T-RFLP (terminal-restriction fragment length polymorphism) analysis. While these approaches are sometimes still used (especially T-RFLP; von Netzer et al. 2013; Gründger et al. 2015; Luo et al. 2016), the majority of publications regarding anaerobic hydrocarbon-containing environments now describe the use of amplicon sequencing (primarily based on the 16S rRNA gene) to determine microbial diversity. Here, DNA is extracted from mixed samples and subject to a PCR reaction using a primer set that targets specific variable regions of the 16S rRNA gene (often the V3-V4 or V6–V8 regions; http://jgi.doe.gov/our-science/science-programs/metagenomics/; An et al. 2013a). Although there are limitations to this approach (such as primer bias, not all diversity captured, Singer et al. 2016), targeted metagenomic sequencing has become very affordable per base pair sequenced (www.genome.gov/sequencingcosts) and is now usually included as a standard approach for most environmental microbiology studies to identify key taxa within a given community and predict their putative functions. In general, the majority of proposed anaerobic hydrocarbon-degrading bacteria have been found to affiliate within the Proteobacteria or Firmicutes, but genera within these phyla can vary substantially (Head et al. 2014; Mouser et al. 2016). Other members of diverse phyla (e.g., *Chloroflexi*, *Spirochaetes*, *Bacteroidetes*) are usually associated with mixed hydrocarbon-impacted communities (Strąpoć et al. 2011; Kleinsteuber et al. 2012; Mouser et al. 2016). In methanogenic hydrocarbon-degrading environments, the archaeal taxa are predominantly methanogens that utilize hydrocarbons, acetate, and/or methylated/methoxy substrates (Strąpoć et al. 2011; Kleinsteuber et al. 2012; Head et al. 2014; Mayumi et al. 2016). In hot environments such as thermogenic oil reservoirs (>50 °C), bacterial members such as those affiliating with *Thermotogales*, *Synergistales*, *Deferribacterales*, or *Thermoanaerobacterales* and thermophilic methanogens like *Methanothermobacter* are believed to be involved in anaerobic hydrocarbon degradation (Orphan et al. 2000; Gieg et al. 2010), but definitive evidence is required. However, it has been experimentally verified that the thermophilic archaeon *Archaeoglobus fulgidus* can metabolize long-chain alkanes and alkenes under sulfate-reducing conditions (Khelifi et al. 2010, 2014).

Obtaining 16S rRNA gene-based community profiles across a variety of similar environments can help determine whether similar taxa can be considered

characteristic of that habitat; e.g., does a core microbiome exist? A few studies have addressed this question using phylogenetic profiling data collected from similar environments. For example, Wilson et al. (2016) conducted a comparative analysis of 95 different anoxic samples collected from six different oil sands tailings ponds (managed by three different operators in different ways, e.g., by treatment with different chemical additives) to determine whether a core microbiome was associated with these highly engineered anaerobic hydrocarbon-degrading environments. Each individual tailings pond contained its own core biome that presumably reflected the selective pressures placed on the extant communities due to different pond management strategies (Wilson et al. 2016). An analysis of all 95 tailings pond samples revealed that the core microbiome consisted of only two to five OTUs that included *Comamonadaceae*, *Hydrogenophilaceae*, and/or *Anaerolineaceae* as the bacterial members and *Methanosaeta* and *Methanoregula* as the archaeal members. It was postulated that these limited taxa play key roles in the various anaerobic processes and/or harbor functional abilities that are common across all tailings ponds such as hydrocarbon degradation and methanogenesis (Wilson et al. 2016). To examine whether shale-associated fluids (typically saline in nature) harbor similar or distinctive taxa, Mouser et al. (2016) conducted a nonparametric multidimensional scaling (NMDS) analysis of 16S rRNA gene sequences retrieved from the limited datasets available for this environment, including data collected before and following a hydraulic fracturing operation (a method used to recover gas or fluids from shale formations). They found that microbial communities in the source waters used for fracturing were very different and revealed few halotolerant organisms. However, there was a large shift in the microbial community profiles in the flowback waters in as little as 1–14 days post fracturing (Mouser et al. 2016) wherein the dominant taxa were primarily known halotolerant microorganisms such as *Marinobacter*, *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Arcobacter*, and *Marinilabilia*. After 2–4 weeks post fracturing, the diversity was found to decrease substantially, with *Halanaerobium* (a firmicute) becoming heavily enriched – this trend was seen across all fracturing operations analyzed. Other taxa such as *Halomonas* and *Marinobacter* were found to be more broadly distributed throughout the course of fluid flowback and were hypothesized to be active degraders of hydrocarbons or related substrates associated with shales (Mouser et al. 2016; Daly et al. 2016).

Network or co-occurrence analysis is another approach often used to determine potential interactions between microbial community members and can be based on 16S rRNA gene sequences (Barberán et al. 2012; Williams et al. 2014) or metagenomic datasets (Li et al. 2016). Common correlation coefficients used to identify positive or negative interactions between pairs of microorganisms within each dataset include Pearson's r (a measure of the linear dependence) and Spearman's ρ (a nonparametric measure of rank) (Barberán et al. 2012). While it not has yet met widespread application to study hydrocarbon biodegradation (e.g., An et al. 2013a; Fowler et al. 2016), network analysis may offer new insight to help understand community functions, especially with the increasing number of metagenomes available for sequencing comparison. For example, in

an assessment of 160 phylogenetic datasets generated from oil reservoirs, coal seams, oil sands, and oil sands tailings ponds (~14,000 OTUs), An et al. (2013a) conducted a 16S rRNA co-occurrence analysis on the order level and found two major positive co-occurrence networks that metabolize hydrocarbons in a mutually exclusive manner: network A that consisted almost entirely of anaerobic taxa and network B that consisted of facultative anaerobes and aerobes. Accompanying metagenomic surveys also detected a higher frequency of aerobic catabolic genes in samples harboring greater proportions of network B taxa, calling into reconsideration the notion that hydrocarbon resource environments are exclusively anoxic (An et al. 2013a). It should be noted that metagenomic-based network analysis requires additional computational considerations due to the high complexity and (sometimes) incomplete nature of the datasets (Li et al. 2016).

In lieu of determining microbial diversity based on amplification of the 16S rRNA gene, several researchers have used a targeted metagenomics approach based on the key hydrocarbon activation genes for benzylsuccinate synthase (*bss*A) and alkylsuccinate synthase (*ass*A/*mas*D) to identify the anaerobic hydrocarbon-degrading potential in hydrocarbon-containing environments. Beller and colleagues initially designed a primer set based on a Betaproteobacterial (nitrate reducer) *bss*A sequence in order to interrogate a hydrocarbon-contaminated site for toluene biodegradation potential (Beller et al. 2002). Subsequently, Winderl et al. (2007) expanded on this work by designing primer sets that more broadly included diverse *bss*A genes associated with iron and sulfate reducers and successfully used these to identify hydrocarbon degraders in a tar oil-contaminated aquifer system (Winderl et al. 2008). Similarly, following the discovery of the *ass*A/*mas*D genes responsible for alkane activation, Callaghan et al. (2010) designed several primer sets based on the AK-01 *ass*A and *bss*A gene sequences and were used to successfully detect both genes in variety of enrichment cultures, river sediments, and contaminated aquifer samples. Johnson et al. (2015) also found *ass*A and *bss*A genes in Chesapeake Bay estuarine sediments revealing the potential for anaerobic alkane and aromatic hydrocarbon biodegradation in these environments. The *ass*A genes have been detected in a variety of produced waters from crude oil reservoirs where putative hydrocarbon-degrading anaerobic taxa and/or putative metabolites were also detected (Li et al. 2012; Zhou et al. 2012; Bian et al. 2015) and in coalbed methane site fluids (Wawrik et al. 2012). von Netzer et al. (2013) further refined the *bss*A primer sets to be applicable to be even more diverse sequences associated with a broader range of environments including hydrocarbon-contaminated aquifers, cold seeps, and hydrothermal vent systems. Gittel et al. (2015) and Stagars et al. (2016) independently developed new primer sets for the *ass*A/*mas*D genes based on a variety of known alkane-degrading isolates and available sequences and used these to interrogate numerous different hydrocarbon seep environments from across the globe, revealing that these environments harbor an unprecedented diversity of anaerobic alkane-degrading ability.

6 Hydrocarbon-Impacted Environments: Shotgun Metagenomics

While the majority of studies to date have used targeted metagenomics to assess microbial community composition or specific functional properties, there are now some reports on the use of shotgun metagenomics to determine the overall potential functionality of communities within anoxic hydrocarbon-containing environments. Table 3 provides examples of where a shotgun metagenomic approach was used to assess the genetic composition of samples from a variety of such environments. The importance of anaerobic hydrocarbon biodegradation in these environments and findings resulting from their metagenomic sequencing are briefly described below.

6.1 Fossil Energy Reservoirs

It is well known that deep subsurface crude oil-containing reservoirs harbor thriving subsurface microbial communities wherein a variety of saturate and aromatic hydrocarbons can serve as the key carbon and energy substrates (Head et al. 2014). In fact, most of the world's crude oil in reservoirs have been biodegraded to some extent over geological time; this is believed to have primarily occurred under syntrophic, methanogenic conditions because isotopic signatures of methane in gas caps overlying reservoirs (where biodegraded oil is found) is primarily biogenic in nature (Head et al. 2014). More recently, microorganisms associated with shale or coal reservoirs have been identified (Strąpoć et al. 2011; An et al. 2013a; Lawson et al. 2015; Mouser et al. 2016). The carbon substrates within these fossil energy reservoirs are less defined but also contain complex, organic carbon-rich substrates; organic solvent extracts of coal or shale have revealed a variety of components such as alkanes, PAHs, heterocyclic compounds, aromatic acids and alcohols, and alkanoic acids that can feasibly support extant microbial communities (Orem et al. 2010; Strąpoć et al. 2011; Lawson et al. 2015). In addition, Mayumi et al. (2016) recently discovered that methoxy compounds found in coal can be used directly by some methanogens to produce methane. Understanding the microbial activities within petroliferous reservoirs not only provides insight into life processes occurring within the deep subsurface but can have important applications for microbially enhanced energy recovery wherein entrained hydrocarbon or related substrates can be converted to CH_4 as a clean-burning energy source (An et al. 2013a; Head et al. 2014; Lawson et al. 2015).

While the microbial community compositions (16S rRNA gene analysis) for several crude oil reservoirs, coal seams, and shale gas systems have been determined (e.g., reviews by Head et al. 2014; Mouser et al. 2016), several reports now describe a metagenomic analysis of samples from these hydrocarbon-containing environments (Table 1). Kotlar et al. (2011) and Lewin et al. (2014) were among the first to perform metagenomic sequencing of two hot (80–85 °C), deep (~ 2.5 km below the

Table 3 Examples of anoxic hydrocarbon-containing environmental samples studied using a shotgun metagenomic approach and a summary of key findings related to anaerobic hydrocarbon biodegradation

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Fossil energy reservoirs			
Crude oil reservoir produced water from 2.5 km below sea floor (85 °C and 250 bar); not exposed to seawater injection (well I) Norwegian continental shelf Microbial diversity of hot oil reservoirs	Fluids retrieved from deep hot sediments (well I) were dominated by sulfate/sulfur-reducing bacteria, with lesser abundance of methanogenic (primarily *Methanococcus*) taxa	Not reported	Kotlar et al. (2011)
Crude oil reservoir produced water from 2.5 km below sea floor (85 °C and 250 bar); not exposed to seawater injection (well II) Norwegian continental shelf Microbial diversity of hot oil reservoirs, comparisons between well I (Kotlar et al. 2011) and well II	Fluids retrieved from deep hot sediments from well II, physically separated from well I, were dominated by archaea (*Thermococcus* and *Pyrococcus*, both noted for S metabolism) with lesser abundance of bacteria (primarily Deltaproteobacteria); both wells I and II showed similar taxa overall but in different abundances and similar gene compositions	Not reported	Lewin et al. (2014)
Crude oil reservoir, produced water sample, 30 °C, water flooded Medicine Hat, Alberta, Canada Functional microbial potential within hydrocarbon resource environments	Dominant taxa affiliated with *Clostridiales*, *Syntrophobacterales*, *Methanomicrobiales*, and *Methanosarcinales*, all known to be associated with methanogenic oil biodegradation; gene analysis revealed an enrichment of anaerobic hydrocarbon-degrading genes compared to aerobic hydrocarbon-degrading genes	Short Read Archive, SRX210984	An et al. (2013a)
Crude oil reservoir, produced water samples Alaska North Slope oilfields, Alaska, USA Physiological potential of microbial communities in petroleum reservoirs	Reconstructed genomes for several anaerobic microbes including candidate phyla; identified multiple *ass*A, *bss*A, and benzoate reductase genes indicating potential for anaerobic hydrocarbon biodegradation; nitrogen-fixing genes associated with methanogens	Raw reads deposited at Genbank, SRP057267	Hu et al. (2016)
Crude oil reservoir, crude oil samples Qinghai and Daqing oilfields, China Microbial community composition and functioning in oil reservoirs; comparisons with metagenomes from 2 oil reservoirs and other environments (948 total)	Bacteria dominated by Proteobacteria (*Pseudomonas, Acinetobacter, Marinobacter*) and Firmicutes while Archaea dominated by methanogens; genes for both aerobic and anaerobic hydrocarbon biodegradation found in oilfield samples	Short Read Archive, BioProject PRJNA251580	Nie et al. (2016)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Deep shale formations disturbed by hydraulic fracturing, sampled over a period of 328 days Appalachian basin shales – Marcellus (Pennsylvania) and Utica (Ohio), USA Subsurface microbiology and biogeochemistry in deep engineered systems; changes following hydraulic fracturing	Enrichment of halotolerant communities following hydraulic fracturing; reconstruction of 31 genomes demonstrated potential for methanogenesis from methylated substrates, for fermentation of chemical additives used for hydraulic fracturing (that can include hydrocarbons), and for sulfur cycling; only aerobic hydrocarbon-degrading genes were detected	Short Read Archive BioProject PRJNA308326	Daly et al. (2016)
Shale samples from Marcellus shale, including source water, and flowback water after 1 and 9 days Pennsylvania, USA Assess microbial community response following hydraulic fracturing	Flowback water samples were more enriched in anaerobic taxa than in original source water, along with an increase in gene abundances for numerous metabolic processes; hydrocarbon-degrading gene analysis not examined	MG-RAST, 4525703.3, 4525704.3, 4525705.3	Mohan et al. (2014)
Coal seams, 4 produced water and 3 cuttings samples from different depths Alberta, Canada Functional microbial potential within hydrocarbon resource environments	An analysis for aerobic and anaerobic hydrocarbon-degrading genes revealed a predominance of aerobic genes, suggesting that aerobic processes are more common in deep coal seams than expected	Short Read Archive, SRX210870, SRX210867, SRX210868, SRX210875, SRX210869, SRX211003, SRX211004	An et al. (2013a)
Deep bituminous coal seam sample from the Mannville Group Alberta, Canada Understanding microbial communities and functions in coal seams	Metagenomic sequencing allowed for the reconstruction of a major taxon associated with this coal sample (*Celeribacter*) which harbored genes for aerobic degradation of aromatics, glycogen storage, and fermentation pathways suggesting aerobic/anaerobic scheme for coal substrate metabolism leading to methanogenic substrates	Short Read Archive, SRR573886	Lawson et al. (2015)
Deep-sea cold seeps and hydrothermal vents			
Crude oil seep, core samples from 0–4 cm and 10–15 cm depths Tonya seep, Coal Oil Point in Santa Barbara Channel, California, USA Potential for aerobic and anaerobic oxidation of methane and other hydrocarbons	Genes for aerobic methane oxidation found at 0–4 cm, while genes for (reversed) methanogenesis found at 1–15 cm depth along with anaerobic methane oxidizing taxa (ANME-1, ANME-2, ANME-3) and sulfate-reducing syntrophic partners	Genbank, SRP005641	Håvelsrud et al. (2011)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Crude oil cold seep sample Coal Oil Point in Santa Barbara Channel, California, USA Microbial diversity and processes around hydrocarbon seeps	Dominance of anaerobic methane oxidizers (ANME-1, ANME-2, ANME-3) and requisite genes for (reverse) methanogenesis were found	IMG/M project ID 45292	Hawley et al. (2014a, b)
Pockmarked sediments (potential hydrocarbon-releasing cold seeps) overlying a known petroleum reservoir (Troll field) and “control” sediments with no hydrocarbon influence North Sea, Troll (hydrocarbon) and Oslofjord (no hydrocarbon) areas, Norway Microbial community understanding in relation to geochemical parameters in cold seep systems; potential for anaerobic methane/ hydrocarbon degradation	Some known hydrocarbon biodegradation genes were overrepresented in the pockmarked samples compared to the “control” sediments. Several were for aerobic processes but also included genes for benzoyl-CoA reductase which catalyzes aromatic ring reduction. In contrast, genes for AOM were not enriched, suggesting little methane is occurring from the pockmarked sediments. Overabundance of autotrophic nitrifiers was evident in pockmarked vs. control sediments	Genbank, SRP009243	Håvelsrud et al. (2012)
Oil-immersed, hydrocarbon-releasing deep-sea hydrothermal vent Guaymas Basin, California, USA Ecological functions and activities of microbial communities in hydrothermal vents	Several thermophilic sulfate reducers were identified along with *bss*A and *ass*A genes demonstrating the potential for anaerobic hydrocarbon biodegradation; enhanced expression of these genes was also found in a subsequent metatranscriptomic analysis	MG-RAST, 4510962.3	He et al. (2013, 2015)
Hydrothermal vent microbial mats (red mat, 18 °C; white mat 70 °C) Hellenic Volcanic Arc, Greece Microbial diversity and processes around hydrothermal vents	Detected a variety of genes associated with the anaerobic biodegradation of aromatic hydrocarbons including for fumarate addition (*bss*A), hydroxylation (ethylbenzene dehydrogenase), and benzoyl-CoA reductases; these genes were more abundant in the cooler red mat	IMG/M, 3300002231 & 3300002242	Oulas et al. (2016)
Hydrocarbon-impacted marine environments			
Marine sediments near Deepwater Horizon blowout, and one distant sample unimpacted by hydrocarbons Gulf of Mexico, USA Characterize microbial communities and assess	Metagenomic analysis of cored anoxic seabed sediments (2) collected from near the blowout site, as compared to a distant unaffected sample (1) revealed an enriched in sulfate-reducing taxa, and an enrichment/detection of anaerobic hydrocarbon-degrading genes (e.g., *bss*A) in a sample closest to the blowout site; PCR amplification	*ass*A and *bss*A sequences deposited in GenBank, JX135105-JX135128	Kimes et al. (2014)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
potential for hydrocarbon metabolism in anoxic marine sediments associated with hydrocarbon-contamination	revealed the presence of *ass*A and *bss*A only in the hydrocarbon-contaminated sediments		
Hydrocarbon-impacted terrestrial environments			
Oil sands tailings ponds, anoxic zone, 3 samples Northeastern Alberta, Canada Functional microbial potential within hydrocarbon resource environments	Taxa affiliated primarily with known syntrophs and methanogens; genes associated with both anaerobic and aerobic aromatic hydrocarbon biodegradation were detected	Short Read Archive, SRX210980, SRX210872, SRX210871	An et al. (2013a)
Oil sands tailings, anoxic zone Northeastern Alberta, Canada Assessment of anaerobic hydrocarbon biodegradation potential	Metagenomic analysis of unenriched anoxic tailings samples revealed genes associated with acetate- and H_2-using methanogenesis, fumarate addition to aromatic hydrocarbons, (*bss*A gene), and genes for the ATP-independent ring reduction of benzoate	MG-RAST, 4492774.3; Short Read Archive, SRX210871	An et al. (2013b)
Hydrocarbon and chlorinated solvent-contaminated aquifer sediments, 15 m depth Kwazulu-Natal, South Africa Determine microbial communities and functions in contaminated aquifers	Dominance of *Bacteroides* and Betaproteobacteria; protein recruitment plots showed close similarities to known hydrocarbon degraders strain EbN1 and *Dechloromonas aromatica* RCB; some anaerobic benzoate degradation genes detected	Not reported	Abbai and Pillay (2013)

seafloor) crude oil reservoir samples retrieved from the Norwegian North Sea that were ~3 km apart and physically separated, and analysis focused mainly on the recovered taxa. They found similar gene profiles and anaerobic taxa across both wells, albeit the microbial members were of different relative abundances (Lewin et al. 2014). Nie et al. (2016) performed metagenomic sequencing on crude oils obtained from two distinct Chinese oilfields and compared these with the Norwegian metagenomic datasets (Kotlar et al. 2011; Lewin et al. 2014). The Chinese field metagenomes were found to be abundant in lipid metabolism genes, along with genes for known aerobic (e.g., *alk*B) and anaerobic (*ass*A/*bss*A) hydrocarbon metabolism in accordance with the identification of both aerobic and anaerobic taxa. In contrast, the Norwegian samples that were characterized primarily by anaerobic taxa contained only the anaerobic hydrocarbon-degrading genes (Nie et al. 2016). Hu et al. (2016) conducted shotgun metagenomic sequencing on several produced water samples from Alaska North Slope oilfields characterized by varying temperatures (24–27 °C or 80–83 °C) and whether or not they had a history of

souring. Immense diversity was discovered but decreased as fluid temperature increased. Dozens of nearly complete genomes were reconstructed from this metagenomics dataset. In the hottest sample (80–83 °C), *Thermoanaerobacter*, *Desulfonautics*, *Archaeoglobus*, and *Thermodesulfobacterium* were the primary organisms detected, for which near-complete genomes were reconstructed and were not found to contain any genes associated with anaerobic hydrocarbon metabolism. In contrast, reconstructed genomes from the cooler reservoir samples revealed the presence of several taxa known to be associated with anaerobic hydrocarbon biodegradation (*Clostridia*, *Clostridiales*, *Desulfotomaculum*, *Syntrophobacterales*) along with a relatively high abundance of candidate phyla such as OP9 (Atribacteria) and OD1 (Parcubacteria). Notably, many gene sequences for benzylsuccinate synthase, alkylsuccinate synthase, and benzoyl-CoA reductase were associated with these taxa, indicating the potential for in situ anaerobic hydrocarbon biodegradation in these anoxic crude oil reservoirs (Hu et al. 2016).

Some shale and coal reservoir samples have also been subject to metagenomic sequencing (Table 3). Although some coal-bearing environments are dominated by anaerobic signatures (taxa and genes) (Wawrik et al. 2012; Gründger et al. 2015), some of these have exhibited aerobic signatures in addition to anaerobic signatures, going against the more commonly accepted belief that these are exclusively anoxic environments. In a 16S rRNA gene survey and metagenomic analysis of hydrocarbon resource environments (that included coal cuttings, cores, and produced waters, crude oil reservoir produced water, and samples from oil sands and oil sands tailings ponds), taxa with known aerobic respiration along with aerobic hydrocarbon-degrading genes (e.g., for mono- and dioxygenases) were detected to some extent in all samples. Unexpectedly, the coal samples had the highest gene counts for aerobic hydrocarbon biodegradation, including genes for aerobic methane oxidation (An et al. 2013a). Based on these findings, it was proposed that oxygen may intermittently be available to subsurface environments through meteoric waters or through the slow diffusion of oxygen from the coal itself. Metagenomic analyses of other coalbed methane samples have also found genes for the aerobic transformation of a variety of monoaromatic compounds (that are known coal degradation by-products) along with genes for fermentative and methanogenic pathways (Lawson et al. 2015). The reconstruction of a nearly complete genome of a *Celeribacter* sp. that had genes for the aerobic degradation of aromatics, glycogen storage, and fermentation pathways led the authors to propose that such bacteria can degrade coal components during oxygen ingress, store the carbon as glycogen, and then ferment this during periods of anoxia producing methanogenic substrates leading to subsequent methane production from coalbeds (Lawson et al. 2015). In fractured shale formations, it also appears that aerobic, but not anaerobic, hydrocarbon-degrading genes are present (Daly et al. 2016); thus some aerobic hydrocarbon metabolism may occur in these kinds of reservoirs. However, genes for fermentation of a variety of other substances (including chemicals used in fracturing operations) and for methanogenesis indicate that methane-producing consortia are also key in shale deposits (Daly et al. 2016).

6.2 Hydrocarbon Seeps and Hydrothermal Vents

In contrast to confined fossil energy reservoirs (that are bound by impermeable cap rocks allowing for the accumulation of economically recoverable amounts of crude oil and gas), hydrocarbons can also be steadily released through natural cold seeps or hydrothermal vent systems (Farwell et al. 2009; Boetius and Wenzhöfer 2013; Teske et al. 2014). These unconfined structures release either gaseous hydrocarbons (primarily methane, or C_1–C_4 alkanes) or a mix of gaseous and liquid alkanes ($>C_4$) that, along with other potential substrates (such as inorganic sulfur compounds), support the proliferation of both macro- and microbiological life-forms (Boetius and Wenzhöfer 2013, Teske et al. 2014). Notably, the process of anaerobic methane oxidation (AOM) has been well documented at both cold seeps and at hydrothermal vents (Orcutt et al. 2008; Knittel and Boetius 2009; Teske et al. 2014). The microorganisms involved in AOM are vital for keeping global methane emissions from oceans at bay by acting as natural methane biofilters (Knittel and Boetius 2009; Boetius and Wenzhöfer 2013); this process has been estimated to consume ~300 Tg methane/year within marine systems (Hawley et al. 2014a).

To date, metagenomic analyses have been performed on samples recovered from some natural hydrocarbon-releasing systems. Coal Oil Point in the Santa Barbara Channel (California, USA) has been a well-studied natural hydrocarbon seep area because it releases heavy oil that creates oil slicks on the ocean surface, along with significant amounts of methane (Hornafius et al. 1999; Farwell et al. 2009). In order to study the potential for AOM at this seep area, both Håvelsrud et al. (2011) and Hawley et al. (2014a, b) conducted a metagenomic analysis of seep sediments. Both studies revealed the presence of known AOM taxa (anaerobic methanotrophic archaea, or ANME, and sulfate reducers) along with genes associated with sulfate reduction and with “reverse” methanogenesis, a known mechanism of anaerobic methane oxidation (Hallam et al. 2004). These studies thus showed the potential for AOM at this oil-releasing cold seep.

Guaymas Basin, located in the Gulf of California (Baja California), harbors an active hydrothermal vent system (Teske et al. 2016). Here, buried sedimentary organic matter is hydrothermally transformed to a variety of hydrocarbons (and other components such as organic acids and non-hydrocarbon gases) that are continuously emitted from hydrothermal vent features. This hydrothermal vent area is of high microbiological interest as it is characterized by steep temperature gradients and diverse redox zones that can feasibly support diverse microbial processes including AOM (Teske et al. 2014; Kleindienst et al. 2014). The Guaymas Basin hydrothermal vent system has also been the source of several hydrocarbon-degrading sulfate-reducing isolates (Table 1) including strain BuS5 (propane and butane utilizer, activation by fumarate addition; Kniemeyer et al. 2007), *Desulfothermus naphthae* TD3 (alkane utilizer; Rueter et al. 1994), strain EbS7 (ethylbenzene utilizer, activation by fumarate addition; Kniemeyer et al. 2003), and the newly reported butane-degrading mixed culture that activates *n*-butane via CoM (Laso-Pérez et al. 2016). In an investigation of sulfate-reducing diversity and anaerobic hydrocarbon-utilizing functions at the Guaymas Basin and a cooler seep (Amon mud volcano), Kleindienst

et al. (2014) showed that seep-associated sulfate reducers capable of utilizing either butane or dodecane were phylogenetically affiliated with the *Desulfosarcina*/*Desulfococcus* clade (to which BuS5 belongs, along with sulfate-reducing partners associated with AOM). Indeed, several reports have now also identified diverse *ass*A genes associated with the anaerobic oxidation of higher alkanes both in the Guaymas Basin and in other hot and cold seeps (von Netzer et al. 2013; Stagars et al. 2016). Recent metagenomic sequencing of a Guaymas Basin vent sample revealed the presence of a number of thermophilic sulfate- or sulfur-utilizing taxa including members of the *Archaeoglobaceae*, *Thermococcaceae*, *Desulfobacteraceae*, and *Thermodesulfobacteraceae* along with several *ass*A and *bss*A gene sequences associated with known sulfate-reducing hydrocarbon-degrading strains (He et al. 2013, 2015). Collectively, these studies have clearly demonstrated that this hydrothermal vent system is an area of active anaerobic hydrocarbon biodegradation beyond AOM. Further, the detection of anaerobic hydrocarbon-degrading genes in a geographically distant hydrothermal vent system (Hellenic Volcanic Arc; Oulas et al. 2016) has underlined the importance of anaerobic hydrocarbon-biodegrading activities associated with hydrocarbon-releasing thermal vent areas.

6.3 Contaminated Marine Environments

It is now firmly established that the world's oceans host diverse microbial life (Hazen et al. 2016). Owing to the fact that approximately 600,000 tons of hydrocarbons leak into marine systems through natural seeps each year, microbial communities in many marine environments have the ability to utilize hydrocarbons and hence are "primed" to respond to oil influxes (Kimes et al. 2014; Hazen et al. 2016). This phenomenon was clearly observed following the Deepwater Horizon blowout in the Gulf of Mexico in 2010 that released approximately four million barrels of crude oil and ~250,000 metric tons of natural gas (mostly methane, with lesser amounts of C_2–C_4 alkanes) at a depth of ~1500 m below the sea surface. The Gulf of Mexico is a marine system that is rife with natural seeps (emitting an estimated 0.4–1 million barrels oil/year); thus the extant microbial community responded rapidly to the released oil (Orcutt et al. 2008; Hazen et al. 2016). To date, most research has focused on the response of aerobic microorganisms (for detailed reviews, see Kimes et al. 2014; Joye et al. 2014; King et al. 2015; Hazen et al. 2016). For example, multiple lines of evidence demonstrated that within a few weeks following the accident, microorganisms within the impacted deepwater column were found to have aerobically biodegraded several of the spilled light oil components (Hazen et al. 2010). Members of the *Oceanospirillales*, *Colwellia*, and *Cycloclasticus* were among the first taxa enriched in the deep oil plume, differing substantially from uncontaminated waters collected at the same depths, followed by a succession of other taxa (Kimes et al. 2014; Hazen et al. 2016). Further metagenomic and metatranscriptomic studies of samples collected following the spill revealed the key aerobic microbial players that contributed to the aerobic biodegradation of particular crude oil components in the water column, identifying, for example, that members of the *Oceanospirillales*

were key *n*-alkane and cyclic alkane degraders and *Colwellia* were key ethane and propane degraders (Redmond and Valentine 2012; Mason et al. 2012). Studies also showed that some of the released hydrocarbons were deposited onto the seafloor either as hydrocarbons or as marine snow (a mixture of oil, microorganisms, and extracellular polymeric substances; Joye et al. 2014; Kimes et al. 2014; Chanton et al. 2015). Metagenomic sequencing of 64 seabed surface sediment samples revealed that the oiliest sediments contained the greatest enrichment of *Colwellia* and an unclassified Gamma proteobacterium, along with genes for aerobic aliphatic hydrocarbon biodegradation, demonstrating a capacity for aerobic hydrocarbon degradation at the seafloor surface sediments (Mason et al. 2014). In a separate study, *Cycloclasticus* was found to be associated with the snow floc areas on the seabed, along with members of the *Desulfobacteraceae* and *Desulfobulbaceae* in some samples, suggesting the development of anaerobic "patches" in the sediments (Yang et al. 2016). To date, only one study has reported on potential anaerobic hydrocarbon transformations in seabed sediments associated with the Deepwater Horizon spill. Kimes et al. (2013) collected three core samples (1.5–3 cm below seabed surface) from near the blowout wellhead and conducted a metagenomic analysis to assess the response of the hydrocarbon-exposed sediment communities (compared to a hydrocarbon-free sample). Most notably, the metagenomic survey revealed an enrichment of several *bss*A and *ass*A genes associated with the sample collected closest to the wellhead along with the detection of primarily Deltaproteobacteria, showing that the anoxic sediments harbored the potential for anaerobic hydrocarbon degradation. Metabolite determinations also revealed the presence of benzylsuccinates in the sediments, strengthening the argument that anaerobic hydrocarbon degradation was active in the hydrocarbon-impacted sediments associated with the Deepwater Horizon blowout (Kimes et al. 2013).

6.4 Contaminated Terrestrial Environments

Although there have been numerous studies examining the anaerobic in situ bioremediation of hydrocarbons in contaminated groundwater aquifers (e.g., Beller 2000; Beller et al. 1995, 2002, 2008; Gieg et al. 1999; Griebler et al. 2004; Winderl et al. 2007, 2008; Parisi et al. 2009; Callaghan et al. 2010; Jobelius et al. 2011; Essaid et al. 2011; Morasch et al. 2011; Meckenstock et al. 2015), there have been surprisingly few reports to date describing a metagenomic dataset from this kind of environment (Table 3). Abbai and Pillay (2013) used a metagenomics approach to examine the microbiological and functional composition of two borehole samples retrieved from an aquifer system contaminated with industrial chemicals including aromatic hydrocarbons. While the majority of taxa and hydrocarbon-degrading genes detected were aerobic in nature (e.g., oxygenases), some identified taxa were mostly closely associated with known hydrocarbon-degraders although anaerobic hydrocarbon-degrading genes (e.g., *ass*A, *bss*A) were not found or reported (Abbai and Pillay 2013). Although not aquifer systems, terrestrial-based oil sands tailings ponds that store solid and liquid wastes from surface bitumen mining in northeastern Alberta,

Canada, are predominantly anoxic (Penner and Foght 2010; Ramos-Padrón et al. 2011), harboring methanogenic consortia capable of biodegrading a variety of alkanes and aromatic hydrocarbons as demonstrated in several enrichment cultures (e.g., Siddique et al. 2006, 2007; Tan et al. 2015a, b; Abu Laban et al. 2015) and in situ (Stasik et al. 2015). In accordance with observations in enrichments, a metagenomic analysis of unenriched anoxic tailings samples revealed genes associated with acetotrophic and hydrogenotrophic methanogenesis, fumarate addition (*bss*A), and ATP-independent ring reduction of benzoate (An et al. 2013b). These metagenomic findings provided additional evidence that methanogenic consortia are capable of biotransforming hydrocarbons associated with oil sands tailings ponds.

7 Hydrocarbon Metabolomics

The term "metabolome" has been defined as a collection of all of the biochemical molecules produced by a given cellular system, and the field of science involving the analysis of the metabolome is called "metabolomics." In the field of anaerobic hydrocarbon metabolism, "hydrocarbon metabolomics" or "hydrocarbon metabolite profiling" generally refers to evaluating environmental samples for the presence of specific, signature hydrocarbon metabolites that are only detected if anaerobic biodegradation is occurring within a given environment. Thus, while metagenomics reveals the genetic *potential* for biochemical reactions, metabolomics can be considered as the "ultimate proof" that a biochemical reaction has occurred. Using a metabolomic approach to deduce in situ anaerobic hydrocarbon biodegradation has been a topic of recent reviews (e.g., Agrawal and Gieg 2013; Callaghan 2013a) and is the focus of a separate chapter in this manual (Gieg and Toth 2016), so here we present only a high-level overview on this topic.

There are a handful of anaerobic mechanisms known to mediate anaerobic hydrocarbon activation for which signature metabolites (and in some cases genes and enzymes) have been identified, including fumarate addition, carboxylation, and hydroxylation (see above and reviews by Widdel and Musat 2010; Heider and Schühle 2013; Musat 2015; Rabus et al. 2016). Of these pathways, metabolites stemming from fumarate addition reactions (benzylsuccinates, alkylsuccinates and naphthylmethylsuccinates; Fig. 1) can be considered as the best diagnostic indicators of anaerobic in situ hydrocarbon metabolism (in part) due to their unequivocal relationship with their parent molecule, their absence in fuel mixtures, and their relative stability in the environment (NRC 1993; Beller 2000). As such, finding these signature metabolites in hydrocarbon-containing environments provides unequivocal evidence that anaerobic hydrocarbon biodegradation is occurring. Fumarate addition metabolites have now been detected in an array of hydrocarbon-containing environments, including in groundwater systems (e.g., Beller 2000; Beller et al. 1995, 2002, 2008; Elshahed et al. 2001; Gieg and Suflita 2002; Martus and Püttman 2003; Griebler et al. 2004; Gieg et al. 2009; Parisi et al. 2009; Jobelius et al. 2011), petroleum reservoirs (e.g., Duncan et al. 2009; Gieg et al. 2010; Bian et al. 2015), coal seams (Wawrik et al. 2012), and in oil-contaminated marine sediments (Kimes

et al. 2013). Note, however, that fumarate addition reactions are not known to occur for unsubstituted aromatic hydrocarbons like benzene, naphthalene, or other unsubstituted PAHs and heterocycles, making it difficult to use a metabolomic approach to diagnose their in situ biodegradation. For example, anaerobic benzene biodegradation yields either benzoate or phenol as an early intermediate (Fig. 1). Since both of these intermediates can also be formed aerobically (Assinder and Williams 1990), and benzoate is a central metabolic intermediate of numerous aromatic substrates (Fuchs et al. 2011), their detection in a field site cannot be definitively linked to the anaerobic biodegradation of benzene. Similarly, down-gradient metabolites following initial fumarate addition reactions (e.g., toluic acids from xylenes, naphthoic acids from naphthalenes, and fatty acids from alkanes) can also be products of aerobic metabolism (Mahajan et al. 1994). Thus, conclusively diagnosing the in situ anaerobic metabolism for some hydrocarbons can be inherently challenging.

Another of the most important limitations to consider when employing hydrocarbon metabolomics is that the absence of intermediate products cannot be interpreted as an absence of degradation, as metabolites are transient during active catabolism and therefore can be difficult to detect by conventional instrumentation (Callaghan 2013a; Gieg and Toth 2016). Therefore, it is critical that multiple diagnostic approaches be employed when evaluating in situ anaerobic biodegradation in field investigations (Gieg et al. 1999; Weiss and Cozzarelli 2008; Beller 2000; Bombach et al. 2010; Morasch et al. 2011). For example, using a functional gene approach, such as the detection or quantification of *ass*A or *bss*A (by PCR or qPCR), can also compliment a metabolomic analysis and has been used to determine the prospects for in situ anaerobic biodegradation in some hydrocarbon-containing environments (Beller et al. 2002, 2008; Callaghan et al. 2010; Oka et al. 2011; Wawrik et al. 2012; Li et al. 2012; Zhou et al. 2012; Bian et al. 2015). Along with biomarker and genomic tools, recent studies have also evaluated the application of proteomics and metatranscriptomics to characterize anaerobic hydrocarbon biodegradation (e.g., Selesi et al. 2010; Konopka and Wilkins 2012; Embree et al. 2014). These approaches offer real-time snapshots of the functional expression of hydrocarbon-catabolizing genes, but have yet to be widely applied to assessing anaerobic hydrocarbon biotransformation in situ.

8 Research Needs

The earliest investigations into anaerobic hydrocarbon biodegradation focused primarily on understanding this process in anoxic hydrocarbon-contaminated environments such as groundwater aquifers. However, through biogeochemical observations and the use of molecular biology and genomics approaches, it is now apparent that this process occurs in highly diverse environments that additionally include subsurface fossil energy reservoirs, marine sediments, hydrocarbon seeps, and hydrothermal vent systems. The study of isolates or highly enriched cultures obtained from several of these environments has shed enormous light on the

mechanisms of hydrocarbon metabolism in the absence of oxygen, bolstered by information garnered from their genome (and/or transcriptome/proteome) sequences. However, the roles of and interactions among microorganisms in most natural environments, including hydrocarbon-containing ecosystems, are poorly understood. Metagenomics (and other meta-omics approaches) can provide an abundance of information to help define and understand natural biogeochemical processes. While there are now some available metagenomic datasets for microbial communities inhabiting diverse hydrocarbon-containing environments (Table 3), there is still a great need for many more datasets generated from these hydrocarbon-containing environments in order to better understand their governing ecological principles. For example, while the anaerobic hydrocarbon biodegradation within contaminated aquifers has been studied for a long time and in great detail, only one metagenomic dataset has been described for this environment (Abbai and Pillay 2013). Recently, Anantharaman et al. (2016) conducted a terabase-scale metagenomic study of aquifer sediments (not hydrocarbon impacted) from which they were able to reconstruct >2500 individual genomes that allowed for the discovery of many new phyla and a proposed understanding of how microbial community members interact to carry out critical biogeochemical reactions; conducting these sorts of studies for hydrocarbon-containing environments is a clear research need. Further, amassing a large number of metagenomic datasets from environments with similar ecological pressures (such as anoxia and the presence of hydrocarbons) can, for example, allow for comparative analyses in order to elucidate the metabolic traits that define these environments (such as syntrophy, Tan et al. 2015a; Oberding and Gieg 2016).

That said, it should be noted that while metagenomics can provide enormous amounts of informative genetic information, it is an approach that describes metabolic *potential* – experimentation is still required to observe this potential. Thus, obtaining hydrocarbon-degrading isolates or highly enriched cultures and characterizing their functions through physiological experimentation, along with defining their genomes, transcriptomes, proteomes, and metabolomes, are ongoing research needs. Such information from model hydrocarbon degraders can help guide the interpretation of metagenomic datasets and “ground-truth” metagenomic-based predictions through experimentation. In all, metagenomic approaches are only starting to be used to assess anaerobic hydrocarbon biodegradation in many environments but coupled with model experimental systems have the potential to reveal a more comprehensive understanding about this process in diverse hydrocarbon-containing ecosystems.

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