

ORIGINAL ARTICLE

Comparative analysis of metagenomes from three methanogenic hydrocarbon-degrading enrichment cultures with 41 environmental samples

Boonfei Tan^{1,4,5}, S Jane Fowler^{2,4,6}, Nidal Abu Laban^{1,2}, Xiaoli Dong^{3,7},
Christoph W Sensen^{3,8}, Julia Foght¹ and Lisa M Gieg²

¹Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada; ²Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada and ³Visual Genomics Centre, Faculty of Medicine, Calgary, Alberta, Canada

Methanogenic hydrocarbon metabolism is a key process in subsurface oil reservoirs and hydrocarbon-contaminated environments and thus warrants greater understanding to improve current technologies for fossil fuel extraction and bioremediation. In this study, three hydrocarbon-degrading methanogenic cultures established from two geographically distinct environments and incubated with different hydrocarbon substrates (added as single hydrocarbons or as mixtures) were subjected to metagenomic and 16S rRNA gene pyrosequencing to test whether these differences affect the genetic potential and composition of the communities. Enrichment of different putative hydrocarbon-degrading bacteria in each culture appeared to be substrate dependent, though all cultures contained both acetate- and H₂-utilizing methanogens. Despite differing hydrocarbon substrates and inoculum sources, all three cultures harbored genes for hydrocarbon activation by fumarate addition (*bssA*, *assA*, *nmsA*) and carboxylation (*abcA*, *ancA*), along with those for associated downstream pathways (*bbs*, *bcr*, *bam*), though the cultures incubated with hydrocarbon mixtures contained a broader diversity of fumarate addition genes. A comparative metagenomic analysis of the three cultures showed that they were functionally redundant despite their enrichment backgrounds, sharing multiple features associated with syntrophic hydrocarbon conversion to methane. In addition, a comparative analysis of the culture metagenomes with those of 41 environmental samples (containing varying proportions of methanogens) showed that the three cultures were functionally most similar to each other but distinct from other environments, including hydrocarbon-impacted environments (for example, oil sands tailings ponds and oil-affected marine sediments). This study provides a basis for understanding key functions and environmental selection in methanogenic hydrocarbon-associated communities.

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Introduction

Since the dawn of the industrial age, anthropogenic activities have resulted in contamination of various soil, water and subsurface environmental systems

with organic compounds including hydrocarbons. Methanogenic biodegradation of hydrocarbons is important in many environments such as contaminated groundwater, sediments and organic chemical waste disposal sites that are devoid of electron acceptors such as oxygen, nitrate, iron(III) and sulfate (Chang *et al.*, 2005, Essaid *et al.*, 2011; Lykidis *et al.*, 2011). Methanogenic hydrocarbon biodegradation is also an important biogeochemical process in subsurface fossil energy reservoirs that, over geological time, has contributed to the formation of natural gas deposits and/or the conversion of light oil to heavy oil (Jones *et al.*, 2008).

Hydrocarbon mineralization *via* methanogenesis involves the coordinated metabolism of syntrophic (fermentative) bacteria that catalyze the initial attack on hydrocarbon substrates with subsequent conversion to methanogenic substrates, and methanogens that produce CH₄ (and CO₂ and/or H₂O) in a thermodynamically-interdependent manner (Gieg *et al.*, 2014). Many previous studies have shown that

Correspondence: L Gieg, Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, Canada T2N 1N4.

E-mail: lmgieg@ucalgary.ca

⁴These two authors contributed equally to this work

⁵Current address: Center for Environmental Sensing and Modeling, Singapore-MIT Alliance for Research and Technology, 1 Create Way, #09-03 Create Tower, Singapore 138602.

⁶Current address: Department of Environmental Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark.

⁷Current address: Department of Geoscience, University of Calgary, 2500 University Dr. NW, Calgary, Alberta, Canada T2N 1N4.

⁸Current address: Graz University of Technology, Institute of Molecular Biotechnology, Petersgasse 14, 8010 Graz, Austria.

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microbial communities in methanogenic hydrocarbon-associated environments (for example, Chang *et al.*, 2005; Kasai *et al.*, 2005; Sun and Cupples, 2012; Wawrik *et al.*, 2012; An *et al.*, 2013) or enrichment cultures (Ficker *et al.*, 1999; Fowler *et al.*, 2012) can be highly diverse. Literature-wide compilations examining such communities (Gray *et al.*, 2011; Strapoc *et al.*, 2011; Kleinstüber *et al.*, 2012) have revealed that they frequently contain hydrogenotrophic (for example, *Methanoculleus*, *Methanolinea*, *Candidatus Methanoregula*, *Methanospirillum*), acetotrophic (for example, *Methanosaeta*), and/or methylotrophic methanogens (for example, *Methanobolus*; Wuchter *et al.*, 2013). Deltaproteobacteria (for example, *Syntrophus/Smithella*, *Desulfovibrio*, *Geobacter*) and Firmicutes (for example, *Desulfotomaculum*, *Desulfosporosinus*, *Pelotomaculum*) are commonly abundant bacteria, and are often identified as the putative hydrocarbon-activating organisms (Gray *et al.*, 2011; Kleinstüber *et al.*, 2012). Members of the Spirochaetes, Bacteroidetes, Chloroflexi and Betaproteobacteria are also frequently found, but generally in lower abundance (Strapoc *et al.*, 2011; Kleinstüber *et al.*, 2012). Methanogenic hydrocarbon-degrading co-cultures, consisting of a single bacterium and archaeon, are difficult to obtain (Sieber *et al.*, 2012), and therefore the specific roles of this wide diversity of organisms in methanogenic hydrocarbon metabolism remain cryptic. The specific community structure may depend on the hydrocarbon substrates, the available nutrients and/or specific biogeochemical characteristics. Genes encoding anaerobic hydrocarbon-degrading enzymes (for example, *bssA*, *assA*) have been detected in hydrocarbon-degrading methanogenic cultures (for example Aitken *et al.*, 2013; Cheng *et al.*, 2013; Fowler *et al.*, 2012; Washer and Edwards, 2007) as well as genes involved in syntrophic processes such as H₂ and formate transfer, and flagella, amino-acid and coenzyme biosynthesis (Kato *et al.*, 2009; Walker *et al.*, 2009). Despite recent reports of hydrocarbon-degrading co-cultures of sulfate reducers and methanogens (Callaghan *et al.*, 2012; Lyles *et al.*, 2014), the diversity of key enzymes and genes involved in methanogenic hydrocarbon metabolism are not yet well described.

In this study, we carried out metagenomic sequencing of three methanogenic cultures enriched from two different environments (an oil sands tailings pond and a hydrocarbon-contaminated aquifer) and on different hydrocarbon substrates. We examined the diversity of taxa and genes involved in various anaerobic hydrocarbon biodegradation pathways in the cultures to determine whether differences would be observed based on different inoculum sources and hydrocarbon substrates. Further, we used a comparative metagenomic approach to examine how certain metabolic genes and functions compared among the cultures themselves and with other environments including hydrocarbon-impacted sites. We hypothesized that the three cultures would be more functionally similar to each other than to

other enrichment cultures and environments. We sought to identify the functions that distinguish these cultures from other hydrocarbon-impacted and anaerobic samples and enrichments to discern the hallmark features of methanogenic hydrocarbon-degrading communities.

Materials and methods

Methanogenic cultures and incubation conditions

The three methanogenic hydrocarbon-degrading cultures (*naphtha-degrading culture* (NAPDC), *short chain alkane-degrading culture* (SCADC) and *toluene-degrading culture* (TOLDC)) analyzed in this study were enriched from hydrocarbon-impacted environments on different hydrocarbons as the sole carbon and energy source(s) (Table 1). These cultures were maintained on the same substrates since their initial enrichment. Some metabolic features of the TOLDC and SCADC cultures have been reported (Fowler *et al.*, 2012; Tan *et al.*, 2013), while NAPDC is newly described here. NAPDC and SCADC were enriched (see Supplementary Information) from methanogenic mature fine tailings collected from Mildred Lake Settling Basin (MLSB), an oil sands tailings pond in Alberta, Canada that contains diverse low and high molecular weight hydrocarbons from unrecovered bitumen and naphtha (Siddique *et al.*, 2006). NAPDC was enriched with 0.2% (v/v) naphtha (CAS no. 64742-49-0), a petroleum product comprising monoaromatic hydrocarbons and C₆-C₁₀ alkanes that is present in many oil sands tailings ponds; NAPDC cultivation methods are provided in Supplementary Information. SCADC was enriched on 0.1% v/v C₆-C₁₀ *n*-alkanes with minor proportions of 2-methylpentane, 3-methylpentane and methylcyclopentane, as described by Tan *et al.* (2013). TOLDC was established from aquifer sediments contaminated with gas condensate (containing predominantly C₅-C₁₅ hydrocarbons, Gieg *et al.*, 1999) that was enriched solely on 0.01% (v/v) toluene (Fowler *et al.*, 2012). The three cultures were incubated statically in the dark under mesothermic conditions (20–28 °C) and transferred to defined minimal salts freshwater medium at regular intervals (Table 1). Gas chromatography was used to monitor substrate depletion and methane production in the cultures (Fowler *et al.*, 2012; Supplementary Information).

DNA extraction for 16S rRNA gene pyrotag sequencing and metagenomic sequencing

DNA extraction from TOLDC and SCADC for amplification and pyrotag sequencing of 16S rRNA gene amplicons has been described (Fowler *et al.*, 2012; Tan *et al.*, 2013). Total DNA from NAPDC used for 16S rRNA gene pyrosequencing was extracted and PCR amplified performed as described by Foght *et al.* (2004) and Tan *et al.* (2013). All 16S rRNA gene pyrotag sequencing

Table 1 Source of hydrocarbon-degrading methanogenic cultures and enrichment conditions

	NAPDC	SCADC	TOLDC
<i>In situ conditions in environment</i>			
Sample environment	Mature fine tailings in oil sands tailings pond ^a	Mature fine tailings in oil sands tailings pond ^a	Gas condensate-contaminated aquifer sediments ^b
Location	Alberta, Canada	Alberta, Canada	Colorado, USA
Depth (m below surface)	31 m	35 m	1.5 m
Bulk pH	7–8	7–8	7–8
Temperature	12–20 °C	12–20 °C	15–20 °C
Redox condition	Anoxic	Anoxic	Anoxic
<i>Incubation conditions for enrichment cultures</i>			
Initial inoculum %	50% (v/v)	50% (v/v)	30% (v/v)
Number of transfers	2	4	> 10
Inoculum proportion transferred	10% (v/v)	50% (v/v)	30% (v/v)
Substrate	0.2% (v/v) Naphtha	0.1% (v/v) C ₆ -C ₁₀ <i>n</i> -alkanes ^c	0.01% (v/v) Toluene
Culture medium	Anoxic mineral medium ^d	Anoxic mineral medium ^d	Anoxic mineral medium ^e
Initial medium pH	7–8	7–8	7–8
Initial headspace gas	20/80% (v/v) CO ₂ /N ₂	20/80% (v/v) CO ₂ /N ₂	20/80% (v/v) CO ₂ /N ₂
Incubation temperature	28 °C	28 °C	20–25 °C
Incubation time (before DNA extraction)	120 days	120 days	150 days
Light exposure	Dark	Dark	Dark

Abbreviations: NAPDC, *naphtha-degrading culture*; SCADC, *short chain alkane-degrading culture*; TOLDC, *toluene-degrading culture*.

^aMildred Lake Settling Basin, Alberta, Canada (Siddique *et al.*, 2007).

^bFt. Lupton, Colorado, USA (Fowler *et al.*, 2012).

^cIncludes minor proportions of 2-methylpentane, 3-methylpentane and methylcyclopentane (Tan *et al.*, 2013).

^dPer Widdel and Bak (1992) (anoxic freshwater mineral salts medium).

^ePer McInerney *et al.* (1979) (anoxic freshwater mineral salts medium).

reads were generated using the same ‘universal’ primer set, 926 F and 1392 R, targeting the V6-V8 regions of the 16S rRNA gene of bacteria and archaea (Engelbrektson *et al.*, 2010).

For high throughput pyrosequencing of total DNA without amplification, 500 ml of TOLDC and NAPDC or 800 ml of SCADC were filtered, extracted with phenol-chloroform, and purified using cesium chloride centrifugation (Wright *et al.*, 2009). Additionally, total DNA was obtained from two oil sands tailings ponds located in Alberta, Canada: MLSB (the same tailings pond used for enrichment of the NAPDC and SCADC cultures), and Tailings Pond 6 (TP6; Ramos-Padrón *et al.*, 2011). These tailings samples were collected and handled as described by An *et al.* (2013) before total DNA isolation using the bead-beating methods described by Ramos-Padrón *et al.* (2011) for TP6 and Foght *et al.* (2004) for MLSB. DNA was sequenced on a half plate using the GS FLX Titanium Sequencing Kit XLR70 (Roche Diagnostics, Laval, QC, Canada) at McGill University and the Génome Québec Innovation Centre, Montreal, Canada. Detailed information about library construction is provided by An *et al.* (2013).

Downstream processing of 16S rRNA gene and metagenomic pyrosequencing reads

All 16S rRNA gene pyrotag data sets were submitted simultaneously to Phoenix 2 (Soh *et al.*, 2013) for quality control, cluster analysis using the average neighbor joining method at 0.05 distance cutoff, and

taxonomic classification using RDP classifier with SILVA Small Subunit rRNA Database release 108 (<http://rdp.cme.msu.edu/>). A lack of consensus for similarity thresholds used in cluster analysis exists (for example, 97% or 95%). In this study, a 95% similarity threshold for operational taxonomic unit (OTU) clustering was used to accommodate potential biases introduced through homopolymers and sequencing artifacts produced by 454 pyrotag sequencing (Kunin *et al.*, 2010).

All metagenomic 454 pyrosequencing reads were subjected to quality control and *de novo* assembly using a Newbler v2.6-based pipeline developed in-house, as previously described (Tan *et al.*, 2013). All quality-controlled metagenomic reads were submitted to MG-RAST for automated gene calling and annotation. Unassembled metagenomic reads were also subjected to taxonomic classification using the MG-RAST classifier with the following parameters: M5NR annotation, *e*-value $\leq 1 \times 10^{-10}$, alignment ≥ 50 bp, identity $\geq 60\%$ (Meyer *et al.*, 2008), and homology-based binning using SOrt-ITEMS (Haque *et al.*, 2009). Additionally, unassembled reads were mapped to selected reference genomes (Supplementary Table S2) using Geneious Pro 7.05 (Biomatters Ltd., Auckland, New Zealand) with default settings. Metagenomic sequences from NAPDC, SCADC and TOLDC are available on the Short Read Archive under the SRA accession numbers SRX210874, SAMN01828453 and SRX210873. Those for the pyrotag data are SRX831148 (SCADC), SRX831147 (NAPDC) and SRX831099 (TOLDC).

Comparative analysis of functional categories in metagenomic data sets

Unassembled metagenomic sequences from NAPDC, SCADC and TOLDC (Metagenome Group 1; Supplementary Table S1) were annotated in MG-RAST V2 using SEED with the following parameters: e -value $\leq 1 \times 10^{-10}$; identity $\geq 60\%$; alignment length ≥ 50 bp (Meyer *et al.*, 2008). Additional metagenomes used in the comparison were chosen according to their environment type in MG-RAST. Both terrestrial and marine environments were selected, as the methanogenic cultures were derived from terrestrial environments previously associated with ancient marine environments before geological uplift. Eight additional metagenomes representing anaerobic dechlorinating enrichment cultures and hydrocarbon-impacted sediments (Metagenome Group 2; Supplementary Table S1) and 33 metagenomes from soil, sludge and marine and freshwater environments (Metagenome Group 3; Supplementary Table S1) were compared using principal component analysis based on the relative abundance of SEED functional categories (normalized against the total gene count of each metagenome) using R with the ade4 package (www.r-project.org). Metagenome Group 2 was further compared with Group 1 using STAMP (Parks and Beiko, 2010). For subsystem categories with rare counts (for example, $\leq 0.1\%$ of total counts), a fixed number of pseudocounts proportional to the total gene count was added to all categories across all groups used in comparisons to prevent selection of rare categories (Hug *et al.*, 2012). For all comparisons, unassembled metagenomic reads were used unless stated otherwise.

Comparative analysis of anaerobic hydrocarbon degradation and other metabolic pathways

Specific genes encoding proteins involved in various anaerobic hydrocarbon-degrading pathways (Figure 2, Supplementary Table S3) and a variety of other metabolic pathways (Supplementary Figures S4 and S5) were sought in NAPDC, SCADC and TOLDC metagenomes using Hidden Markov Models (HMMs). Where available, Pfam profile HMMs (Finn *et al.*, 2010) were used to identify protein families; in-house HMMs were made for genes for which no Pfam models existed and a minimum of four representative sequences were available in GenBank. To construct HMMs, amino-acid sequences of a gene were aligned using T-Coffee, and multiple alignments were manually curated using GeneDoc (Notredame *et al.*, 2000). Edited alignments were used as an input to HMMER to create HMMs (Eddy, 1998). Models were then used to screen assembled metagenomic data sets (including singletons) with an e -value of $< 1 \times 10^{-10}$. For genes with < 4 representative sequences available, tBLASTn searches were performed on each of the metagenomes (e -value $< 1 \times 10^{-10}$). Both HMM and BLAST-based methods were used, as each employs different methodologies in finding target genes, thereby minimizing the chances of missing

genes of interest. Assembled sequences, which include singletons, were used for this analysis, as detection of full-length or near full-length genes was difficult using unassembled reads (since many of these can include intergenic regions or partial genes) and precluded gene quantitation. Therefore, due to differences in sequencing depth in the three metagenomes, and likelihood of key genes not being sequenced, we used 'identification of the majority of genes in a pathway' (or enzyme-encoding subunits) as a more suitable indicator of the presence of a pathway or enzyme than individual gene abundance.

Phylogenetic analysis of putative fumarate addition enzymes

Illumina data from SCADC (Tan *et al.*, 2013) were re-assembled *de novo* in the current study using CLC Genomics Workbench (CLC-Bio, Boston, MA, USA) with the default settings. Amino-acid sequences of genes encoding enzymes for fumarate addition to hydrocarbons (for example, *assA*, *bssA* and *nmsA*) were recovered from the SCADC Illumina sequence assembly using tBLASTn. All recovered sequences were aligned with fumarate addition genes detected in TOLDC and NAPDC metagenomes plus reference sequences from GenBank using MUSCLE V3.3 (Edgar, 2004). Poorly aligned regions were manually edited and indels were removed. Phylogenetic analysis was performed using PhyML (Guindon and Gascuel, 2003) with WAG model and 100 bootstrap replicates. Trees were visualized and edited in FigTree V14.0 (Rambaut, 2012).

Results and discussion

Features of the enrichment cultures and their metagenomes

In this study, we first compared the metagenomes of three hydrocarbon-degrading methanogenic enrichments that were derived from different locations, enriched for varying lengths of time, and/or amended with different hydrocarbon substrates. Such variations allowed us to determine whether varying selective pressures have an effect on the genetic potential for anaerobic hydrocarbon metabolism in methanogenic enrichment cultures.

As shown in Table 1, TOLDC was enriched from a hydrocarbon-impacted aquifer, and has been maintained solely on toluene as the carbon and energy source for > 10 years (Fowler *et al.*, 2012, 2014). SCADC and NAPDC were newer enrichments (~ 2 years old at time of analyses) that were derived from a methanogenic oil sands tailings pond, enriched on short-chain alkanes (SCADC), or a complex hydrocarbon mixture (naphtha) that contains both aromatic and aliphatic hydrocarbons (NAPDC). Supplementary Figure S1 shows that NAPDC consumed toluene, xylenes and *iso*-alkanes concomitant with methane production over 103 days. For all cultures, substrate-unamended controls produced low amounts of CH_4 (Fowler *et al.*, 2012;

Supplementary Figure S1) indicating that the added hydrocarbons were the sources of methane.

Metagenomic 454 pyrosequencing of DNA from NAPDC, SCADC and TOLDC generated ~370 000, 670 000 and 550 000 quality-controlled reads, respectively (Table 2). Rarefaction analysis and assembly statistics revealed that TOLDC was more deeply sequenced than either NAPDC or SCADC, and that none of the cultures was sequenced to saturation (Supplementary Figure S2). The metagenomes assembled into approximately 8500 (NAPDC), 15 000 (SCADC) and 11 000 (TOLDC) contigs, with ~162 000, 327 000 and 179 000 singletons, respectively, and had GC contents of 49–53% (Table 2). The average assembled sequence lengths ranged from 1118 to 1863 bp but contigs varied greatly in length (200–28 000 bp). This likely reflects the relative abundance of different community members or the presence of closely related strains (for example, microdiversity), with more abundant members being better assembled due to a higher proportion of reads. The longest contigs ranged from 25.8 to 28.3 Kb, which is relatively short versus other comparable metagenomes (Hug *et al.*, 2012), suggesting that these metagenomes reflect particularly biodiverse communities.

Community compositions of methanogenic hydrocarbon-degrading cultures

The microbial community compositions of NAPDC, SCADC and TOLDC were determined in two ways; by

pyrotag sequencing of amplified 16S rRNA genes (Table 3), and by taxonomic profiling of unassembled metagenomic reads (Figure 1; Supplementary Figure S3). Overall, the total numbers of OTUs determined by 16S rRNA gene amplicon pyrosequencing ($\geq 95\%$ similarity) were 153 (NAPDC), 320 (SCADC) and 147 (TOLDC), including 65, 167 and 65 singletons, respectively. This is consistent with species richness as predicted by rarefaction analysis using the unassembled metagenomic reads (Supplementary Figure S2). The microbial communities predicted based on shotgun metagenomic sequencing (Figure 1; Supplementary Figure S3; PCR-independent) were largely consistent with those detected by pyrotag sequencing (Table 3) at the intra-taxon level. Some inter-taxon discrepancies were observed when comparing pyrotag sequencing (OTU assignment) and taxonomic profiling of metagenomes (Supplementary Figure S3), which is likely caused by primer bias or suboptimal PCR conditions. Nonetheless, the recovery of prevalent features in each metagenome using random shotgun metagenomic sequencing provides a basis for comparing the three metagenomes.

The archaeal reads in all three metagenomes, determined using 16S rRNA amplicon pyrosequencing, were dominated by Methanomicrobia, represented by Methanosarcinales (two OTUs affiliated with *Methanosaeta*; total 53–61% of archaea) and Methanomicrobiales (two *Methanoculleus* OTUs; total 16–36%), plus one *Methanolinea* OTU that was abundant (27%) only in TOLDC (Table 3). These

Table 2 Features of the metagenomes generated by 454 pyrosequencing of three hydrocarbon-degrading methanogenic enrichment cultures

	NAPDC	SCADC	TOLDC
Number of raw reads post-quality control (QC)	368 209	667 134	550 247
Unassembled reads post-QC (bp)	130 215 413	230 716 341	215 982 194
Mean read length post-QC (bp)	353.65	345.83	392.52
<i>Newbler assembly</i>			
Total length of contigs (bp)	9 473 370	17 382 962	20 280 655
Number of contigs	8471	15 274	10 888
Range of contig lengths (bp)	200–26 000	200–26 000	200–28 000
Mean contig length (bp)	1118	1138	1863
Largest contig (bp)	25 813	26 073	28 253
Number of singletons	161 851	326 382	179 067
N50	1330	1343	2813
Number of predicted proteins	133 107	261 378	170 842
Number of rRNA genes ^a	23/54/155	46/135/267	29/104/189
Number of tRNA genes	816	1322	891
<i>MG-RAST data (assembled)</i>			
MG-RAST ID	4 492 772.3	4 492 619.3	4 492 778.3
Metagenome size (bp)	64 676 632	127 644 733	89 513 841
Average sequence length (bp)	379 ± 352	373 ± 310	471 ± 647
Number of sequences	170 322	341 656	189 955
GC content (%)	49 ± 8%	50 ± 9%	53 ± 9%
Number of predicted ORFs	162 642	325 794	187 741
ORFs with predicted function	94 562	184 014	105 547
Alpha diversity ^b	662	771	907

Abbreviations: NAPDC, naphtha-degrading culture; SCADC, short chain alkane-degrading culture; TOLDC, toluene-degrading culture.

^a5S, 16S and 23S, respectively.

^bNumber of distinct species in a given metagenome sample as calculated by MG-RAST.

Table 3 The six most abundant bacterial and archaeal operational taxonomic units (OTUs) in enrichment cultures, determined by performing PCR amplification, sequencing and phylogenetic analysis of 16S rRNA genes

Phylum	Taxonomic affiliation ^a	Percentage of reads in each taxon		
		NAPDC	SCADC	TOLDC
<i>Bacterial OTUs</i>				
Firmicutes	<i>Anaerobacter</i>	9.5	3.0	30.2
Firmicutes	Lachnospiraceae	0.0	0.0	23.8
Firmicutes	Peptococcaceae	19.5	0.2	0.0
Firmicutes	<i>Desulfotomaculum</i>	15.3	0.0	0.0
Firmicutes	<i>Sedimentibacter</i>	0.0	0.0	11.1
Firmicutes	<i>Desulfosporosinus</i>	4.0	0.0	3.0
Spirochaetes	Spirochaetaceae	0.6	6.5	2.2
Spirochaetes	Spirochaetaceae	2.7	14.3	0.2
Deltaproteobacteria	<i>Smithella</i>	0.6	11.6	0.0
Deltaproteobacteria	<i>Syntrophus</i>	0.1	6.8	0.0
Deltaproteobacteria	<i>Geobacter</i>	2.8	2.6	0.1
Deltaproteobacteria	<i>Desulfovibrio</i>	0.6	4.2	0.0
Deltaproteobacteria	<i>Desulfobacterium</i>	0.1	3.6	0.0
Synergistetes	<i>Thermanaerovibrio</i>	4.2	2.5	0.0
Chloroflexi	Anaerolineaceae	0.0	0.0	5.4
Candidate division	Candidate division OP11	2.4	0.0	0.0
Candidate division	Candidate division OP8	0.0	0.0	3.3
<i>Archaeal OTUs</i>				
Euryarchaeota	<i>Methanosaeta</i>	51.5	55.9	50.6
Euryarchaeota	<i>Methanosaeta</i>	5.4	5.4	2.2
Euryarchaeota	<i>Methanoculleus</i>	32.1	21.3	15.1
Euryarchaeota	<i>Methanoculleus</i>	3.6	2.4	0.4
Euryarchaeota	<i>Methanolinea</i>	0.4	1.3	27.3
Euryarchaeota	<i>Candidatus Methanoregula</i>	0.6	4.8	0.6
Euryarchaeota	<i>Methanomicrobia</i>	1.4	1.4	0.0
Euryarchaeota	WCHA1	0.2	0.3	3.0

Abbreviations: NAPDC, naphtha-degrading culture; SCADC, short chain alkane-degrading culture; TOLDC, toluene-degrading culture.
^aEach OTU was assigned a taxonomic affiliation using RDP classifier. Only the lowest identified taxon is shown.

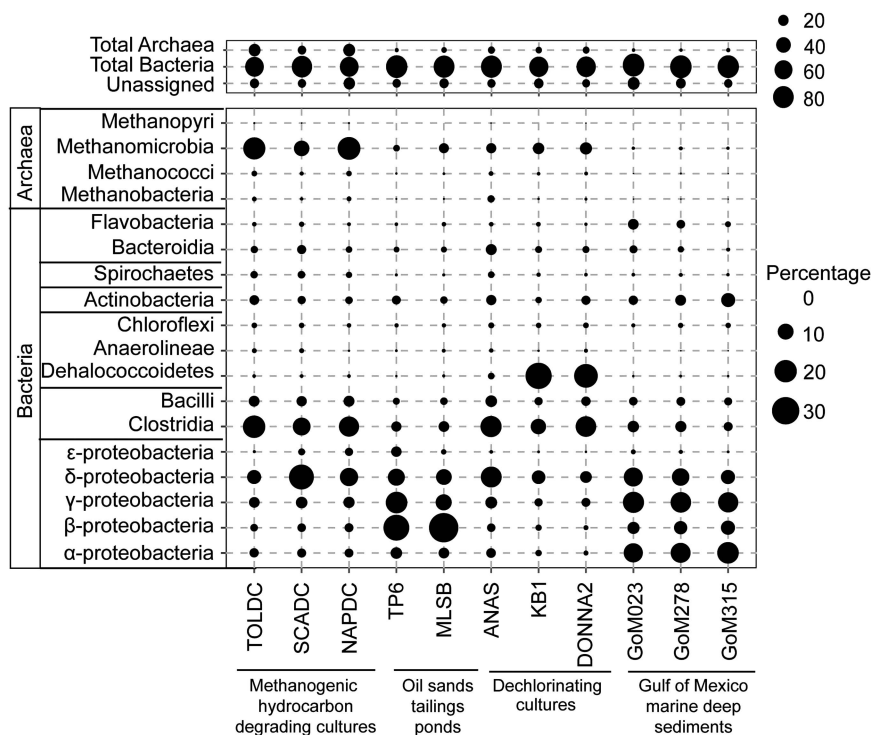


Figure 1 Relative abundance of microbial classes detected in metagenomes from NAPDC, TOLDC, SCADC and eight relevant environments (Supplementary Table S1), based on MG-RAST assignment of unassembled 454 metagenomic reads and expressed as the proportion of total reads in each metagenome.

methanogens are associated with acetotrophic and hydrogenotrophic methanogenesis, suggesting that both pathways contribute to methane production in the cultures.

The bacterial communities in NAPDC and TOLDC were dominated by members of the phylum Firmicutes, followed by Proteobacteria, Bacteroidetes, Actinobacteria and Spirochaetes (Figure 1; Table 3; Supplementary Figure S3). Despite similarities in bacterial community structure at the phylum level, there are noteworthy distinctions between the dominant OTUs affiliated with the Firmicutes as determined by pyrotag sequencing of amplicons (Table 3). The dominant Firmicutes-related OTUs in NAPDC belonged to the Peptococcaceae (*Desulfotomaculum* and *Desulfosporosinus*), whereas other Clostridiales (*Anaerobacter* and Lachnospiraceae) were most abundant in TOLDC (Table 3). This is in contrast to the recent finding that *Desulfosporosinus* sp. is a key toluene activating organism in TOLDC (Fowler *et al.*, 2014). Binning of metagenomic contigs from TOLDC (Fowler, 2014) suggests that there is an overrepresentation of *Anaerobacter* and Lachnospiraceae, and an underrepresentation of *Desulfosporosinus* sp. and specific members of the Deltaproteobacteria in the TOLDC 16S rRNA pyrotag data set. This is likely due to PCR bias, as *Desulfosporosinus* and Deltaproteobacteria were well represented in the TOLDC metagenome (Fowler, 2014). Another possibility is that the over-represented organisms contain multiple rRNA operons; for example, *Clostridium acetobutylicum* (closely related to *Anaerobacter* sp.) harbors eleven 16S rRNA gene copies (Acinas *et al.*, 2004). Further sequencing and genome reconstruction of TOLDC could reveal why this bias impacted TOLDC but not other cultures. The bacterial community in SCADC, as determined using metagenomic reads (Figure 1), mainly comprised Deltaproteobacteria and Clostridia, although the latter were not abundant in amplified sequences (Table 3) likely due to PCR bias. Not surprisingly, SCADC and NAPDC shared more OTUs with each other than with TOLDC (for example, Deltaproteobacteria, Table 3), consistent with their common environmental origin and substrate pressure (for example, the presence of alkanes in the enrichment substrates; Table 1).

The high abundance of Firmicutes in TOLDC and NAPDC is consistent with recent reports of anaerobic monoaromatic hydrocarbon-degrading cultures that implicate Firmicutes (for example, Peptococcaceae) as primary hydrocarbon degraders (Abu Laban *et al.*, 2009; Winderl *et al.*, 2010; Sun and Cupples, 2012; Fowler *et al.*, 2014) or playing key roles alongside Deltaproteobacteria (Ficker *et al.*, 1999; Ulrich and Edwards, 2003; Sakai *et al.*, 2009). In contrast, members of the Deltaproteobacteria, particularly *Syntrophus/Smithella* sp., have been implicated in methanogenic alkane degradation, particularly of longer-chain alkanes (Zengler *et al.*, 1999; Gray *et al.*, 2011; Cheng *et al.*, 2013). Indeed, all of the

known strictly anaerobic (for example, sulfate-reducing/syntrophic) alkane degraders are Deltaproteobacteria (Mbadanga *et al.*, 2011; Agrawal and Gieg, 2013; Callaghan, 2013). However, the implication of a Peptococcaceae in degradation of low molecular weight *n*-alkanes (for example, propane) under sulfate-reducing conditions (Kniemeyer *et al.*, 2007) and *iso*-alkanes under methanogenic conditions (Abu Laban *et al.*, in press; Tan *et al.*, 2014B) supports a role for Firmicutes in anaerobic alkane degradation. Based on the dominant OTUs in these cultures and the phylogenetic distribution of genes involved in fumarate addition, the primary hydrocarbon degraders in NAPDC, SCADC and TOLDC appeared to consist of different species from a few bacterial taxa (Table 3; Supplementary Figure S3). Indeed, the genomes of novel putative hydrocarbon degraders including *Smithella* spp. and Peptococcaceae capable of fumarate addition have been obtained from SCADC using single-cell amplified genomes (Tan *et al.*, 2014B, 2014C). Novel species capable of anaerobic hydrocarbon degradation are likely also present in NAPDC and TOLDC, as metagenomic reads from these cultures did not map well to reference genomes of known hydrocarbon degraders or their close relatives (Supplementary Table S2).

The high diversity (for example, 150–320 OTUs) present in NAPDC, SCADC and TOLDC after multiple culture transfers (particularly TOLDC, which has been passaged on toluene for >10 years) is likely due to the presence of secondary fermenters and syntrophs that are essential to the growth of the community. These organisms (for example, Clostridiales, Spirochaetes, *Anaerolinea*, *Desulfovibrio*; Figure 1, Table 3) may function in the transformation of hydrocarbon degradation products (such as fatty acids) to methanogenic substrates, maintaining a low redox potential and hydrogen partial pressure, scavenging or recycling of waste products and dead biomass within the enrichments, and/or providing essential nutrients such as vitamin B12 and amino acids for other community members (Ahring *et al.*, 1991; Beller and Edwards, 2000; Gray *et al.*, 2011; Walker *et al.*, 2012). The stability and persistence of diverse microorganisms in methanogenic hydrocarbon-degrading environments after long-term incubation suggests that these organisms are important for syntrophic growth of the communities.

Anaerobic hydrocarbon activation genes

Figure 2a shows the pathways and genes/enzymes known to be involved in the anaerobic metabolism of various hydrocarbons (for example, reviewed by Foght, 2008; Callaghan, 2013). Characterized hydrocarbon activation mechanisms (Figure 2) include addition to fumarate (pathways 1, 2, 3, 5 and 7), hydroxylation (pathway 1) and carboxylation (pathways 4 and 6). Thus, the three enrichment cultures were examined for the presence of genes associated

with these pathways by using HMMs and BLAST to infer their complete hydrocarbon substrate spectrum (Figure 2; Supplementary Table S3).

Fumarate addition reactions

Examination of the culture metagenomes revealed several full-length and/or partial genes associated

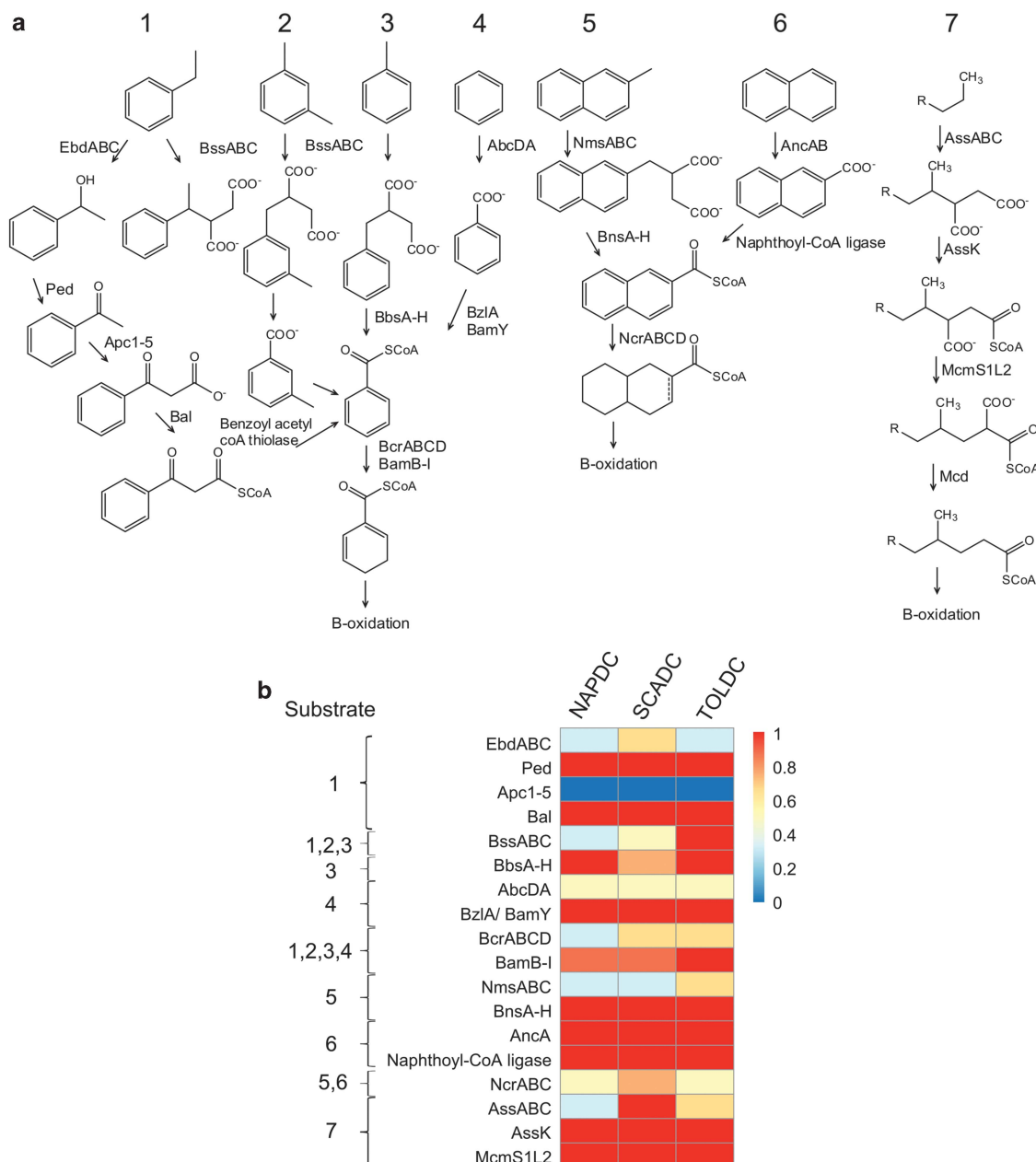


Figure 2 Enzymes involved in anaerobic hydrocarbon degradation and the corresponding genes detected in the metagenomes of NAPDC, SCADC and TOLDC. **(a)** Known pathways (substrates) for anaerobic hydrocarbon metabolism and associated enzymes. **(b)** Heatmap showing the presence of various genes and putative genes involved in anaerobic hydrocarbon metabolism in the metagenomes. The color scale indicates the inferred enzyme completeness, in the case of enzymes with multiple subunits. Enzyme abbreviations: EbdABC, ethylbenzene dehydrogenase; Ped, (S)-1-phenylethanol dehydrogenase; Apc1 to Apc5, acetophenone carboxylase; Bal, benzoylacetate-CoA ligase; BssABC, benzylsuccinate synthase trimer; BbsEF, succinyl-CoA:(R)-benzylsuccinate CoA transferase; BbsG, (R)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxyl(phenyl)methyl]- succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase; AbcDA, anaerobic benzene carboxylase; BzIA/BamY, benzoate-CoA ligase; BcrABCD, benzoyl-CoA reductase (ATP-dependent); BamB to BamI, benzoyl-CoA reductase (ATP-independent); NmsABC, naphthyl-2-methyl-succinate synthase; BnsEF, naphthyl-2-methyl-succinate CoA transferase; BnsG, naphthyl-2-methyl-succinate dehydrogenase; BnsH, naphthyl-2-methylene-succinyl CoA hydratase; BnsCD, naphthyl-2-hydroxymethyl-succinyl CoA dehydrogenase; BnsAB, naphthyl-2-oxomethylsuccinyl CoA thiolase; Anca, anaerobic naphthalene carboxylase; NcrABC, 2-naphthoyl CoA reductase; AssABC, alkylsuccinate synthase; AssK, AMP-dependent synthetase and ligase; Mcm, methylmalonyl CoA mutase; Mcd, methylmalonyl-CoA decarboxylase. Based on the models used, Mcd and benzoyl-CoA thiolase were abundant in all metagenomes. It was not known whether any of the genes identified were associated with hydrocarbon-degrading pathways or more general decarboxylase and thiolase functions, thus they have been omitted from the heatmap to curtail over-interpretation of the data.

with fumarate addition (*bssA* for methylated monoaromatics, *assA* for alkanes, *nmsA* for methyl-naphthalene) showing that all cultures have the potential to activate multiple substrates by fumarate addition.

Putative *assA* genes were identified in NAPDC and SCADC, three of which (*Smithella*_SCADC, NAPDC_ *assA1* and SCADC_ *assA3*) shared high homology with each other and a putative *assA* gene identified in *Smithella* sp. ME-1 (Embree et al., 2014; Tan et al., 2014A) (Figure 3). Five distinct putative *assA* genes were identified in SCADC (Figure 3), reflecting the diversity of alkane substrates degraded by this

culture (Tan et al., 2013). A partial putative *assA* was also detected in TOLDC, but it was not closely affiliated with *assA* reference sequences nor SCADC and NAPDC *assA* genes (Figure 3). Instead, the amino-acid sequence of TOLDC_ *assA1* gene had a best BLAST hit to *assA* in *Desulfoglaeba alkanexedens* ALDC (ADJ51097, similarity 44.5%). Apparently this gene has persisted in TOLDC despite long-term enrichment on toluene, and either belongs to a bacterium with unique taxonomic affiliations, encodes an enzyme having a substrate spectrum yet to be identified, and/or may even be involved in toluene activation (Rabus et al., 2011).

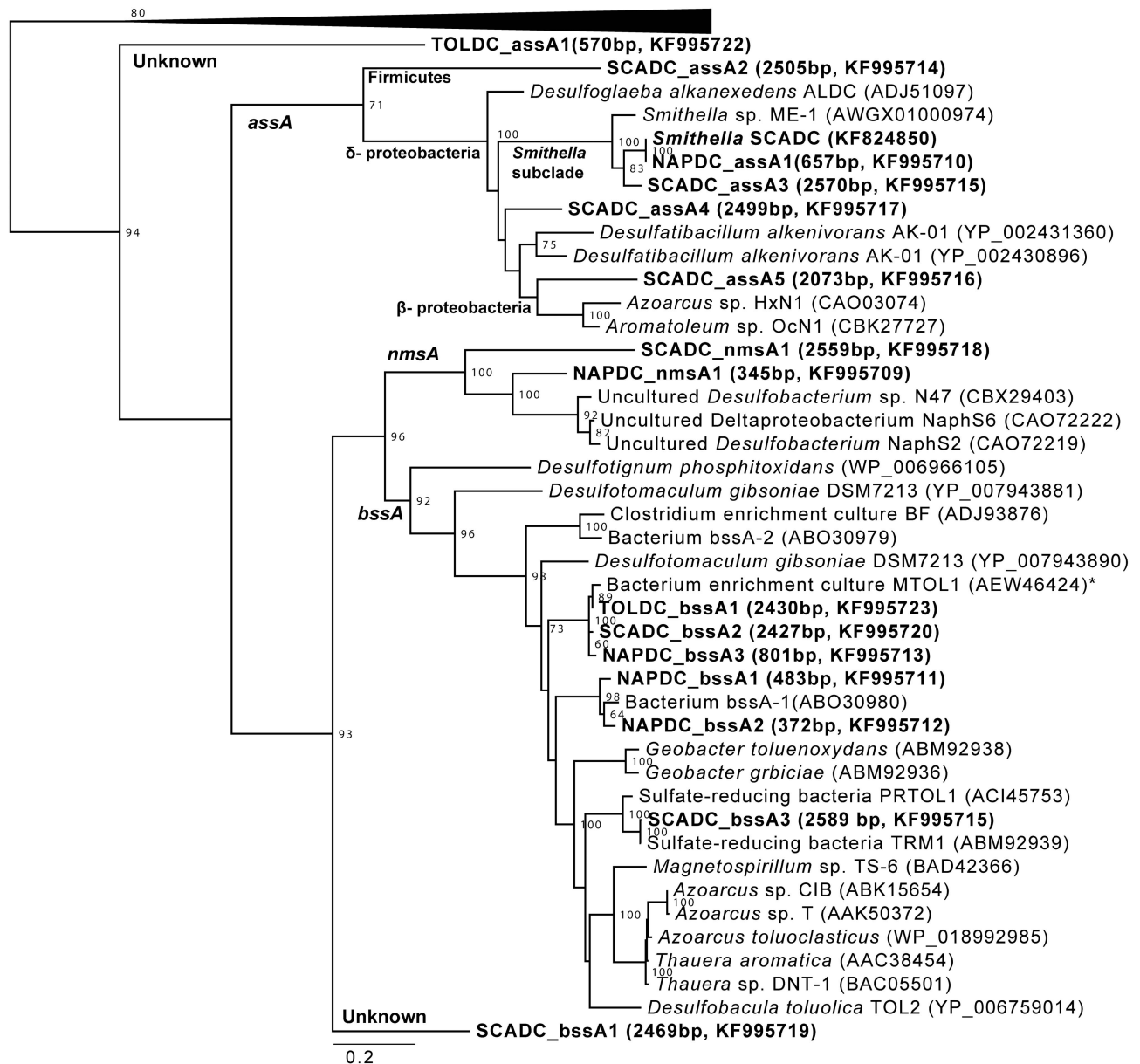


Figure 3 Maximum likelihood tree of translated full-length and partial *assA*, *bssA*, and *nmsA* homologs recovered from TOLDC, SCADC and NAPDC metagenomes (bold font). Bootstrap support $\geq 60\%$ is indicated. Full-length translated pyruvate formate lyase (PFL) sequences were used as an outgroup (collapsed in figure). Sequence length of genes recovered from TOLDC, SCADC and NAPDC, and their corresponding GenBank accession numbers are indicated in parentheses. A tree with the same overall topology was obtained when including only full-length sequences with gaps (not shown).

Full-length *bssA* genes having high sequence homology to one another were also detected (SCADC_ *bssA*2, TOLDC_ *bssA*1 and NAPDC_ *bssA*3; Figure 3), implying similar substrate spectra and/or phylogeny (for example, Firmicutes). Detection of *bssA* in the TOLDC metagenome is consistent with a previous report of benzylsuccinate formation from toluene by this culture (Fowler *et al.*, 2012). The NAPDC and SCADC metagenomes contained additional *bssA* genes most closely related to those recovered from a methanogenic toluene-degrading culture (ABO30980) and sulfate-reducing environments (ACI45753, ABM92939). SCADC also contained a deeply branching *bssA* gene (SCADC_ *bssA*1) with best a BLAST hit to *bssA* from *Desulfobacula toluolica* (YP_006759359, similarity 55%) rather than pyruvate formate lyase or *assA* genes (Figure 3), despite enrichment on aliphatic hydrocarbons. The detection of deeply branching putative *assA* and *bssA* genes is especially interesting because recent evidence suggests that an archaeal species (*Archaeoglobus fulgidus* strain VC-16) activates long chain alkanes by fumarate addition using a homology of pyruvate formate lyase that is phylogenetically distant from canonical *assA* and *bssA* (Khelifi *et al.*, 2014). Although genes matching the HMM for *bssA* were detected in all cultures, *bssB* and *bssC* genes encoding the other two subunits of benzylsuccinate synthase were not detected in NAPDC (Figure 2, Supplementary Table S3), underlining the importance of screening for all enzyme subunits to indicate the presence of functions (as performed here), especially for metagenomes suffering from low sequencing coverage.

Whereas all three metagenomes contained *assA* and *bssA*, only NAPDC and SCADC contained *nmsA* (pathway 5, Figures 2 and 3). Recent evidence suggests that some fumarate addition genes that cluster with *nmsA* may function in the activation of toluene or other monoaromatics rather than polycyclic aromatic hydrocarbons (Acosta-González *et al.*, 2013; von Netzer *et al.*, 2013); thus, the physiological role of *nms*-like genes in SCADC and NAPDC is unclear as polycyclic aromatic hydrocarbon degradation has not been tested with these cultures. Previous studies noted that *bssA* and *nmsA* exhibit a high degree of homology (for example, Selesi *et al.*, 2010), thus enzymes involved in fumarate addition might potentially co-activate several structurally diverse substrates. For example, *Azoarcus* sp. HxN1, which activates *n*-alkanes by using Ass (Mas), can co-activate *cyclo*-alkanes in the presence of *n*-alkanes (Wilkes *et al.*, 2003). Similarly, other Ass enzymes can activate toluene in the presence of *n*-alkanes (Rabus *et al.*, 2011), whereas Bss can catalyze fumarate addition to both toluene and xylenes (Beller and Spormann, 1997).

A point of interest in comparing the fumarate addition genes in these cultures is the relatively high abundance and sequence divergence of alpha gene subunits in NAPDC and SCADC compared with

TOLDC (Figures 2 and 3). This may reflect the degree of enrichment of the cultures, with NAPDC and SCADC being established relatively recently, vs TOLDC that was established > 10 years ago. Another contributing factor may be the diversity of hydrocarbon substrates during enrichment: NAPDC was established on a mixed alkane and monoaromatic hydrocarbon substrate (naphtha) and SCADC was enriched on a mixture of alkanes, whereas TOLDC was only exposed to toluene. The initial environments may also have dictated the genetic diversity (oil sands tailings ponds vs contaminated aquifer). However, other enrichments derived from the same aquifer sediments as TOLDC were shown to degrade a broad range of hydrocarbons such as other monoaromatics, *n*- and *cyclo*-alkanes, and polycyclic aromatic hydrocarbons (Gieg *et al.*, 1999; Townsend *et al.*, 2003 and 2004; Berdugo-Clavijo *et al.*, 2012), thus we speculate that long-term enrichment on a sole hydrocarbon is more likely the reason for lower gene diversity in TOLDC. The presence of multiple *assA/bssA/nmsA* genotypes in these cultures is of interest because hydrocarbon contaminants often exist as diverse, structurally complex hydrocarbon mixtures. Thus, a microbial community that can degrade multiple substrates may be useful for *in situ* bioremediation and biostimulation. However, the presence of enzyme subunits does not necessarily denote functionality, and some enzymes may activate multiple substrates as discussed above. Further physiological studies are needed to define the complete range of substrates that these cultures can activate by fumarate addition but these results suggest that all three cultures harbor the genetic ability to degrade a range of hydrocarbon substrates.

Anaerobic pathways downstream of fumarate addition

Following toluene activation by fumarate addition, benzylsuccinate is converted to benzoyl-CoA by a multi-subunit enzyme encoded by the *bbsABC-DEFGH* genes (pathway 3, Figure 2). Analogous genes (*bns*) have also been reported in the degradation of 2-methylnaphthalene (Figure 2, pathway 5, Selesi *et al.*, 2010). We found matches to all HMMs for *bbs* genes in TOLDC and for the majority of *bbs* genes in NAPDC and SCADC (Figure 2), showing that these genes are present in all cultures regardless of the hydrocarbon substrate used for enrichment. BLAST matches were found for all of the *bns* genes in all three cultures (Figure 2, Supplementary Table S3), although it is unknown whether these genes are involved in the degradation of 2-methylnaphthalene by the cultures. Anaerobic degradation of monoaromatic compounds converges at the central intermediate benzoyl-CoA (Figure 2) that is then further metabolized by ring reduction. Reductive dearomatization can occur *via* an ATP-dependent benzoyl-CoA reductase found in facultative organisms (*bcrABCD*), or *via* an ATP-independent reductase found in strict anaerobes (*bamBCDEFGHI*; Carmona

et al., 2009). We found matches to the alpha and delta subunits of the ATP-dependent ring reductases in all three cultures, but beta and gamma subunits were detected only in SCADC and TOLDC (Figure 2, Supplementary Table S3). Virtually all genes encoding the strictly anaerobic ring reductase (*bamB-I*) were also found in the three cultures, an expected result as the bacterial communities consist mainly of strict anaerobes, rather than facultative organisms (Table 3).

Following alkane activation (pathway 7; Figure 2), alkylsuccinates are activated to a CoA derivative, followed by carbon-skeleton rearrangement and decarboxylation (Wilkes et al., 2002). In *D. alkenivorans* AK-01, these reactions are presumably catalyzed by AssK, a putative methylmalonyl-CoA mutase, and a putative methylmalonyl-CoA carboxyl transferase before β -oxidation (Callaghan et al., 2012). We found BLAST or Pfam matches to these genes in all three metagenomes (Figure 2, Supplementary Table S3). However, because CoA-synthetases, along with methylmalonyl-CoA mutase and carboxylase (involved in the methylmalonyl-CoA pathway) are widespread in microbes, their presence cannot be solely associated with *n*-alkane degradation. It has been proposed that, before carbon skeleton rearrangement, the diastereomers produced from fumarate addition undergo epimerization catalyzed by alpha-methylacyl-CoA racemase to produce one intermediate for carbon skeleton rearrangement (Jarling et al., 2012). In a partial *ass* operon found in the genome of Peptococcaceae from SCADC (Tan et al., 2014B), putative genes involved in fumarate addition and downstream pathways clustered together, including alpha-methylacyl-CoA racemase, methyl malonyl-CoA mutase and β -oxidation, suggesting that alpha-methylacyl-CoA racemase may indeed be involved in *n*-alkane degradation. The putative alpha-methylacyl-CoA racemase gene in Peptococcaceae SCADC was used as a probe in tBLASTn searches to screen assembled contigs from TOLDC and NAPDC and to reveal homologs that have identity (~80%) but low sequence coverage (<50%). Unfortunately, the operon structure of the genes involved in *n*-alkane degradation in TOLDC and NAPDC is cryptic due to the short length of the associated contigs, so similarity to operons from known alkane degraders cannot be confirmed yet.

Alternate anaerobic hydrocarbon activation mechanisms

Hydroxylation and carboxylation are alternate mechanisms of anaerobic hydrocarbon activation. Genes for ethylbenzene hydroxylation (pathway 1, Figure 2) have been described (Ebd, encoded by *ebdAB*) (Rabus and Widdel, 1995; Kniemeyer and Heider, 2001) and it was recently proposed that *Desulfococcus oleovorans* Hxd3 activates alkanes by hydroxylation at C2 using an enzyme homologous to Ebd to produce a secondary alcohol (Callaghan,

2013). BLAST searches of TOLDC, SCADC and NAPDC assembled contigs using Ebd sequences from *D. oleovorans* Hxd3 revealed that *ebd* homologs are not abundant in these metagenomes. All genes identified had higher similarity to dehydrogenases with other functions (for example, DMSO dehydrogenase and nitrate reductase) than to Ebd, suggesting that these genes are not involved in ethylbenzene and alkane hydroxylation in the three cultures.

Putative genes encoding a benzene carboxylase (*abcDA*) (pathway 4, Figure 2) were recently identified under iron- (Abu Laban et al., 2010) and nitrate-reducing conditions (Luo et al., 2014) along with analogous genes encoding a putative naphthalene carboxylase (*ancA*) and naphthoyl-CoA ligase under sulfate-reducing conditions (pathway 6, Figure 2) (Bergmann et al., 2011). Several genes with homology to *abcA* were found in the three metagenomes, however, no delta subunit (*abcD*) genes were identified (Figure 2, Supplementary Table S3). The *ancA* gene was also detected in all three metagenomes (Figure 2) and matches to the CoA-ligases of benzene and naphthalene carboxylation were also identified (pathway 4, Figure 2), but none were detected on contigs adjacent to putative benzene carboxylase genes, as previously observed (Abu Laban et al., 2010; Luo et al., 2014). Overall, metagenomic analysis of the three cultures showed the genetic potential for carboxylation as a hydrocarbon activation strategy for non-substituted aromatic hydrocarbons but this activity remains to be tested in the cultures.

Other metabolic pathways

The metagenomes were interrogated for metabolic pathways including methanogenesis, fumarate regeneration, β -oxidation, carbon fixation, the glyoxylate cycle and alternate electron-accepting pathways (Supplementary Figures S4 and S5). Notably, genes were found for acetotrophic and hydrogenotrophic pathways of methane production in the cultures, but not the methylotrophic pathway (Supplementary Figure S5). These findings along with other pathways analyzed in this study are discussed further in the Supplementary Information.

Comparison of function among TOLDC, SCADC and NAPDC metagenomes

To examine the individual contributions of NAPDC, SCADC and TOLDC to their aggregate functional profile, we performed a three-way comparison of the relative abundance of SEED functional categories according to the method of Hug et al. (2012) (Figure 4a). Key functions related to methanogenesis, anaerobic hydrocarbon metabolism, redox condition regulation and hydrogen and formate transfer (important for syntrophy) were shared among the three cultures (Figure 4a). Features that were highly enriched in any single metagenome were related to

non-specific functions associated with specific taxa present in each of the cultures (Supplementary Table S4). The observation that NAPDC, SCADC and TOLDC share similar genetic potentials despite being enriched from two distinct environments on different hydrocarbon substrates for different incubation periods (~2 years vs >10 years) indicates that the cultures comprise communities having flexible taxonomic composition and high functional redundancy. A similar conclusion was reached recently for a very different anoxic environment (termite hindgut) wherein, despite the presence of diverse taxa in different termite species, common functionality was observed (He *et al.*, 2013).

Taxonomic and functional comparison of enrichment cultures with other environments and cultures

To investigate the effects of environmental selection on functional potential in methanogenic microbial communities, the three metagenomes from the hydrocarbon-degrading cultures were compared with 41 metagenomes available through MG-RAST including two oil sands tailings ponds (MLSB and TP6), three Gulf of Mexico (GoM) marine sediment samples collected from the Deepwater Horizon oil spill area (Kimes *et al.*, 2013), three dechlorinating enrichment cultures (the only other anaerobic pollutant-degrading enrichment culture metagenomes available on MG-RAST at time of analysis), and 33 metagenomes from a range of environments with varying physiochemical properties (Supplementary Table S1). A comparison of this nature assumes that random shotgun sequencing has recovered the prevalent genetic composition of the microbial community in any given environmental sample, validated by the taxonomic composition expected in their respective niches (for example, Proteobacteria in GoM and Dehalococcoidetes in dechlorinating cultures). Nonetheless, comparisons using functional categories (COG, KEGG, SEED) may effectively remove discovery of novel functions which otherwise are annotated as hypothetical proteins, and are not examinable due to unassigned functions. This study investigated the overall functional properties and dissimilarities among the 41 environmental metagenomes, and may not provide insights into functions of the rare biosphere due to unequal sequence coverage in the metagenomes.

Taxonomic analysis of metagenomes from hydrocarbon-impacted environments including the methanogenic cultures, tailings ponds, GoM sediments, and dechlorinating cultures revealed that all of these cultures contained methanogenic archaea (albeit in different proportions; Figure 1) indicating that they are anoxic environments or contain anoxic microenvironments. However, the bacterial communities supporting methanogenesis differed considerably in diversity and relative abundance (Figure 1).

Using principal component analysis based on the abundance of SEED functional categories, the

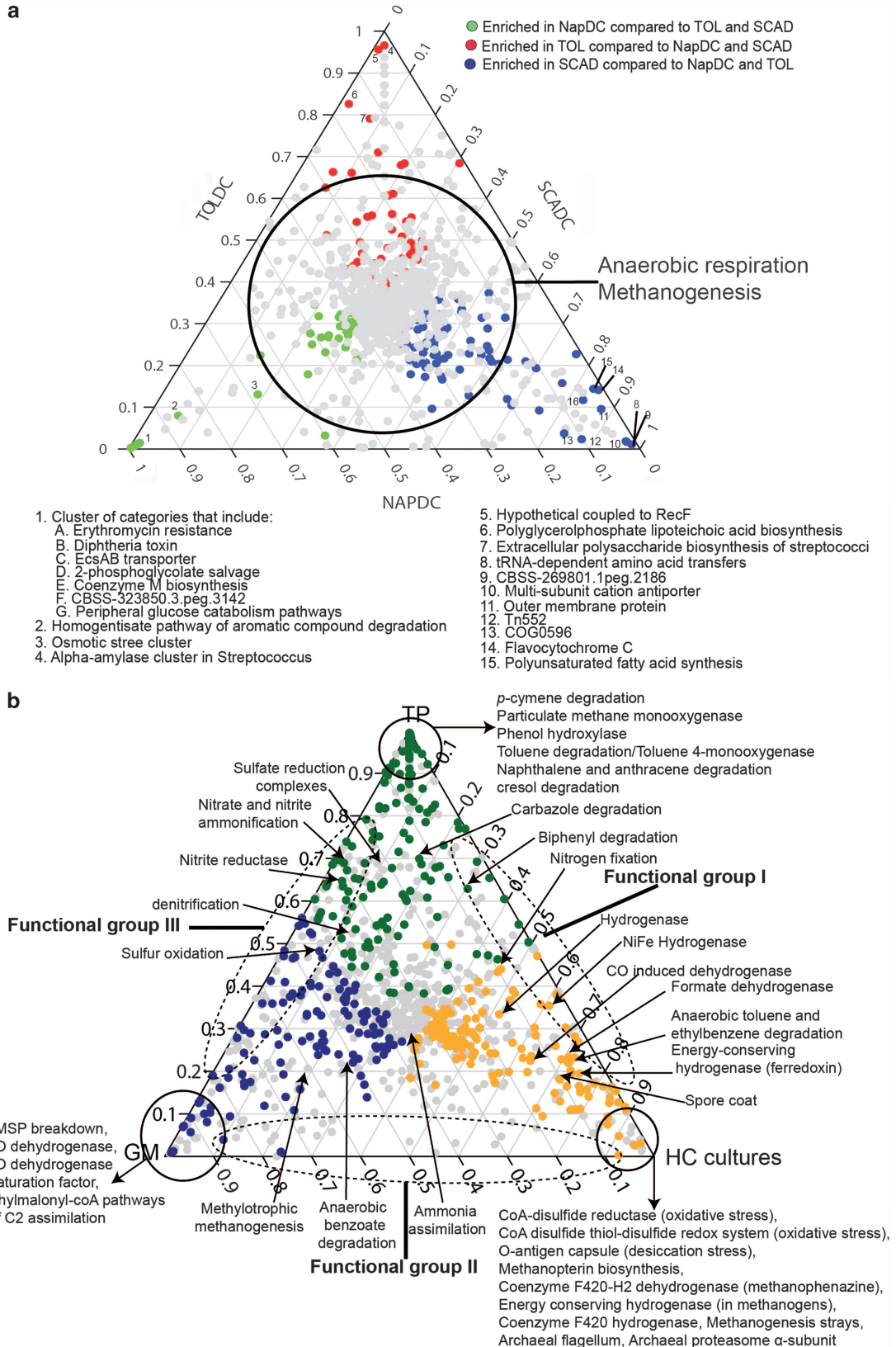
metagenomes from each type of environment or enrichment culture were found to be more functionally related to each other than to metagenomes from other environments (Figure 5), presumably due to selective pressures imposed by diverse environmental conditions. The 44 metagenomes clustered into four distinct groups: (1) terrestrial environments including different soil types; (2) sludge; (3) marine environments including lagoon and marine sediments and the GoM deep sediment samples; and (4) methanogenic hydrocarbon-degrading enrichments, tailings ponds and dechlorinating enrichment cultures (Figure 5). NAPDC, SCADC and TOLDC were more functionally similar to each other than to any other environments, including the tailings pond (MLSB) from which NAPDC and SCADC were enriched (Figure 5). The cultures also grouped closely with methanogenic dechlorinating cultures enriched from environments that were co-contaminated with chlorinated compounds and hydrocarbons (Hug *et al.*, 2012). This grouping presumably reflects the process of enrichment compared with unenriched samples from tailings and other environmental samples. All three GoM metagenomes grouped more closely with marine samples and lagoon sediments than with hydrocarbon-impacted environments, despite the recent exposure of at least two of the GoM sediments to hydrocarbons during the Deepwater Horizon oil spill (Kimes *et al.*, 2013).

To further interrogate the functions enriched in TOLDC, NAPDC and SCADC vs hydrocarbon-impacted environments, we performed a three-way comparison of pooled metagenomes from the cultures, tailings ponds and the GoM marine sediments (Figure 4b). Features specifically enriched in the three cultures included those associated with hydrogen and formate transfer, methanogenesis and regulation of redox conditions (for example, CoA-disulfide redox systems) (HC vertex, Figure 4b), processes that are important for syntrophy and methanogenesis. GoM metagenomes shared little functional potential with the methanogenic hydrocarbon-degrading enrichments, as shown by the paucity of data points along the bottom axis (Functional Group II; Figure 4b). This is consistent with the taxonomic profiles of GoM metagenomes that were characterized by an abundance of Alpha- and Gammaproteobacteria, distinctly different from the methanogenic enrichments and oil sands tailings ponds (Figure 1). The GoM metagenomes did share some similarities in functional potential with tailings ponds (Functional Group III; Figure 4b), particularly related to sulfur cycling (likely due to the abundance and types of sulfur compounds present in both environments) and denitrification processes. Despite the exposure of two of the GoM samples to hydrocarbons following the Deepwater Horizon oil spill, there was a notable absence of enriched functions specifically related to hydrocarbon metabolism (Figure 4b).

Both MLSB and TP6 are located in the same geographic region and contain residual naphtha, components of which can serve as substrates for

methanogenic communities (Siddique *et al.*, 2006, 2007). On the basis of principal component analysis (Figure 5), the genetic potentials of the two tailings

ponds were more similar to each other than to SCADC and NAPDC that were enriched from MLSB. Both tailings ponds harbor microbes genetically capable of



anaerobic alkane and aromatic hydrocarbon metabolism by fumarate addition (An *et al.*, 2013). In addition, three-way comparison revealed that, in agreement with the recent report by An *et al.* (2013), the metagenomes of the tailings ponds are also enriched in functions associated with aerobic metabolism of pollutants and hydrocarbons (Figure 4b), likely due to the presence of oxic surface water layers in the ponds and sedimentation of aerobic or facultative microbes from the water into the underlying tailings. Indeed, the ponds harbor a high abundance of Beta- and Gammaproteobacteria including *Burkholderia*, *Pseudomonas* and *Rhodococcus* (Figure 1), genera associated with aerobic hydrocarbon metabolism (Das and Chandran, 2011). In deeper strata of TP6, the microbial communities consist mainly

of syntrophs, sulfate reducers and methanogens (Ramos-Padrón *et al.*, 2011). The addition of gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) to this pond stimulates sulfate reduction (Ramos-Padrón *et al.*, 2011), a function that was enriched in tailings ponds relative to the methanogenic cultures (Figure 4b). The oil sands tailings ponds were found to share some functions with the methanogenic hydrocarbon-degrading cultures, particularly those associated with anaerobic or methanogenic metabolism (Functional Group I, Figure 4b). For example, previous analyses revealed the presence of genes associated with fumarate addition in TP6 (An *et al.*, 2013) and in clone libraries from GoM sediments (Kimes *et al.*, 2013), suggesting that this is a widespread mechanism of hydrocarbon activation in anoxic environments.

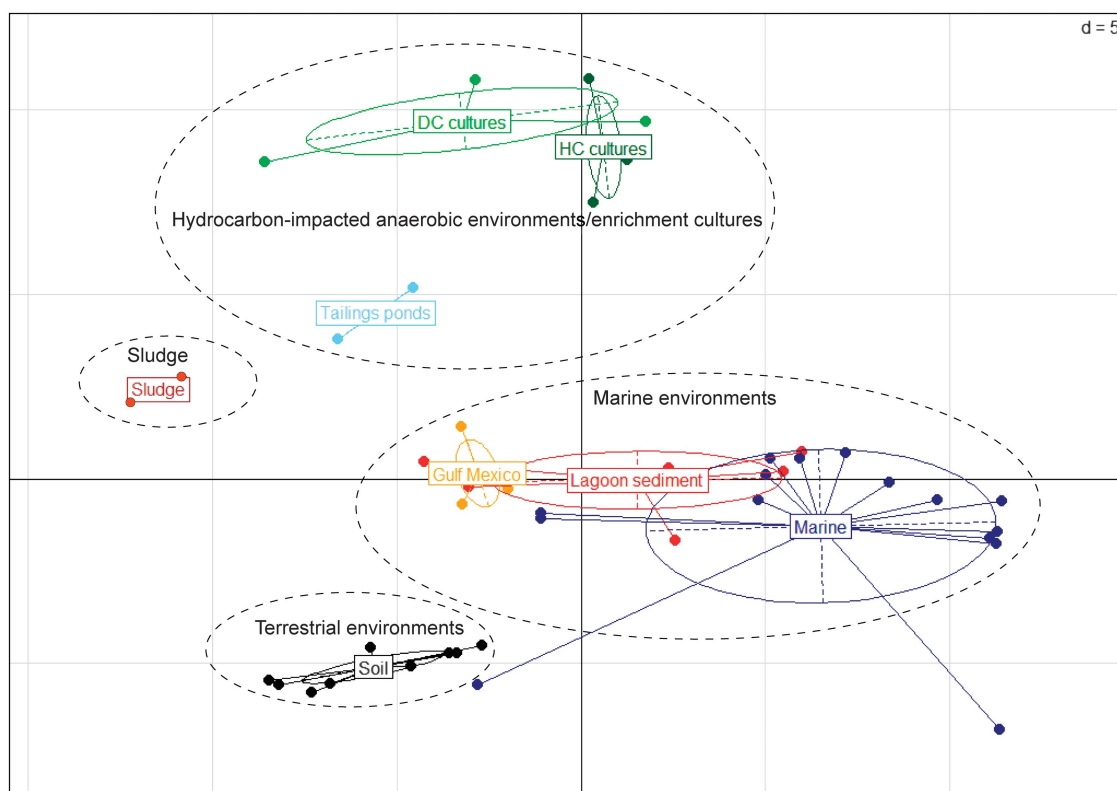


Figure 5 Principal component analysis of NAPDC, SCADC and TOLDC plus 41 additional relevant metagenomes available in MG-RAST (Supplementary Table S1); symbols are colored according to their environment of origin. All counts were normalized against total annotated sequences of each metagenome. Metagenomes from related environments are enclosed with broken lines.

Figure 4 Ternary plots showing three-way comparisons of functional categories in SEED subsystems level 3 shared among individual metagenomes or groups of metagenomes. (a) Comparison of individual NAPDC, SCADC and TOLDC metagenomes. (b) Comparison of metagenome groups from hydrocarbon-impacted communities: HC (TOLDC, SCADC and NAPDC); GM (three metagenomes from Gulf of Mexico deep marine sediments); and TP (two metagenomes from oil sands tailings ponds). Each point on the ternary plot represents a subsystem category in the three metagenomes or groups of metagenomes, with the proportion of each SEED being normalized to a value of 1. Data points are colored according to the source of each metagenome or group; gray dots represent functional categories present at low, statistically non-significant ($P < 0.01$) abundance. Points located near the vertices are enriched within the metagenome or group associated with that vertex, points along each axis are shared only between the two vertices associated with that axis (labelled as Functional Groups I, II and III in b), and points located near the center of the plot have similar proportions in all three metagenomes or groups (that is, show no specific enrichment; Hug *et al.*, 2012).

Conclusions

Metagenomic analysis of three distinct methanogenic hydrocarbon-degrading enrichment cultures (NAPDC, SCADC and TOLDC) revealed that despite differences in enrichment pressures and putative hydrocarbon-degrading taxa, all harbored multiple functional genes for different mechanisms of anaerobic hydrocarbon activation (fumarate addition and carboxylation) and subsequent conversions. The diversity and abundance of fumarate addition genes increased in the cultures enriched with a mixture of hydrocarbon substrates. However, it remains difficult to assess the effect of any given substrate on the enrichment of a particular fumarate addition gene as the cognate enzymes may exhibit relaxed substrate specificity (Rabus *et al.*, 2011). Comparative metagenomic analyses showed that the functional profiles of the methanogenic hydrocarbon-degrading cultures were similar to each other but were distinct from hydrocarbon-impacted environments including the tailings pond from which two of the cultures were derived. Specialized functions related to methanogenesis, anaerobic hydrocarbon metabolism, regulation of redox conditions and assorted hydrogenases and formate dehydrogenases were shared among the culture metagenomes and enriched compared with other metagenomes suggesting that these are hallmark features of methanogenic hydrocarbon degradation. Because the methanogenic hydrocarbon-degrading cultures were functionally similar, the study of such cultures can provide fundamental information about environmentally important syntrophic processes in hydrocarbon-associated methanogenic environments.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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