RESEARCH ARTICLE

Biodegradation of low-molecular-weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates and community patterns

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Abstract

We evaluated the ability of the native microbiota in a low-temperature, sulfidic natural hydrocarbon seep (Zodletone) to metabolize short-chain hydrocarbons. *n*-Propane and *n*-pentane were metabolized under sulfate-reducing conditions in initial enrichments and in sediment-free subcultures. Carbon isotope analysis of residual propane in active enrichments showed that propane became enriched in ^{13}C by 6.7 (± 2.0)‰, indicating a biological mechanism for propane loss. The detection of *n*-propylsuccinic and isopropylsuccinic acids in active propane-degrading enrichments provided evidence for anaerobic biodegradation via a fumarate addition pathway. A eubacterial 16S rRNA gene survey of sediment-free enrichments showed that the majority of the sequenced clones were phylogenetically affiliated within the *Deltaproteobacteria*. Such sequences were most closely affiliated with clones retrieved from hydrocarbon-impacted marine ecosystems, volatile fatty acid metabolizers, hydrogen users, and with a novel Deltaproteobacterial lineage. Other cloned sequences were affiliated with the *Firmicutes* and *Chloroflexi* phyla. The sequenced clones were only distantly ($< 95\%$) related to other reported low-molecular-weight alkane-degrading sulfate-reducing populations. This work documents the potential for anaerobic short-chain *n*-alkane metabolism for the first time in a terrestrial environment, provides evidence for a fumarate addition mechanism for *n*-propane activation under these conditions, and reveals microbial community members present in such enrichments.

Introduction

Alkane mixtures of various lengths (C_1–C_{100}) are major components of various fossil fuel formations (Hunt, 1995; Philp, 2005) as well as of hydrocarbon seeps (Whelan *et al.*, 1988; Clifton *et al.*, 1990), and subseafloor gas hydrates (Kvenvolden, 1995). High-molecular-weight liquid and solid alkanes are present as the main constituents of crude oil, while lower molecular weight alkanes are mainly encountered in thermogenic and biogenic natural gas (Hunt, 1995). In addition, methane is biogenically produced in globally significant quantities in wetlands, rice fields, and rumina of herbivores (Wahlen, 1993; Kinnaman *et al.*, 2007).

The aerobic degradation of alkanes with various chain lengths has been studied and documented extensively (Leahy & Colwell, 1990; Hanson & Hanson, 1996; Berthe-Corti & Fetzner, 2002; Van Hamme *et al.*, 2003). This process involves the stepwise oxidation of an alkane into an alcohol, aldehyde, and carboxylic acid before β-oxidation to produce intermediates that enter the tricarboxylic acid cycle. Anaerobic alkane degradation has received considerable attention in the last two decades (reviewed in Heider *et al.*, 1998; Widdel & Rabus, 2001; Boll *et al.*, 2002; Heider, 2006), mainly to document the occurrence of this metabolism in hydrocarbon-contaminated, oxygen-depleted environments and subsequently to develop strategies for the remediation of such ecosystems. As such, a handful of enrichments and

FEMS MICROBIOLOGY ECOLOGY

isolates capable of liquid C_6–C_{16} *n*-alkanes have been described (Aeckersberg *et al.*, 1991; Rueter *et al.*, 1994; So & Young, 1995; Caldwell *et al.*, 1998; Ehrenreich *et al.*, 2000; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2006; Callaghan *et al.*, 2009). The mechanisms by which such compounds are metabolized have also been actively researched during the last decade (So & Young, 1999; Kropp *et al.*, 2000; So *et al.*, 2003; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2006). Most studies have shown that *n*-alkanes are activated by fumarate addition to the corresponding alkylsuccinic acids in the initial metabolic step (Kropp *et al.*, 2000; Rabus *et al.*, 2001; Cravo-Laureau *et al.*, 2005; Callaghan *et al.*, 2006), although some cultures clearly activate *n*-alkanes by a different mechanism (e.g. carboxylation) (So *et al.*, 2003; Callaghan *et al.*, 2009).

With the exception of anaerobic methane oxidation (Boetius *et al.*, 2000; Raghoebarsing *et al.*, 2006; Beal *et al.*, 2009), the anaerobic metabolism of short-chain alkanes (e.g. C_2–C_5) has received far less attention. Such low-molecular-weight alkanes are present in significant quantities in hydrothermal, as well as other marine vents (e.g. hydrocarbon seeps in Gulf of Mexico, Guaymas basin) (Whelan *et al.*, 1988; Formolo *et al.*, 2004; Kniemeyer *et al.*, 2007) and in terrestrial habitats (e.g. natural gas fields of thermogenic origin) (Jarvie *et al.*, 2007; Kinnaman *et al.*, 2007; Mochimaru *et al.*, 2007). Recently, Kniemeyer *et al.* (2007) isolated a mesophilic Deltaproteobacterium capable of anaerobic, sulfate-dependent propane and butane metabolism, and described multiple sediment-free enrichments dominated by *Deltaproteobacteria* or *Firmicutes* that are capable of propane and butane degradation at 12 °C, and propane degradation at 60 °C, respectively. These cultures, derived from natural marine hydrocarbon seeps, were shown to metabolize the gaseous hydrocarbons by fumarate addition (Kniemeyer *et al.*, 2007). The identification of alkylsuccinates indicative of the anaerobic metabolism of C_1–C_4 alkanes in a hydrocarbon-contaminated aquifer or in oilfield fluids has also offered *in situ* evidence for this metabolism (Gieg & Suflita, 2002; Duncan *et al.*, 2009). However, little information is currently available on the global occurrence of this phenomenon (e.g. in terrestrial as opposed to marine habitats), the rate of degradation of such hydrocarbons, whether specific hydrocarbons are preferentially degraded, and the phylogenetic and metabolic diversity of the microorganisms capable of mediating such a process.

In Zodletone spring in southwestern Oklahoma, gaseous alkanes (methane, ethane, and propane) are continuously ejected from a deep underground formation, together with anaerobic, sulfide-rich ground water at the source of the spring. The spring source sediments thus represent an ideal habitat to examine the potential of nonmarine microbial communities to metabolize short-chain alkanes under anaerobic conditions. Here, we evaluated the ability of the native microbiota to degrade short-chain alkanes under sulfate-reducing conditions. We provide quantitative, isotopic, and metabolic evidence for the anaerobic degradation of short-chain alkanes, and identify microbial community members in the enrichments using 16S rRNA gene-based analysis.

Materials and methods

Site description

Zodletone spring is located in Kiowa County in southwestern Oklahoma. The spring flows for 20 m at a rate of approximately 8 L min^{-1}, where it then empties into nearby Saddle Mountain Creek (Younger, 1986). The spring is characterized by high concentrations of dissolved sulfide (8–10 mM at the source), which maintains anaerobic conditions throughout the spring. Sulfate is present at lower concentrations (about 50 μM) in source water. The source is a contained area (approximately 1 m^2) overlaid by water at a depth of about 50 cm and filled with biomass and soft sediments to a depth of at least 15 cm. A detailed site description of the spring geochemistry and microbial community can be found elsewhere (Havens, 1983; Sanders, 1998; Elshahed *et al.*, 2003; Senko *et al.*, 2004).

Enrichments' setup

Enrichment cultures were set up in an anaerobic glove bag (Coy, Grass Lake, MI) in 40-mL serum bottles using 10 g of source sediment and 15 mL of spring water under sulfate-reducing and methanogenic conditions. The ground water was buffered with sodium bicarbonate (0.04 M) and amended with 10 mL of vitamins (Tanner, 1989) and 10 mL of minerals (Brandis & Thauer, 1981; Odom & Wall, 1987). Ferrous sulfate (15 mM) was added only to the sulfate-reducing incubations. Bottles were sealed with Teflon-lined stoppers, and the headspace was flushed with N_2/CO_2 (80/20). The bottles were then amended with 4.0 mL (0.16 mmol) of ethane, propane, or butane or 1.0 μL (8.7 μmol) of neat liquid pentane (Sigma-Aldrich, St. Louis, MO), and incubated at room temperature (22 °C, approximating the *in situ* temperature of the Zodletone spring source) in the dark. Additional enrichments without the addition of a hydrocarbon substrate were maintained to account for background sulfate reduction and methane production. Enrichments showing that 80–90% of the alkane substrate was degraded and was subcultured by transferring 1.0 mL of liquid from the active enrichment into an anaerobic Balch tube containing 9.0 mL of an anaerobically prepared (Bryant, 1972; Balch & Wolfe, 1976) mineral medium containing (ml or g L^{-1}) NH_4Cl (0.27), KH_2PO_4 (0.03), NaCl (11.7), $MgCl_2 \cdot 6H_2O$ (1.22), $CaCl_2 \cdot 2H_2O$ (0.44), vitamin solution (6.25) (Tanner, 1989), mineral solution (6.25) (Brandis & Thauer, 1981;

Odom & Wall, 1987), and 0.1% resazurin (0.5), as well as ferrous sulfate (final concentration, 15 mM) to prevent possible inhibition of alkane-degradation activity due to sulfide toxicity (Beller & Reinhard, 1995). Transferred incubations were amended with an alkane substrate as described above. All bottles and tubes were monitored for sulfate and substrate loss as described below. Subcultured enrichments continuing to show alkane loss were then serially diluted (up to 10^{-5}).

Isotope fractionation

In order to unequivocally assess the biological transformation of propane under sulfate-reducing conditions, we determined the $\delta^{13}C$ value of propane gas used as a substrate (Sigma-Aldrich), and compared it with residual propane in propane-degrading enrichments, as well as the propane from sulfate-amended sterile controls and propane from live enrichments that were not amended with sulfate, and where no propane degradation was observed. $\delta^{13}C$ Isotope ratios were determined using GC and isotope ratio mass spectrometry (Finnigan MAT 252 IRMS) as described previously (Schoell, 1983; Sofer, 1984; Rodriguez Maiz & Philp, 2009).

Analytical methods

Substrate loss was monitored using a Shimadzu GC-14A GC (Shimadzu Scientific Instruments Columbia, MD), equipped with a Chemipak C-18 column (Sigma-Aldrich) and a flame ionization detector set at 125 °C. The oven temperature was set at 30 °C to measure ethane and propane and set at 100 °C to measure butane and pentane. The injector temperature was maintained at 100 °C. Sulfate loss was monitored using a Dionex DX500 ion chromatography system (Dionex Corp. Sunnyvale, CA) equipped with an AS4A column as described previously (Caldwell *et al.*, 1998).

Active subcultured enrichments were used for the detection of metabolic intermediates. Cultures (10.0 mL) were acidified to a pH < 2 using 12 N of HCl. Cultures were then extracted three times using 15 mL of ethyl acetate, and the combined organic layer was subsequently concentrated by rotary evaporation and under a stream of nitrogen to 0.2 mL. The concentrated extract was then derivatized using 50 μL of *N*, *O*-bis(trimethylsilyl)trifluoroacetimide according to the manufacturer's instructions (Pierce Chemicals, Rockford, IL). Potential metabolic intermediates in the concentrated and derivatized samples were identified using GC-MS on an Agilent HP 6890 GC and an Agilent 5973 MS as described in detail previously (Duncan *et al.*, 2009). Peaks were identified by either comparing their mass spectral profiles with chemical standards or by detailed examination of their mass spectral profile and characteristic fragmentation patterns as described previously (Gieg & Suflita, 2002; Rios-Hernandez *et al.*, 2003).

Community analysis

DNA was extracted from 1.5 mL of active diluted enrichments, using the MoBio Power Soil DNA Isolation Kit (MO BIO Laboratories Carlsbad, CA) according to the manufacturer's instructions. Bacterial 16S rRNA gene was amplified using 8F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 805R 5′-GACTACCAGGGTATCTAATCC-3′ primers as described previously (Elshahed *et al.*, 2003). Amplified products were then cloned into *Escherichia coli* using a TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA) and sequenced at Washington University at St. Louis Genome Center (St. Louis, MO).

The sequences obtained were initially compared with the GenBank nr database and checked using BLAST (Altschul *et al.*, 1990). Sequences with more than 97% similarity were considered to be of the same operational taxonomic unit (OTU). Zodletone sequences and GenBank-downloaded sequences were aligned using the CLUSTALX program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using representatives of closely related reference sequences to highlight the phylogenetic affiliation of clones obtained in this study. Evolutionary distance trees were constructed using PAUP (Version 4.01b10; Sinauer associates, Sunderland, MA).

Nucleotide sequences accession numbers

The sequences generated in this study were deposited in GenBank under accession numbers GU211106–GU211166.

Results and discussion

Hydrocarbon biodegradation

Enrichments for ethane, propane, butane, and pentane biodegradation were constructed using Zodletone spring source sediment and ground water under methanogenic and sulfate-reducing conditions. No appreciable substrate loss was observed in any of the methanogenic enrichments, even though the bioconversions of all tested *n*-alkanes to methane are energetically favorable reactions (e.g. $\Delta G^{\circ\prime}$ for ethane is $-34.5\,kJ\,mol^{-1}$; that for pentane is $-100\,kJ\,mol^{-1}$). In the sulfate-reducing enrichments, no appreciable ethane or butane loss was observed (data not shown). Significant loss ($> 70\%$) of the substrate was observed only in propane- and pentane-amended enrichments and we thus focused on these enrichments for further study.

In initial enrichments under sulfate-reducing conditions, *n*-propane degradation commenced after approximately 80 days of incubation, and the majority of added propane

(> 90%) was metabolized after 467 days (approximately 0.125 mmol consumed) (Fig. 1a). Sulfate loss was observed in both the initial propane-amended and the substrate-unamended incubations (0.571 and 0.364 mmol, respectively). This phenomenon was reported previously during the initial enrichment of environmental samples on hydrocarbon substrates (Gieg *et al.*, 1999) and is likely due to the metabolism of other substrates present in the initial sediment material. Thus, in the initial propane-amended enrichments, the net sulfate reduction was about 67% (0.2 mmol net sulfate lost) of the theoretical value expected (0.31 mmol) from the sulfate-dependant complete oxidation of propane to CO_2 according to the following equation (after Kniemeyer *et al.*, 2007):

$$C_3H_8 + 2.5\,SO_4^{2-} + 2\,H^+ \rightarrow 3\,HCO_3^- + 2.5\,H_2S + H_2O$$
$$(\Delta G^{\circ\prime} = -102\,\text{kJ mol}^{-1}\text{ propane}) \quad (1)$$

These results agree with previous reports indicating that 70–90% of a hydrocarbon is typically used for energy production, while about 30% is used for biomass (Jahn *et al.*, 2005). The correlation of propane degradation coupled with sulfate reduction in the initial incubations was confirmed as propane and sulfate losses were again observed following a transfer (10% v/v) of the culture. In the subcultures, approximately 70% (0.03 ± 0.01 mmol) of the propane added was lost after 286 days of incubation. The stoichiometry of the mol sulfate reduced per mol propane lost (after correction for sulfate loss in substrate-unamended controls) was 3.0 mmol of sulfate mmol^{-1} propane, representing approximately 120% of the theoretically expected value. These subcultures were evaluated for metabolic byproducts, and replicates were further serially diluted up to 10^{-5} to obtain sediment-free cultures. After 124 days of incubation, approximately 50% of the added propane was lost in the 10^{-1} dilution and this sample was used for microbial community analysis (discussed below).

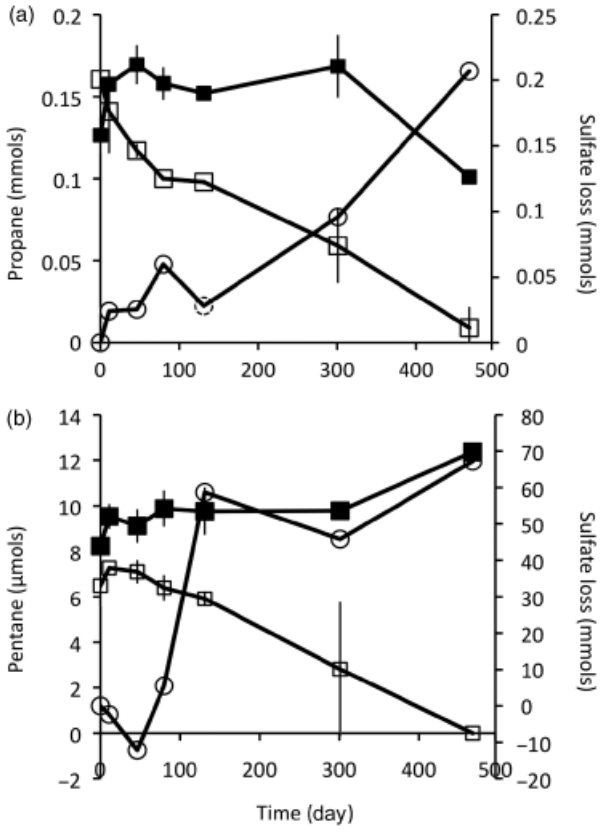

Fig. 1. *n*-Propane and *n*-pentane degradation under sulfate-reducing conditions in propane (a) and pentane (b) enrichments from Zodletone spring source sediments and ground water. Substrate concentration in active enrichments (-□-); substrate concentration in sterile controls (-■-); net sulfate loss (-O-).

For the initial *n*-pentane-amended incubations, the lag time before the onset of degradation was almost the same as for the propane-amended incubations (~80 days). In one replicate incubation, pentane was 90% metabolized after 301 days (giving rise to the high degree of error among the replicates for pentane measurements at 301 days; Fig. 1b). After 467 days, approximately 7 µmol pentane had been consumed in the initial incubations. As with the propane-amended enrichments, sulfate reduction was observed in both pentane-amended and substrate-unamended incubations (0.432 and 0.364 mmol, respectively). Again, background sulfate reduction was likely due to the presence of other degradable substrates in the sediments used for incubations. The net sulfate reduction (0.07 mmol) in this case was greater than (~10 mol sulfate mol^{-1} pentane) the theoretical value expected (4 mol sulfate mol^{-1} pentane) from the sulfate-dependant complete oxidation of pentane to CO_2 according to the following equation:

$$C_5H_{12} + 4\,SO_4^{2-} + 3\,H^+ \rightarrow 5\,HCO_3^- + 4\,H_2S + H_2O$$
$$(\Delta G^{\circ\prime} = -191\,\text{kJ per mol pentane}) \quad (2)$$

Despite the incongruent mass balance in the initial incubations, pentane degradation coupled with sulfate reduction could be confirmed by the continued activity upon subculturing. Pentane-amended subcultures (10% v/v) consumed 85% (3.9 ± 1.2 µmol) of the pentane added after 390 days of incubation (data not shown). The corrected mol sulfate per mol pentane stoichiometry was ~7 : 1, which was still in excess of the theoretically expected value (4 : 1) according to Eqn. (2), and suggested that other substrates were also transferred along with the initial inoculum. It was clear, though, that pentane was consumed and the subcultures were examined for the presence of known anaerobic pentane metabolites.

Serial dilution of successful replicate pentane subcultures was conducted to obtain sediment-free enrichments and pentane degradation ensued. After 471 days, > 80% of added pentane was degraded in the 10^{-3} dilution, which was subjected to 16S rRNA gene-based community analysis (discussed below).

Isotopic fractionation in propane-degrading sulfate-reducing enrichments

Because of the relatively long time period observed for complete propane degradation (approximately 1.3 years, Fig. 1) and the uncertainty associated with the propane/sulfate mass balance calculations, we sought to further ascertain the biological origin of propane degradation in sulfate-reducing enrichments by isotopic fractionation. Biological degradation of organic compounds results in the enrichment of the heavy carbon isotope ^{13}C in the residual substrate, expressed as $\delta^{13}C$ (Ahad *et al.*, 2000; Meckenstock *et al.*, 2004). This occurs as bonds between heavier isotopes are slightly more difficult to cleave (Hoefs, 2009). Enrichment of $\delta^{13}C$ can thus be used to definitively demonstrate the degradation of organic compounds. The experiments required appropriate controls. The residual propane in the subcultured, propane-degrading enrichments was found to be enriched in the heavy carbon isotope [$\delta C^{13} = -16.03‰$ (± 2.00)] compared with propane used as the substrate [$\delta^{13}C = -22.75‰$ (± 0.92)] (Table 1), attesting to its biological transformation. Furthermore, analysis of the isotopic ratio of CO_2 after incubation of the active propane-degrading enrichment indicated that carbon dioxide was further enriched in ^{12}C. (Table 1), providing evidence that the propane was being mineralized to light carbon dioxide.

Activation of *n*-propane through the formation of alkylsuccinate intermediates

We tested for the presence of potential anaerobic intermediates of propane and pentane metabolism by GC-MS. Two compounds eluting within a minute of each other were detected in propane-degrading enrichments with mass spectral features consistent with those of *n*-propylsuccinic acid and isopropylsuccinic acid (Fig. 2). These metabolites were not detected in substrate-unamended controls (data not shown). The MS profiles of both compounds showed nearly identical ion fragmentation patterns with a characteristic M^+-15 ion of 289 and other ions distinctive for alkylsuccinates (*m*/*z* 262, 217, 172, 147, and 73) (Gieg & Suflita, 2002; Rios-Hernandez *et al.*, 2003). The identity of *n*-propylsuccinic acid in the propane-amended culture extracts was confirmed by comparing its GC-MS profile with that of a TMS-derivatized standard. In a propane-degrading culture enriched from marine sediments, both isomers of propylsuccinic acid were also detected (Kniemeyer *et al.*, 2007). These findings, coupled with the detection of propylsuccinic acid in other petroleum-laden environments (Gieg & Suflita, 2002; Duncan *et al.*, 2009), suggest that fumarate addition is a key mechanism for the activation of *n*-propane in disparate anaerobic environments. Although further downstream metabolites (Kniemeyer *et al.*, 2007) were not detected, the propylsuccinic acids were likely further metabolized to lower molecular weight products that could enter into central metabolic pathways and ultimately drive energy production.

Comparable pentylsuccinate formation was not found when the pentane-degrading enrichments were similarly analyzed by GC-MS nor could other predicted metabolites be detected (such as those that might arise from carboxylation). It remains unclear whether the formation of diagnostic metabolites was simply below the detection limits of the analytical procedures or whether pentane utilization was by an alternate, unknown mechanism.

Microbial community patterns in propane- and pentane-degrading enrichments

16S rRNA gene-based analysis was conducted to identify the prominent bacterial taxa within the propane- and pentane-degrading enrichments. A total of 63 and 67 clones were sequenced from the propane- and pentane-degrading enrichments, respectively.

Members of the *Deltaproteobacteria* dominated both propane and pentane clone libraries (68.2% and 79.8% of clones, respectively), followed by members of *Chloroflexi*, *Firmicutes*, and candidate division MBMPE71 (Table 2, Fig. 3).

Table 1. Differences in the carbon isotope ratios ($\delta^{13}C$) of propane and carbon dioxide in active propane-degrading, sulfate-amended enrichments compared with the propane (from tank), propane-fed sterile control, and a propane-fed enrichment without sulfate

	$\delta^{13}C$ (‰)			
Enrichment conditions	Propane	$\Delta\delta^{13}C$	Carbon dioxide	$\Delta\delta^{13}C$
Propane	−22.75 (±0.92)	–	–	–
Sterile control	−23.28 (±0.54)	−0.53	−10.58 (±0.22)	–
Live enrichment (−sulfate)	−22.35 (±0.35)	+0.40	−13.65 (±0.21)	−3.07
Live enrichment (+sulfate)	−16.03 (±2.00)	+6.72	−17.23 (±0.15)	−6.65

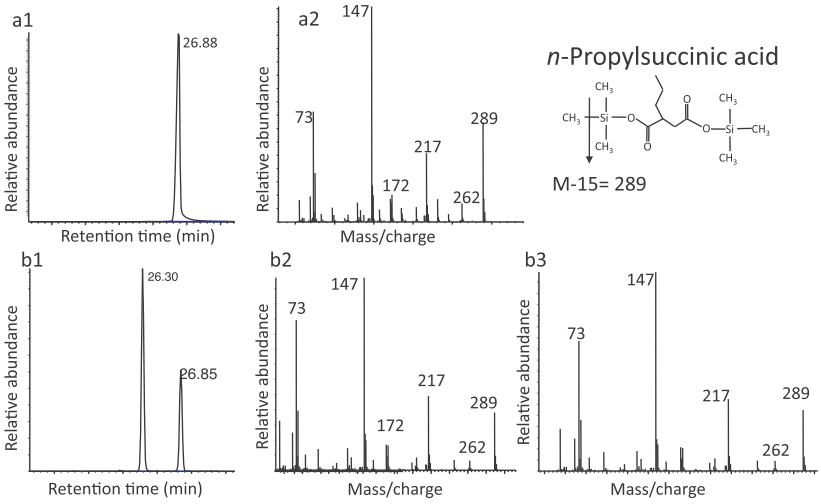

Fig. 2. GC-MS analysis of an *n*-propylsuccinic acid standard and of propane-degrading enrichment culture extracts. (a1) GC partial ion chromatogram following selection for the *m/z* 289 ion of an *n*-propylsuccinic acid standard, (a2) MS of *n*-propylsuccinic acid (retention time, 26.88 min). (b1) GC partial ion chromatogram following selection for the *m/z* 289 ion in the propane-degrading culture extract, (b2) MS for *n*-propylsuccinic acid in the culture extract (retention time, 26.85 min), (b3) MS for isopropylsuccinic acid in the culture extract (retention time, 26.30 min).

Table 2. Community composition of subcultured propane (10^{-1})- and pentane (10^{-3})-degrading enrichments

Putative phylum assignment	Propane-degrading enrichment (total number of clones)*	Pentane-degrading enrichment (total number of clones)*
Deltaproteobacteria	43	54
Desulfovibrionaceae	11	18
Desulfobacteraceae	17	24
Syntrophaceae	10	4
Desulfarculaceae	–	8
Desulfobulbaceae	2	–
Desulfuromonaceae	3	–
Firmicutes	5	2
Clostridiaceae	1	–
Peptococcaceae	4	1
Thermoanaerobacterales	–	1
Chloroflexi	9	4
Caldineaceae	9	4
Other	6	7
MBMPE71	1	4
Gammaproteobacteria	2	1
Bacteroidetes	1	–
Deferribacteres	1	1
Planctomycetes	1	1

*A total of 63 and 67 clones were analyzed in the propane- and pentane-degrading enrichments, respectively.

Collectively, these four phyla constituted 92% and 95.6% of clones in the propane and pentane clone libraries, respectively (Table 2). The Deltaproteobacterial OTUs identified in both enrichments could be assigned to one of four categories: close relatedness to clones from hydrocarbon-associated environments and/or known hydrocarbon degraders, volatile fatty acid (VFA) metabolizers, hydrogen metabolizers, and representatives of a novel Deltaproteobacterial lineage.

Within the propane-degrading enrichments, multiple OTU sequences, designated 'gaseous alkane associated' in Fig. 3, belonged to the *Desulfococcus–Desulfonema–Desulfosarcina* cluster within the family *Desulfobacteraceae*. Specifically, these sequences formed a phylogenetically coherent lineage with a large number of clones identified in studies of globally distributed gaseous hydrocarbon-impacted marine ecosystems including hydrocarbon seeps in the Gulf of Mexico (GenBank accession numbers AM745215, AM745148, AM745130), gas hydrates off the Oregon Coast (Knittel *et al.*, 2003), deep-sea mud volcanoes in the eastern Mediterranean (GenBank accession number AY592396), and in a Japanese trench (Inagaki *et al.*, 2002). To our knowledge, this is the first report that identifies members of this lineage in a nonmarine environment. The sequenced clones from our enrichment culture were only distantly related to the only known pure culture capable of sulfidogenic propane and butane degradation (isolate BuS5; GenBank accession number EF077225, 88.2–95.2% similar) and to clone Butane12-GMe, the most dominant sequence in a cold-adapted butane-degrading enrichment culture from the Gulf of Mexico (GenBank accession number EF077226) (Kniemeyer *et al.*, 2007) (Fig. 3).

Relatives of known hydrocarbon degraders in our pentane-degrading enrichments were closely related to a different lineage within the *Desulfococcus–Desulfonema–Desulfosarcina* cluster in the family *Desulfobacteraceae*. The closest cultured relative to these OTUs (designated 'liquid alkane associated' in Fig. 3) was the 16S rRNA gene of *Desulfococcus oleovorans* strain Hxd3 (95% sequence similarity), with the next closest relatives being $< 88\%$ similar. *Desulfococcus oleovorans* strain Hxd3 is the first described hydrocarbon-degrading sulfate reducer, and is capable of degrading C_{12}–C_{20} *n*-alkanes (Aeckersberg *et al.*, 1991, 1998). Interestingly, strain Hxd3 is one of the few hydrocarbon-degrading isolates thought to use

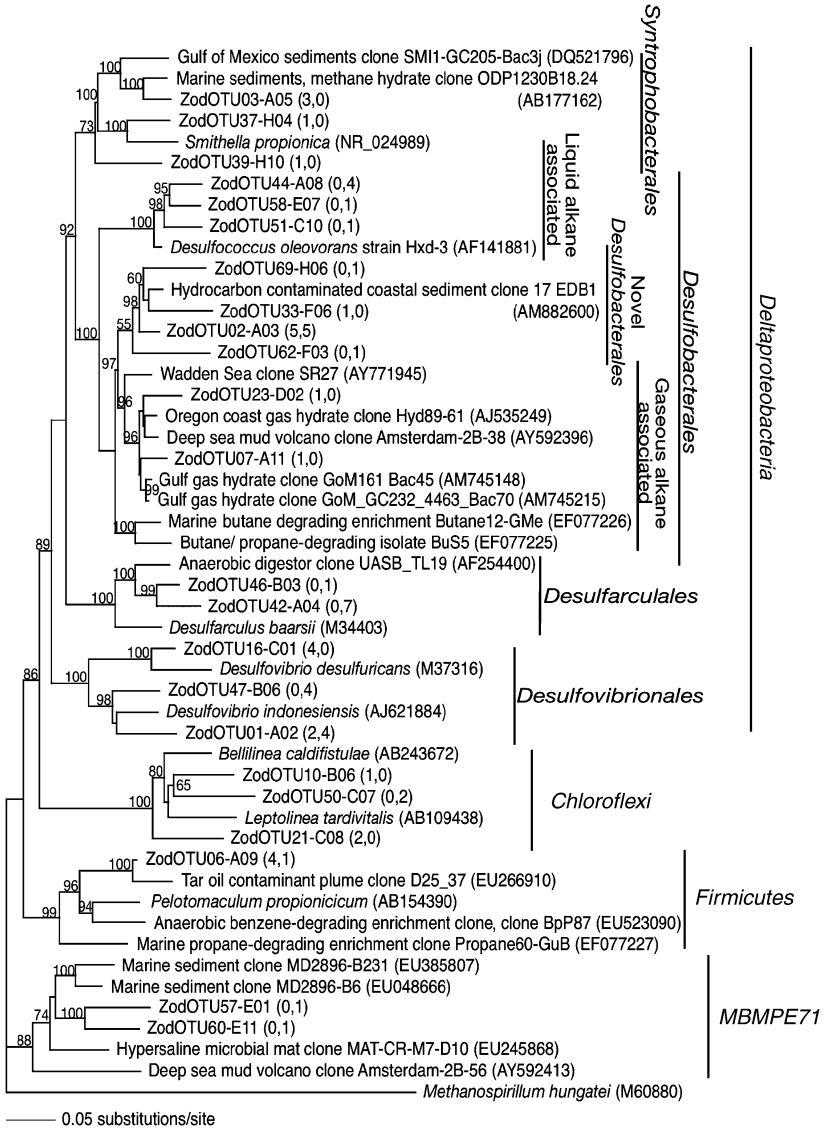

Fig. 3. Distance neighbor-joining tree based on the 16S rRNA gene sequences identified in both the propane and the pentane-degrading sulfate-reducing enrichments from Zodletone spring sediments. Only selected OTUs are included in the tree, and a total inventory of all the sequences identified are presented in Table 2. Bootstrap values (expressed as percentages) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. Sequences retrieved in this study are labeled 'Zod,' and the frequencies of the occurrence of each OTU in the propane- and pentane-degrading enrichments, respectively, are indicated in parenthesis (out of a total of 63 clones from the propane enrichment and 69 clones in the pentane enrichment).

carboxylation instead of fumarate addition to activate hydrocarbons (So *et al.*, 2003).

The OTUs affiliating with VFA-metabolizing lineages within both enrichments were also phylogenetically distinct. Within the propane-using enrichment, relatives of the propionate-degrading bacterium *Smithella propionica* (95%) were identified (order *Syntrophobacterales*, Fig. 3). *Smithella propionica* is a propionate-utilizing bacterium that can grow syntrophically in coculture with a hydrogen-utilizing sulfate reducer (Liu *et al.*, 1999). Within the pentane-degrading enrichments, close relatives of *Desulfarculus baarsii* (order *Desulfoarculales*, Fig. 3) were observed. *Desulfarculus baarsii* is capable of incomplete degradation of higher fatty acids including long-chain fatty acids as well as pentanoic acid (Kuever *et al.*, 2001). The identification of such known fatty acid-degrading microorganisms is not unexpected in anaerobic environments where fatty acid production occurs via the decomposition of organic matter that can include hydrocarbons.

The remaining sequences identified in both enrichments were either members of the order *Desulfovibrionales* or belonged to novel lineages within the family *Desulfobacteraceae* within the *Deltaproteobacteria*. *Desulfovibrionales* are well-known H_2-using sulfate reducers that are ubiquitous in nature (Kuever *et al.*, 2005). The novel *Desulfobacteraceae*-associated lineage (represented by OTUs Zod02-A03, Zod33-F06, Zod62-F03, and Zod69-H06, novel *Desulfobacterales*, Fig. 3) has no pure culture representative, and Zodletone OTUs belonging to this lineage had 91–96% sequence similarity to clones identified from hydrocarbon-impacted

marine ecosystems (Mussmann *et al.*, 2005; Paissé *et al.*, 2008). Members of this lineage were identified in both the propane- and pentane-degrading enrichments (Fig. 3).

In addition to *Deltaproteobacteria*, members of the *Chloroflexi*, *Firmicutes*, and Candidate division MBMPE71 were found in both enrichments. Members of the phylum *Firmicutes* comprised 6.3% of the sequenced clones in the propane-fed enrichments, but only 1.6% of the sequenced clones in the pentane-fed enrichments. In general, the majority of clones belonged to the mostly sulfate-reducing *Desulfotomaculum–-Pelotomaculum* group within the *Peptococcaceae*. Sequences from the propane-degrading enrichments belonging to this group (e.g. Zod06-A09; Fig. 3) were closely related (98% similar) to clones from an oil contaminant plume (Winderl *et al.*, 2008) and only distantly related (89% similarity) to the dominant phylotype in a thermophilic propane-degrading enrichment, propane60-GuB (Kniemeyer *et al.*, 2007).

Chloroflexi OTUs identified in both enrichments were either close relatives of known anaerobic fermenters, for example *Bellilinea caldifistulae* (Yamada *et al.*, 2007) and *Leptolinea tardivitalis* (Yamada *et al.*, 2006) within the order *Anaerolineales* or belonged to uncultured bacterial lineages with clones originating from hydrocarbon-impacted environments (Elshahed *et al.*, 2003) or hydrocarbon-degrading enrichments (Ficker *et al.*, 1999). Sequences belonging to candidate division MBMPE71, in which several OTUs from propane- and pentane-degrading enrichments were identified, are mainly from hypersaline microbial mats (Senbarger *et al.*, 2008), marine sediments (Li *et al.*, 2008), and deep mud volcano Mediterranean sediments (GenBank accession number AY592413).

Using sediments from a terrestrial, anaerobic, low-molecular-weight hydrocarbon seep, we were able to establish enrichment cultures capable of utilizing *n*-propane and *n*-pentane under sulfate-reducing conditions. This is only the second report of such a metabolism and documents the first successful cultivation of microorganisms with such activity from a terrestrial hydrocarbon seep. *n*-Propane degradation was found to proceed via fumarate addition, confirming previous reports of this metabolism by another sulfate-reducing culture (Kniemeyer *et al.*, 2007) and in other terrestrial environments (Gieg & Suflita, 2002; Duncan *et al.*, 2009). Further, microbial community members in the enrichment cultures were identified by 16S rRNA gene sequencing and were found to affiliate with clones retrieved from other hydrocarbon-associated ecosystems or with species that use VFA or H_2 as substrates. However, several of the clones were only distantly related to other known low-molecular-weight alkane-utilizing species (Kniemeyer *et al.*, 2007), attesting to the diversity of organisms associated with such activity. Clearly, from such data, we can only speculate on the roles played by the identified species in our enrichments and further work using other molecular biology based tools (e.g. stable isotope probing) will be necessary to clearly delineate the metabolic functions of the identified species in the *n*-propane- and *n*-pentane-utilizing cultures.

Although hydrocarbon seeps are numerous and widespread (Macdonald *et al.*, 2002), documenting the metabolism of hydrocarbons in such ecosystems has likely remained challenging due to the fastidiousness and slow-growing nature of the associated microbial communities retrieved from such environments. In seminal work involving sulfidogenic propane and butane metabolism using marine-based hydrocarbon seep sediments, Kniemeyer *et al.* (2007) reported incubation times of over a year for some initial incubations, similar to our findings in this work, and doubling times of 4–5 days for established laboratory cultures. Thermodynamic calculations show that *n*-propane and *n*-pentane metabolism is energetically favorable under sulfate-reducing conditions [Eqns (1) and (2)] and such metabolism is thought to explain the disappearance of such compounds along with the formation of methane in many natural marine environments such as gas hydrates (Kniemeyer *et al.*, 2007). Thus, documenting the occurrence of similar processes in terrestrial environments can help provide a microbiological explanation of the various geochemical patterns and processes observed within hydrocarbon-rich terrestrial ecosystems (Kniemeyer *et al.*, 2007) such as the detection of metabolites of gaseous alkane degradation in polluted aquifers (Gieg & Suflita, 2002) and in onshore oil reservoirs (Duncan *et al.*, 2009), the biological source of methane often associated with dry gas caps overlying petroleum reservoirs (Head *et al.*, 2003), and the differences in gas composition in thermogenic and biogenic natural gas reservoirs (Hunt, 1995; Mochimaru *et al.*, 2007).

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References

Aeckersberg F, Bak F & Widdel F (1991) Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch Microbiol* **156**: 5–14.

Aeckersberg F, Rainey FA & Widdel F (1998) Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. *Arch Microbiol* **170**: 361–369.

Ahad JME, Sherwood Lollar B, Edwards EA, Slater GF & Sleep BE (2000) Carbon isotope fractionation during anaerobic biodegradation of toluene: implications for intrinsic bioremediation. *Environ Sci Technol* **34**: 892–896.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Balch WE & Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl Environ Microb* **32**: 781–791.

Beal EJ, House CH & Orphan VJ (2009) Manganese- and iron-dependent marine methane oxidation. *Science* **325**: 184–187.

Beller HR & Reinhard M (1995) The role of iron in enhancing anaerobic toluene degradation in sulfate-reducing enrichment cultures. *Microb Ecol* **30**: 105–114.

Berthe-Corti L & Fetzner S (2002) Bacterial metabolism of *n*-alkanes and ammonia under oxic, suboxic and anoxic conditions. *Acta Biotechnol* **22**: 299–336.

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jorgensen BB, Witte U & Pfannkuche O (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623–626.

Boll M, Fuchs G & Heider J (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* **6**: 604–611.

Brandis A & Thauer RK (1981) Growth of *Desulfovibrio* species on hydrogen and sulphate as sole energy source. *J Gen Microbiol* **126**: 249–252.

Bryant MP (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr* **25**: 1324–1328.

Caldwell ME, Garrett RM, Prince RC & Suflita JM (1998) Anaerobic biodegradation of long chain *n*-alkanes under sulfate-reducing conditions. *Environ Sci Technol* **32**: 2191–2195.

Callaghan AV, Gieg LM, Kropp KG, Suflita JM & Young LY (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl Environ Microb* **72**: 4274–4282.

Callaghan AV, Tierney M, Phelps CD & Young LY (2009) Anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium. *Appl Environ Microb* **75**: 1339–1344.

Clifton CG, Walters CC & Simoneit BRT (1990) Hydrothermal petroleums from Yellowstone National Park, Wyoming, USA. *Appl Geochem* **5**: 169–191.

Cravo-Laureau C, Grossi V, Raphel D, Matheron R & Hirschler-Rea A (2005) Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aiphaticivorans* Strain Cv2803t. *Appl Environ Microb* **71**: 3458–3467.

Davidova IA, Duncan KE, Choi OK & Suflita JM (2006) *Desulfoglaeba alkanexedens* gen. nov., sp. nov., an *n*-alkane-degrading, sulfate-reducing bacterium. *Int J Syst Evol Micr* **56**: 2737–2742.

Duncan KE, Gieg LM, Parisi VA, Tanner RS, Tringe SG, Bristow J & Suflita JM (2009) Biocorrosive thermophilic microbial communities in Alaskan north slope oil facilities. *Environ Sci Technol* **43**: 7977–7984.

Ehrenreich P, Behrends A, Harder J & Widdel F (2000) Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. *Arch Microbiol* **173**: 58–64.

Elshahed MS, Senko JM, Najar FZ, Kenton SM, Roe BA, Dewers TA, Spear JR & Krumholz LR (2003) Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl Environ Microb* **69**: 5609–5621.

Ficker M, Krastel K, Orlicky S & Edwards EA (1999) Molecular characterization of a toluene-degrading methanogenic consortium. *Appl Environ Microb* **65**: 5576–5585.

Formolo MJ, Lyons TW, Zhang C, Kelley C, Sassen R, Horita J & Cole DR (2004) Quantifying carbon sources in the formation of authigenic carbonates at gas hydrate sites in the Gulf of Mexico. *Chem Geol* **205**: 253–264.

Gieg LM & Suflita JM (2002) Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. *Environ Sci Technol* **36**: 3755–3762.

Gieg LM, Kolhatkar RV, McInerney MJ, Tanner RS, Harris SH Jr, Sublette KL & Suflita JM (1999) Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. *Environ Sci Technol* **33**: 2550–2560.

Hanson RS & Hanson TE (1996) Methanotrophic bacteria. *Microbiol Rev* **60**: 439–471.

Havens JS (1983) Reconnaissance of ground water in the vicinity of the Wichita Mountains, southwestern Oklahoma. Oklahoma Geological Survey Circular, Vol. 85, Oklahoma Geological Survey, Norman, OK.

Head IM, Jone DM & Larter SM (2003) Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **426**: 344–352.

Heider J (2006) Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr Opin Chem Biol* **11**: 188–194.

Heider J, Spormann AM, Beller HR & Widdel F (1998) Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* **22**: 459–473.

Hoefs J (2009) *Stable Isotope Geochemistry*. Springer-Verlag, Berlin.

Hunt JM (1995) *Petroleum Geochemistry and Geology*. W.H. Freeman and Co., New York.

Inagaki F, Sakihama Y, Inoue A, Kato C & Horikoshi K (2002) Molecular phylogenetic analyses of reverse-transcribed bacterial rRNA obtained from deep-sea cold seep sediments. *Environ Microbiol* **4**: 277–286.

Jahn MK, Haderlein SB & Meckenstock RU (2005) Anaerobic degradation of benzene, toluene, ethylbenzene, and *o*-xylene in sediment-free iron-reducing enrichment cultures. *Appl Environ Microb* **71**: 3355–3358.

Jarvie DM, Hill RJ, Ruble TE & Pollastro RM (2007) Unconventional shale-gas systems: the Mississippian barnett shale of north-central Texas as one model for thermogenic-gas assessment. *AAPG Bull* **91**: 475–499.

Kinnaman FS, Valentine DL & Tyler SC (2007) Carbon and hydrogen isotope fractionation associated with the aerobic microbial oxidation of methane, ethane, propane, and butane. *Geochem Cosmochem Acta* **71**: 271–283.

Kniemeyer O, Musa F, Sievert SM *et al.* (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulfate-reducing bacteria. *Nature* **449**: 898–902.

Knittel K, Boetius A, Lemke A, Eilers H, Lochte K, Pfannkuche O, Linke P & Amann R (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia Margin, Oregon). *Geomicrobiol J* **20**: 269–294.

Kropp KG, Davidova IA & Suflita JM (2000) Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl Environ Microb* **66**: 5393–5398.

Kuever J, Rainey FA & Widdel F (2001) Genus *Desulfarculus*. *Bergeys Manual of Systematic Bacteriology*, *Vol. 2*, 2nd edn (Brenner DJ & Staley JT, eds), pp. 1004–1005. Springer, New York, USA.

Kuever J, Rainey F & Widdel F (2005) Genus *Desulfovibrio*. *Bergeys Manual of Systematic Bacteriology*, *Vol. 2*, 2nd edn (Brenner DJ & Staley JT, eds), pp. 926–927. Springer, New York, USA.

Kvenvolden KA (1995) A review of the geochemistry of methane in natural gas hydrate. *Org Geochem* **23**: 997–1008.

Leahy JG & Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. *Microbiol Mol Biol R* **54**: 305–315.

Li T, Wang P & Wang PX (2008) Bacterial and archaeal diversity in surface sediments from the south slope of the South China Sea. *Acta Microbiol Sin* **48**: 323–329.

Liu Y, Balkwill DL, Aldrich HC, Drake GR & Boone DR (1999) Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. *Int J Syst Bacteriol* **49**: 545–556.

Macdonald IR, Leifer I, Sassen R, Stine P, Mitchell R & Guinasso N Jr (2002) Transfer of hydrocarbons from natural seeps to the water column and atmosphere. *Geofluids* **2**: 95–107.

Meckenstock RU, Safinowski M & Griebler C (2004) Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Lett* **49**: 27–36.

Mochimaru H, Yoshioka H, Tamaki H, Nakamura K, Kaneko N, Sakata S, Imachi H, Sekiguchi Y, Uchiyama H & Kamagata Y (2007) Microbial diversity and methanogenic potential in a high temperature natural gas field in Japan. *Extremophiles* **11**: 453–461.

Mussmann M, Ishii K, Rabus R & Amann R (2005) Diversity and vertical distribution of cultured and uncultured *Deltaproteobacteria* in an intertidal mud flat of the Wadden Sea. *Environ Microbiol* **7**: 405–418.

Odom JM & Wall JD (1987) Properties of a hydrogen-inhibited mutant of *Desulfovibrio desulfuricans* ATCC 27774. *J Bacteriol* **169**: 1335–1337.

Paissé S, Coulon F, Goñi-Urriza M, Peperzak L, McGenity TJ & Duran R (2008) Structure of bacterial communities along a hydrocarbon contamination gradient in a coastal sediment. *FEMS Microbiol Ecol* **66**: 295–305.

Philp RP (2005) High temperature gas chromatography for the analysis of fossil fuels: a review. *J High Res Chrom* **17**: 398–406.

Rabus R, Wilkes H, Behrends A, Armstroff A, Fischer T, Pierik AJ & Widdel F (2001) Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: evidence for (1-methypentyl)succinate as initial product and for involvement of an organic radical in n-hexane metabolism. *J Bacteriol* **183**: 1707–1715.

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT *et al.* (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918–921.

Rios-Hernandez LA, Gieg LM & Suflita JM (2003) Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. *Appl Environ Microb* **69**: 434–443.

Rodriguez Maiz ND & Philp RP (2010) Geochemical characterization of gases from the Mississippian Barnett Shale, Fort Worth Basin, Texas. *AAPG Bull*, in press.

Rueter P, Rabus R, Wilkest H, Aeckersberg F, Rainy FA, Jannasch HW & Widdel F (1994) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature* **372**: 455–458.

Sanders WE (1998) Rate and mechanism of barite mineralization at Zodletone Mountain, Southwestern Oklahoma. MS Thesis, University of Oklahoma, Norman, OK.

Schoell M (1983) Genetic characterization of natural gases. *Am Assoc Pet Geol Bull* **67**: 2225–2238.

Senbarger TA, Finney M, Rios-Velazquez C, Handelsman J & Ruvkun G (2008) Miniprimer PCR, a new lens for viewing the microbial world. *Appl Environ Microb* **74**: 840–849.

Senko JM, Campbell BS, Henriksen JR, Elshahed MS, Dewers TA & Krumholz LR (2004) Barite deposition resulting from phototrophic sulfide-oxidizing bacterial activity. *Geochim Cosmochim Ac* **68**: 773–780.

So CM & Young LY (1995) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. *Appl Environ Microb* **65**: 2969–2976.

So CM & Young LY (1999) Initial reactions in anaerobic alkane degradation by a sulfate-reducer, Strain AK-01. *Appl Environ Microb* **65**: 5532–5540.

So CM, Phelps CD & Young LY (2003) Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, Strain HxD3. *Appl Environ Microb* **69**: 3892–3900.

Sofer Z (1984) Stable carbon isotope composition of crude oils: application to source depositional environment and petroleum alteration. *AAPG Bull* **68**: 31–49.

Tanner RS (1989) Monitoring sulfate-reducing bacteria: comparison of enumeration media. *J Microbiol Meth* **10**: 83–90.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.

Van Hamme JD, Singh A & Ward OP (2003) Recent advances in petroleum microbiology. *Microbiol Mol Biol R* **67**: 503–549.

Wahlen M (1993) The global methane cycle. *Annu Rev Earth Pl Sc* **21**: 407–426.

Whelan JK, Simoneit BRT & Tarafa ME (1988) C1–C8 hydrocarbons in sediments from Guaymas Basin, Gulf of California – comparison to Peru Margin, Japan Trench, and California Border Lands. *Org Geochem* **12**: 171–194.

Widdel F & Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotech* **12**: 259–276.

Winderl C, Anneser B, Griebler C, Meckenstock RU & Lueders T (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community in distinct redox zones of a tar oil contaminant plume. *Appl Environ Microb* **74**: 792–801.

Yamada T, Sekiguchi Y, Hanada S, Imachi H, Ohashi A, Harada H & Kamagata Y (2006) *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of then classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*. *Int J Syst Evol Micr* **56**: 1331–1340.

Yamada T, Imachi H, Ohashi A, Harada H, Hanada S, Kamagata Y & Sekiguchi Y (2007) *Bellilinea caldifistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum *Chloroflexi* isolated from methanogenic propionate-degrading consortia. *Int J Syst Evol Micr* **57**: 2299–2306.

Younger P (1986) Barite travertine at from southwestern Oklahoma and western-central, Colorado. MS Thesis, Oklahoma State University, Stillwater, OK.