Methanogenic biodegradation of C\textsubscript{9} to C\textsubscript{12} n-alkanes initiated by \emph{Smithella} via fumarate addition mechanism

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Abstract

In the present study, a methanogenic alkane-degrading (a mixture of C\textsubscript{9} to C\textsubscript{12} n-alkanes) culture enriched from production water of a low-temperature oil reservoir was established and assessed. Significant methane production was detected in the alkane-amended enrichment cultures compared with alkane-free controls over an incubation period of 1 year. At the end of the incubation, fumarate addition metabolites (C\textsubscript{9} to C\textsubscript{12} alkylsuccinates) and \emph{assA} genes (encoding the alpha subunit of alkylsuccinate synthase) were detected only in the alkane-amended enrichment cultures. Microbial community analysis showed that putative syntrophic n-alkane degraders (\emph{Smithella}) capable of initiating n-alkanes by fumarate addition mechanism were enriched in the alkane-amended enrichment cultures. In addition, both hydrogenotrophic (\emph{Methanocalculus}) and acetoclastic (\emph{Methanothrix}) methanogens were also observed. Our results provide further evidence that alkanes can be activated by addition to fumarate under methanogenic conditions.

Keywords: Alkanes, Alkylsuccinates, Fumarate addition, Methanogenesis, Oil reservoirs

Introduction

Methanogenic biodegradation of crude oil is a prevalent process occurring in subsurface petroleum reservoirs and has adverse effect on oil quality (Head et al. 2003, 2006; Jones et al. 2008). However, it has been postulated that methanogenic crude oil degradation can be applied for energy recovery in depleted petroleum reservoirs by bio-conversion of residual oil to methane (Gieg et al. 2008). In addition to energy recovery, methanogenic degradation of crude oil is also a major process for bioremediation in the oil-contaminated environments after the depletion of electron acceptors (Amos et al. 2005; Feisthauer et al. 2010, 2012).

\textsuperscript{n}Alkanes are the major constituents of crude oil and also the significant contaminants in oil-polluted environments. Methanogenic biodegradation of n-alkanes requires the initial activation of these substrates before the further degradation (Zengler et al. 1999). Alkane activation by homolytic cleavage of the C–H bond, followed by addition of the resulting radical to the double bond of fumarate with the formation of alkylsuccinates is the most ubiquitous anaerobic n-alkane activation mechanism (Callaghan 2013), which has been demonstrated under sulfate- (Kniemeyer et al. 2007; Kropp et al. 2000) and nitrate-reducing conditions (Rabus et al. 2001). Only a few studies proved fumarate addition occurred under methanogenic conditions with limited detection of initial metabolites alkylsuccinates. Toth and Gieg detected C\textsubscript{1} to C\textsubscript{9} alkylsuccinates and \emph{assA} genes over the incubation time of the methanogenic crude oil-degrading enrichment cultures (Toth and Gieg 2017). Qin et al. identified C\textsubscript{15} and C\textsubscript{16}
alkylsuccinates in methanogenic pentadecane- and hexadecane-degrading enrichment cultures, respectively (Qin et al. 2017). In our recent work, a series of C₁₆ to C₂₀ alkylsuccinates and assA genes were detected in the methanogenic enrichment cultures amended with C₁₆ to C₂₀ n-alkanes (Ji et al. 2019).

Although methanogenic biodegradation of crude oil (Aitken et al. 2013; Gieg et al. 2010; Gray et al. 2011; Jones et al. 2008; Toth and Gieg 2017) and longer n-alkanes (≥ C₁₄) (Liang et al. 2016; Siddique et al. 2011; Wawrik et al. 2016; Zengler et al. 1999; Zhou et al. 2012) has been intensively investigated, methanogenic biodegradation of low molecular weight n-alkanes has not been extensively studied (e.g. activation mechanisms and syntrophic degraders). Members of genus Smithella (in the family Syntrophaceae), implicated in syntrophic alkane degradation, were frequently identified in methanogenic crude oil-degrading enrichment cultures (Gray et al. 2011; Jones et al. 2008; Toth and Gieg 2017). Sherry et al. observed that Smithella was significantly enriched in both the weathered and non-weathered oil-amended (containing C₅ to C₁₀ n-alkanes) methanogenic enrichment cultures, indicating that Smithella can utilize low molecular weight n-alkanes (Sherry et al. 2014). Novel members of the family Peptococcaceae were identified to be the primary degraders in several methanogenic short alkane-degrading (C₅ to C₁₀, n-, iso- and cyclo-alkanes) enrichment cultures derived from oil sands tailings ponds (Abu Laban et al. 2015; Mohamad Shahimin et al. 2016; Mohamad Shahimin and Siddique 2017; Siddique et al. 2015; Tan et al. 2014; Tan et al. 2013). By investigating methanogenic biodegradation of C₇ to C₈ iso-alkanes, Abu Laban et al. proposed a novel family Peptococcaceae activated these substrates by addition to fumarate with the detection of high abundance of Peptococcaceae, Peptococcaceae-related assA gene and fumarate addition metabolites of C₇ to C₈ iso-alkans (Abu Laban et al. 2015). Although a positive expression of assA gene and fumarate addition metabolites of 2-methylpentane and methylcyclopentane were detected in the methanogenic short alkane-degrading (C₆ to C₁₀, n-, iso- and cyclo-alkanes) cultures, Tan et al. still failed to detect initial activation metabolites of n-alkanes (Tan et al. 2015).

Here, we established a methanogenic enrichment culture growing on C₉ to C₁₂ n-alkanes inoculated with production water from a low-temperature petroleum reservoir. Methane production was periodically monitored during the incubation. Microbial community compositions, functional genes (assA and mcrA) and metabolite profiles were analyzed at the end of the incubation period.

Materials and methods
Enrichment cultures
Production water from Xinjiang Kelamayi oil field block 6 (about 21 °C) was collected and stored in a serum bottle with headspace filled with N₂ (99.99% purity). The production water was stored for over 1 year to consume the residual organics. Sterilized basal medium with no electron acceptor (Wang et al. 2011) was dispensed in 120 mL-serum bottles as 48 mL per each. 2 mL of the production water was transferred to each bottle by syringe. Each alkane-amended enrichment culture contained 0.225 mmol of each n-alkane, including n-nonane (C₁₆; ≥ 99%), n-decane (C₁₀; ≥ 99%), n-undecane (C₁₁; ≥ 99%) and n-dodecane (C₁₂; ≥ 99%). Alkane-free control cultures received no n-alkane. The serum bottles were sealed with butyl rubber stoppers. All the cultures were set up in two replications and stationarily incubated at room temperature (around 21 °C) in the dark.

Methane measurements
Methane production was measured using a gas chromatography (GC model 9890B, Shanghai, China) equipped with a flame ionization detection (FID). 200 μL headspace gas taken by a gastight syringe were injected into GC for analysis. Program setting of the GC analysis was: the initial column temperature was set at 50 °C for 2 min, then increased to 130 °C at a rate of 15 °C/min, the temperature of 130 °C sustained for 1 min; the second increase was conducted at a rate of 30 °C/min to 180 °C for 30 min. The temperature of injector and FID was 200 °C. External standard curve of the methane was used for methane concentration calculation (Ma et al. 2018).

Metabolites measurements
To detect acid metabolites in the cultures, about 40 mL of culture aliquots was collected. These culture aliquots were refluxed at 100 °C for 8 h with 50 mL 1 M KOH in a 50% methanol, 50% water mixture for saponification. This was followed by acidification to pH < 2 with HCl. The organic fraction was then extracted with ethyl acetate and derivatized to ethyl esters with 10 mL of ethanol, 10 mL of cyclohexane and 0.2 g of NaHSO₄ (refluxed at 80 °C for 8 h). After rotary evaporation, 10 mL deionized water was added. Metabolites were extracted with 10 mL ethyl acetate for three times and concentrated to about 200 μL. 1 μL sample was injected into GC–MS in a splitless mode for analysis. An Agilent 7890A GC coupled to a MSD 5975C mass detector was used. The injector temperature was 280 °C. The program of GC–MS was followed: the initial temperature was held at 60 °C for 2 min, then increased at a rate of 10 °C/min to 280 °C for 20 min. The MS detector was run in the scan mode from 30 to 1000 mass units.
Diethyl (1-methyloctyl)succinate was synthesized according to Bian et al. (2014). The identification of (1-methyloctyl)succinate in the enrichment cultures was compared with the synthesized authentic standard (Additional file 1: Figure S1). (1-methylnonyl)succinate, (1-methyldecyl)succinate and (1-methylundecyl)succinate were identified by their characteristic fragment ions ([M–45]+ and [M–87]+) (Bian et al. 2014) and relative retention times. Diethyl succinate, diethyl glutarate, diethyl adipate, diethyl suberate and diethyl azelate were synthesized by ethyl esterification of succinic acid, glutaric acid, adipic acid, suberic acid and azelaic acid, respectively. The reaction mixture contained 2 mg of dicarboxylic acid, 10 mL of ethanol, 10 mL of cyclohexane and 0.2 g of NaHSO4. The reaction mixture was refluxed at 80 °C for 8 h. The ethanol and cyclohexane were removed by rotary evaporation, and the residue was treated with 10 mL water. Diethyl products were extracted with extracted with 10 mL ethyl acetate for three times and analyzed by GC–MS in a same program of culture metabolites analysis. α,ω-Dicarboxylic acids in the enrichment cultures were identified by comparison with these authentic standards (Additional file 1: Figure S2). Fatty acids were identified by matching library spectra NIST (https://webbook.nist.gov/chemistry/).

Microbial community analysis
10 mL of culture aliquot were collected for genomic DNA extraction using the AxyPrep™ Bacterial Genomic DNA Maxiprep Kit (Axygen Biosciences, USA). Archaeal and bacterial 16S rRNA genes were amplified using 344F (5′-ACG GGG YGC AGC AGG CGC GA-3′)/915R (5′-GTG CTC CCG GCC GCA TTT CT-3′) (Casamayor et al. 2002) and 515F (5′-GTG CCC AGC GGC GCG G-3′)/907R (5′-CCG TCA ATTCMTTT RAG TTT-3′) (Xiong et al. 2012), respectively. 16S rRNA gene polymerase chain reaction (PCR) and Illumina sequencing were performed as previously described (Ma et al. 2018). Operational taxonomic units (OTUs) were classified using Usearch (Edgar 2013) against the SILVA SSU database 128 (Quast et al. 2013) with the 97% similarity.

**assA and mcrA genes analysis**
Alkylsuccinate synthase gene (assA) and methyl coenzyme-M reductase gene (mcrA) as the key functional genes involved in the methanogenic n-alkane degradation process were investigated. PCR primer sets of assA2F/assA2R (Callaghan et al. 2010) and MLF/MLR (Luton et al. 2002) were used for the PCR amplification of assA and mcrA gene, respectively. PCR cycling conditions for both assA and mcrA gene were conducted as follows: 95 °C for 3 min; 40 cycles of 95 °C for 45 s, 55 °C for 60 s, 72 °C for 2 min; and 72 °C for 10 min. PCR products were purified and cloned, and positive clones were picked for sanger sequencing on ABI 377 automated sequencer (Liang et al. 2015). The valid nucleotide sequences were translated to protein sequences using ORFfinder translation tool (https://www.ncbi.nlm.nih.gov/orffinder/). Protein sequences were classified to OTUs using CD-HIT Suite (Huang et al. 2010) with the 97% similarity. Representative protein sequences were compared with GenBank Database using BLAST to identify similar sequences. Phylogenetic analyses were conducted using MEGA6.0 software with neighbor-joining method and 1000 bootstrap replicates.

Data availability
The sequences generated in this study were deposited in GenBank under accession numbers SAMN08904491 and SAMN08904496 (bacterial and archaeal 16S rRNA genes), MH192396-MH192461 (assA genes), MH192647-MH192713 (mcrA genes). The sequencing data of alkane-free control cultures were available as previously (Ji et al. 2019).

**Results**
**Methane and intermediate metabolites analysis**
Methane production started after 85 days’ incubation in alkane-amended (a mixture of C9 to C12 n-alkanes) enrichment cultures and reached about 33 μmol at the end of the incubation (364 days) (Fig. 1). No methane
was detected in the alkane-free controls (Ji et al. 2019) (Fig. 1).

Potential anaerobic intermediates of \( n \)-alkanes were analyzed by GC–MS. (1-Methyloctyl)succinate (C\(_9\) alkylsuccinate), (1-methylnonyl)succinate (C\(_{10}\) alkylsuccinate), (1-methyldecyl)succinate (C\(_{11}\) alkylsuccinate) and (1-methylundecyl)succinate (C\(_{12}\) alkylsuccinate), generated from fumarate addition to the alkanes \( n \)-nonane, \( n \)-decane, \( n \)-undecane and \( n \)-dodecane respectively, were detected in the alkane-amended enrichment cultures (Fig. 2). All identified metabolites displayed the signature fragments at \( m/z \) 128, 174, [M–45]\(^+\) and [M–87]\(^+\), which are distinctive for alkylsuccinates (Bian et al. 2014) (Fig. 2). The identity of C\(_9\) alkylsuccinate in the alkane-amended enrichment cultures was confirmed by comparing its ion fragmentation patterns and retention time with that of a synthesized standard (Fig. 2, Additional file 1: Figure S1). No alkylsuccinates were identified in the alkane-free controls (Ji et al. 2019).

**Fig. 2** Detection of putative alkylsuccinates in the alkane-amended enrichment cultures. **a** Partial GC–MS \( m/z \) 128 and \( m/z \) 174 selected ion chromatogram showing the presence of (1-methyloctyl)succinate (C\(_9\)), (1-methylnonyl)succinate (C\(_{10}\)), (1-methyldecyl)succinate (C\(_{11}\)) and (1-methylundecyl)succinate (C\(_{12}\)). **b** Mass spectral profiles of (1-methyloctyl)succinate. **c** Mass spectral profiles of (1-methylnonyl)succinate. **d** Mass spectral profiles of (1-methyldecyl)succinate. **e** Mass spectral profiles of (1-methylundecyl)succinate.
Long-chain fatty acids included tetradecanoate, pentadecanoate, hexadecanoate, heptadecanoate, octadecanoate, eicosanoate and docosanoate were detected in the alkane-amended enrichment cultures (Additional file 1: Figure S3). Only hexadecanoate and octadecanoate were detected in the alkane-free controls. Several α,ω-dicarboxylic acids were specifically identified in the alkane-amended enrichment cultures by comparing to the authentic standards with mass spectral profiles and retention times. These included butanedioic (succinic) acid, pentanedioic (glutaric) acid, hexanedioic (adipic) acid, octanedioic (suberic) acid and nonanedioic (azelaic) acid (Additional file 1: Figure S2).

Microbial community compositions
Substantial difference of microbial community compositions was observed between alkane-amended enrichment cultures and alkane-free control cultures at the end of the incubation (364 days). Smithella sp. had the highest relative abundance in the alkane-amended enrichment cultures (Fig. 3a). Other abundant bacterial phylotypes affiliated to Anaerolineaceae, Desulfovibrio, Desulfatibacillus, Proteiniphilum, Thermovirga, and unclassified NB1-n (Fig. 3a). In the alkane-free control cultures, members of Geoalkalibacter and Thermacetogenium became the dominant bacteria (Ji et al. 2019) (Fig. 3a). The archaeal community in the alkane-amended enrichment cultures was dominated by hydrogenotrophic methanogens of Methanocalculus (84%) and Methanothermobacter (10%) (Fig. 3b). Methanothrix (Methanoseta, acetoclastic methanogen) was also detected in the alkane-amended enrichment cultures and comprised about 5% of the total archaeal community (Fig. 3b). The archaeal community in the alkane-free control cultures was essentially comprised by Methanothermobacter (98%) (Ji et al. 2019) (Fig. 3b).

Phylogenetic analysis of assA and mcrA genes
Genes encoding for the alkylsuccinate synthase were only detected in the alkane-amended enrichment cultures. All sequences were clustered into Smithella sub-clade and were most closely related with assA sequence of Smithella sp. SC_K08D17 (Fig. 4). Both alkane-amended enrichment cultures and control cultures detected mcrA genes (Ji et al. 2019). In the alkane-amended enrichment cultures, most mcrA sequences affiliated with Methanocalculus and only one sequence (a total of 67 valid sequences) belonged to Methanothermobacter (Additional file 1: Figure S4).

Discussion
Methanogenic biodegradation of C9 to C12 n-alkanes initiated by addition to fumarate
The detection of corresponding fumarate addition products (C9 to C12 alkylsuccinates) provides convincing evidence that the oxidation of C9 to C12 n-alkanes was initiated by addition to fumarate under methanogenic conditions. It is supported further by the detection of assA genes. Previous studies have reported C1 to C8 alkylsuccinates detected in the production water from oil reservoirs (Agrawal and Gieg 2013; Bian et al. 2015; Duncan et al. 2009; Gieg et al. 2010). And C1 to C9, C15 to C20 alkylsuccinates have been detected under methanogenic conditions associated with microorganisms derived from oil reservoirs (Qin et al. 2017; Toth and Gieg 2017). Here the identification of C9 to C12 alkylsuccinates fills a gap that a series of n-alkanes can be activated by fumarate addition by oilfield-related microorganisms.

Except for fumarate addition products, several dicarboxylic acids were detected in alkane-amended enrichment cultures. These diacids may be cell-associated or secreted from the cells. Oberding and Gieg detected several α,ω-dicarboxylic acids in methanogenic n-octacosane-degrading enrichment cultures (Oberding and Gieg 2018). The authors suggested that these dicarboxylic acids might act as biosurfactants, which could increase substrate accessibility (Oberding and Gieg 2018). Although the origin of these dicarboxylic acids is elusive, the fatty acids as the downstream metabolites involved in fumarate addition pathway (Wilkes et al. 2002), may play a role in alkane emulsification in the current study (Embree et al. 2014).

Key members involved in methanogenic n-alkane degradation
The dominant bacteria in the alkane-amended enrichment cultures were Smithella. Members of Smithella have been detected in numerous methanogenic alkane- and crude oil-degrading enrichment cultures (Cheng et al. 2013; Oberding and Gieg 2018; Siddique et al. 2011; Wawrik et al. 2016; Zengler et al. 1999) and are generally considered as syntrophic n-alkane degraders (Gray et al. 2011). In this study, assA genes closely related to Smithella species was detected, suggesting that Smithella participated in methanogenic n-alkane degradation and initiated alkane activation by fumarate addition reaction.

The abundance of Anaerolineaceae was found to be increased in the alkane-amended enrichment cultures. Microorganisms affiliated to the family Anaerolineaceae have been detected in a vast number of methanogenic alkane-degrading enrichment cultures and were implicated to be responsible for alkane activation in these
cultures (Liang et al. 2015; Liang et al. 2016; Mohamad Shahimin et al. 2016; Mohamad Shahimin and Siddique 2017). However, Anaerolineaceae-related assA genes were not detected in the current culture, consistent with previous studies (Liang et al. 2016; Mohamad Shahimin et al. 2016). It has also been suggested that Anaerolineaceae may serve as a secondary degrader in oxidizing fermentative products from primary degraders (Tan et al. 2015). Based on the results of this study, the role of Anaerolineaceae is currently unknown.

Our results suggest that fumarate addition is a key alkane initial activation mechanism under methanogenic conditions. Smithella were identified as primary syntrophic n-alkane degraders, which can activate C_9 to C_{12} n-alkanes by addition to fumarate. This work expands our knowledge about the biochemical process involved in the methanogenic hydrocarbon biodegradation in petroleum reservoirs and oil-contaminated environments.

![Figure 3](image-url)

**Fig. 3** Microbial community compositions in the alkane-amended enrichment cultures and alkane-free control cultures as determined by Illumina sequencing of 16S rRNA genes. **a** Bacterial population in both cultures. Sequences comprising more than 3% in at least one culture were shown. **b** Archaeal population in both cultures. Sequences comprising more than 1% in at least one culture were shown. The notations in the legend g, f, and o stand for the OTUs assigned to genus, family and order levels, respectively.
**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13568-020-0956-5.

Additional file 1: Figure S1. GC–MS analysis of a diethyl 2-(1-methyloctyl)succinate (C9 alkylsuccinate) standard. (a) GC partial ion chromatogram following selection for the m/z 128 ion of a diethyl 2-(1-methyloctyl) succinate standard, (b) Mass spectral profiles of diethyl 2-(1-methyloctyl) succinate (retention time, 17.60 min). Figure S2. Mass spectral profiles of dicarboxylic acids identified in alkane-amended enrichment cultures. Left panel: compound detected in the alkane-amended enrichment cultures. Right panel: ethyl-derivatized authentic standards. Figure S3. Mass spectral profiles of fatty acids (ethyl derivatives) identified in alkane-amended enrichment cultures. Figure S4. Phylogenetic tree of deduced amino acid sequences of methyl coenzyme-M reductase genes (mcrA) from alkane-amended enrichment cultures (in red). Topology of the tree was obtained by the neighbor-joining method. Bootstrap values (n = 1000 replicates), values below 75% are not shown.

**Abbreviations**

assA: alkylsuccinate synthase gene; mcrA: methyl coenzyme-M reductase gene; C9 alkylsuccinate: (1-methyloctyl)succinate; C10 alkylsuccinate: (1-methylnonyl)succinate; C11 alkylsuccinate: (1-methyldecyl)succinate; C12 alkylsuccinate: (1-methylundecyl)succinate.

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**Authors' contributions**

JHJ and LZ performed all the experiments assisted by PP. JHJ and LZ wrote the manuscript assisted by all co-authors. BZM, JDG designed the study. SMM, JC, YFL, ZZQ, MI assisted JHJ and LZ on statistical analysis and in the discussion on the interpretation of the data. JFL and SZY were committed to all the experiments. All authors read and approved the final manuscript.
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