



Selective inhibition of methanogenesis by sulfate in enrichment culture with production water from low-temperature oil reservoir



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ABSTRACT

CO₂-reducing, acetoclastic and methylotrophic methanogenesis are three main biochemical pathways for biogenic methane production in subsurface environments. In this study, methanol, formate and acetate were used as substrates amended with low-temperature oilfield production fluid to establish six anaerobic enrichment settings (three with addition of sulfate as exogenous electron acceptor, another three without), incubated at room temperature to monitor the biochemical processes involved in the biodegradation of them. Methane was analyzed in the headspace while acetate, propionate and butyrate were measured in the enrichment cultures during the incubation. Methane was produced in all microcosms regardless of whether sulfate was present or not and stoichiometric estimation indicated that accumulated methane accounted for 44%–76% of the expected in the microcosms without addition of sulfate, while in microcosms with sulfate, 11%–63% of methane was recovered. *Methanosarcina* and *Methanomethylovorans* were predominantly detected in enrichment cultures with methanol only, whereas *Methanosaeta* was the most encounter archaea in microcosms with sulfate and acetate addition. Members represented by *Methanomassiliicoccus* dominated in both settings amended with formate or methanol when sulfate was present. Our data showed that methanogenesis was selectively inhibited in the presence of sulfate. The high frequency of *Methanomassiliicoccus* and *Methanosaeta* in response to sulfate amendment yielded insights into the dynamics of the composition of potential functional microorganisms and also into the metabolic flexibility of methanogens residing in low temperature petroleum reservoirs. These results provide fundamental data on the biochemical process of methane formation, and the shift of methanogenic community through sulfate addition in low temperature oil reservoirs.

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1. Introduction

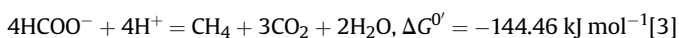
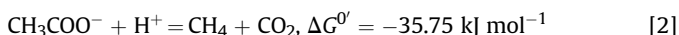
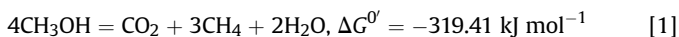
Oil reservoirs, as a large geobioreactor, are rich in a wide range of anaerobic microorganisms, including fermentative bacteria, syntrophic bacteria and methanogens (Magot et al., 2000; Orphan et al., 2000; Grabowski et al., 2005; Gittel et al., 2009; Kaster et al., 2009; Pham et al., 2009; Li et al., 2010, 2011), and as well as large amount of organic substances (Mbadinga et al., 2011). Methanogenesis and sulfate reduction are ubiquitous in oil

reservoirs, among which methanogenesis is considered the main terminal process of subsurface anaerobic degradation of organic matter when sulfate is limited (Jones et al., 2008). The methane produced in oil reservoirs can be thermogenic or biogenic origin. Biogenic methane formation includes acetotrophic, CO₂-reduction, syntrophic acetate oxidation coupled to CO₂-reduction, and methylotrophic methanogenesis (Ferry, 1992). In laboratory-scale experiments and literature, methanogens are able to utilize a variety of substrates including methyl groups (formate, methanol, methylamines etc.), acetate and other low molecular weight fermentation products (Strąpoć et al., 2011). Taking some of the above-mentioned precursors as examples (Thauer et al., 1977; Paulo et al., 2003), methane can be produced through the following

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reactions:



From thermodynamics point of view, conversion of methanol, formate and acetate to methane is feasible. Previous geochemical studies reported CO_2 -reducing and acetoclastic methanogenesis as the predominant subsurface methanogenic pathways for biogenic gas generation, methyltrophic pathway might also exist. So far, anaerobic bioconversion of these methane-producing precursors has been well studied and some reports have showed that the presence of sulfate and/or sulphidogenesis had great inhibition on methanogenesis. However, contradictory reports are also available in that methanogenesis was not inhibited/little affected by the addition of sulfate (Lv et al., 2015). Therefore, microbial conversion of organic substances available, e.g., methanol, formate, acetate to methane in oil reservoir environments is a far more complex process than our current knowledge.

In this study, three typical methanogenic precursors (methanol, formate and acetate) were used as the sole carbon resources, oil field production water was used as the source of microorganisms, incubated at room temperature under two different conditions (with and without sulfate amendment) to evaluate methanogenesis from these substrates. Methane was detected in headspace during incubation and microbial communities in cultures were assessed by construction of bacterial and archaeal 16S rRNA gene clone libraries.

2. Materials and methods

2.1. Oil reservoir conditions and sample collection

Production water samples were collected from an oil-producing well of Xinjiang oil field in West China. Five liters of production water samples were collected directly from the production valve of the pipeline at the well-head into sterile bottles directly after flushing the line initially for approximately 30 min. The bottles were completely filled with oil and water mixture, tightly sealed and immediately transported back to laboratory for further processing.

2.2. Enrichment and culturing techniques

Initial culturing was made by transferring 80 ml of production water into sterilized 120 ml serum bottle containing resazurin (0.1 mg l^{-1}) as redox indicator. Before capped with butyl rubber stoppers, the bottles were flushed with pure N_2 (99.99%) after passing through heated copper filings and were sealed with aluminum crimp seals. After about 3 months of incubation at room temperature ($22 \pm 1 \text{ }^\circ\text{C}$) to consume any residual oxygen and carry over organic carbons, the microcosm was then flushed with pure N_2 to remove CH_4 and CO_2 .

Enrichment cultures were then established by transferring 5 ml of the initial culture content described above into each 120 ml serum bottle containing 50 ml of anaerobic basal medium. The basal medium was prepared according to the description of Lv et al. (2015) previously. For serum bottles incubated with sulfate addition, 4.0 mg l^{-1} of Na_2SO_4 were added into the above basal medium and the medium was then supplemented with 1.0 ml of trace elements and 1.0 ml of vitamins stock solution. The trace elements and vitamins stocks solutions were prepared according to the

description of Wang et al. (2011). Methanol, formate and acetate (final concentration 0.4%, v/v) were added as carbon and energy sources to the microcosms, individually. Treatments used herein were denoted as follows: M0 (control without carbon source or sulfate addition), MM (amended with methanol only), MF (amended with formate only), MA (amended with acetate only), S0 (control without carbon source but with sulfate addition), SM (incubation amended with both methanol and sulfate), SF (incubation amended with both formate and sulfate), and SA (incubation amended with both acetate and sulfate).

2.3. Headspace gas and metabolite analysis

Headspace gas in serum bottle was measured using the method described in Zhou et al. (2012) and Lv et al. (2015). Volatile fatty acids (VFAs) analysis was carried out at the end of the incubation period using the method described previously (Mbadinga et al., 2012; Zhou et al., 2012).

2.4. DNA extraction and PCR amplification

Genomic DNA was extracted from 8 ml of enrichment culture using AxyPrep™ bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., CA, USA) according to the manufacture's protocol. Extracted genomic DNAs were immediately frozen and stored at $-70 \text{ }^\circ\text{C}$ for further use. Partial 16S rRNA genes of bacteria and archaea were amplified using PCR primers and methods described previously (Cheng et al., 2007; Savage et al., 2010; Mbadinga et al., 2012; Zhou et al., 2012).

2.5. Construction of 16S rRNA gene clone libraries

After purification by electrophoresis in 1.8% agarose and recovered by a DNA gel extraction kit (Axygen Biosciences, Inc., CA, USA), the purified 16S rRNA gene fragments were directly cloned into *Escherichia coli* DH5 α cells using pMD19⁺-T Simple cloning vector (Takara, Japan). Obtained white colonies were picked randomly and cultured overnight in 0.8 ml of LB medium in the presence of ampicillin ($50 \text{ } \mu\text{g ml}^{-1}$ CH_4 microcosm⁻¹) at $37 \text{ }^\circ\text{C}$. The insertion of 16S rRNA gene was checked by PCR amplification using the universal primer set M13F (−47)/RV-M plasmid specific primers (Guan et al., 2013), followed by agarose gel electrophoresis with DuRed nucleic acid gel (Fanbo, China) staining. Obtained DNA sequences were checked for vector by VecScreen software (www.ncbi.nlm.nih.gov/tools/vecsreen/) before further analysis.

2.6. Phylogenetic analysis

16S rDNA sequences from each respective clone library were first checked with OrientationChecker (Ashelford et al., 2006), then with BealleroPhon (Huber et al., 2004) and Pintail (Ashelford et al., 2006) to remove chimeric sequences. Sequences with more than 97% similarity were assembled into the same operational taxonomic units (OTUs) using CD-HIT (Li and Godzik, 2006; Fu et al., 2012). For each OTU, the nearest relatives of the representative sequence were identified using the BLAST network service (Altschul et al., 1990; McGinnis and Madden, 2004).

2.7. Nucleotide sequence accession numbers

Partial 16S rDNA sequences of bacteria and archaea obtained in this study were deposited in the GenBank database under accession numbers assigned (Table 1).

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