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Biological souring of crude oil under anaerobic conditions

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ABSTRACT

Seawater injection into oil reservoirs for purposes of secondary oil recovery is frequently accompanied by souring (increased sulfide concentrations). Production of hydrogen sulfide causes various problems, such as microbiologically influenced corrosion (MIC) and deterioration of crude oil. Sulfate-reducing bacteria (SRB) are considered to be major players in souring. Volatile fatty acids (VFAs) in oil-field water are believed to be produced by microbial degradation of crude oil. The objective of this research was to investigate mechanisms of souring, focusing specifically on VFA production via crude oil biodegradation. To this end, a microbial consortium collected from an oil–water separator was suspended in seawater; crude oil or liquid n-alkane mixture was added to the culture medium as the sole carbon source, and the culture was incubated under anaerobic conditions for 190 days. Physicochemical analysis showed that preferential toluene degradation and sulfate reduction occurred concomitantly in the culture containing crude oil. Sulfide concentrations were much lower in the alkane-supplemented culture than in the crude oil-supplemented culture. These observations suggest that SRB are related to the toluene activation and VFA consumption steps of crude oil degradation. Therefore, the electron donors for SRB are not only VFA, but many components of crude oil, especially toluene. Alkanes were also degraded by microorganisms, but did not contribute to reservoir souring.

1. Introduction

Seawater is commonly injected into oil wells in order to increase the oil recovery rate. However, biochemical reduction of sulfate ions present in seawater results in souring, i.e., production of sulfide. Microbes called sulfate-reducing bacteria (SRBs) are involved in the souring. Souring creates severe processing and environmental problems. Control of biogenic H_2S production which improves the quality of the produced oil and decreases the cost of production could be achieved through elimination of sulphate from the water prior to injection, suppression of SRB with biocides or metabolic inhibitors such as nitrite and molybdate, and addition of nitrate to the injection water [1]. Microbial sulfate reduction is an important metabolic activity in many petroleum/hydrocarbon (PHC)-contaminated aquifers, as contamination with mono-aromatic PHCs, e.g., benzene, toluene, and ethylbenzene, is of regulatory concern due to the solubility and toxicity of these compounds [2]. Sulfate reduction can be coupled to the bacterial metabolism of mono-aromatic PHCs, and this process has therefore gathered increasing interest as an intrinsic remediation process [3,4]. SRBs are among the microorganisms present in oil fields that induce souring; most SRBs belong to *Deltaproteobacteria* and *Firmicutes* [5]. In order to control corrosion, steps are taken to remove oxygen from injected water, but this provides an environment conducive to the growth of SRBs that are obligate anaerobes. Seawater contains sulfate (approximately 28 mM) [6], and SRBs derive energy for growth by coupling oxidation of organic electron donors to the reduction of sulfate to form sulfide. The electron donors most commonly used by SRBs in oil fields are volatile fatty acids (VFAs) [7]. VFAs in oil-field water (OFW) are produced by microbial degradation of crude-oil components. Putative hydrocarbon metabolites have been detected in production water taken from oilfields [8]. Crude oil is composed of many components, including alkanes, naphthenes, aromatics, and non-hydrocarbon components. To identify the components of crude oil that contribute to souring, we incubated microbes obtained from an oil well with either crude oil or a mixture of alkanes (C_5–C_{17}, AM) for 190 days.

2. Materials and methods

2.1. Biological conversion of crude oil

The crude oil reservoirs were located at depths of 1300–1500 m. The temperature of the field-water sample on the ground was

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around 25 °C. To store the sample, a 20-L poly-tank was completely filled with water/oil mixture to remove headspace, and kept at 4 °C until use. Microbes in the OFW, which included crude oil, were concentrated by centrifugation at 11,000 × *g* for 15 min in a 500-ml centrifuge tube. Crude oil separated from the OFW by centrifugation was used for the microbial conversion experiment. The pellet was resuspended in 50 ml of filter sterilized (0.22 μm, Millex GV, Millipore) natural seawater collected at Ogasawara (Tokyo, Japan), about 1000 km south of central Tokyo. Thus, the microbes in the OFW were concentrated 50-fold relative to the original sample. Concentration of microbes in the concentrated sample was 4.3×10^8 cells ml^{-1}, as measured by direct counting under a fluorescence microscope after staining with DAPI (4′,6-diamidino-2-phenylindole) [9].

Crude oil (CO), obtained by separation from OFW, and the C_5–C_{17} alkane mixture (AM) were used separately as substrates for the microbial conversion test. AM contained the same volume of each alkane component (C_5–C_{17}). Five milliliters of the mixtures were overlaid on 50 ml of seawater supplemented with microbes collected from OFW in a 70-ml glass vial. The vial was purged with nitrogen gas for 5 min to produce an anaerobic condition. Incubation was carried out at 25 °C, with mixing provided by a magnetic stirrer, for 190 days. Four conditions (CO, CO-M, AM, AM-M) were investigated. To investigate the involvement of molecular oxygen in souring, microaerobic conditions were established: once every 5 days, 10 ml of the seawater in the vial was transferred to the 50-ml plastic tube, saturated with air by vortexing, and returned to the incubation vial (CO-M, AM-M). Every 10 days, 1.8 ml of the seawater was sampled from each vial and was centrifuged at 8000 × *g* for 5 min;the supernatant was used for chemical analysis, and the pellet was used for microbial analysis. The cell pellets were resuspended in distilled water and then centrifuged at the same condition; this process was repeated twice in order to completely remove the seawater from the pellets. Genomic DNA was extracted by bead-beating using the ISO-FECAL method (Nippon Gene, Japan). After incubation with lysis solution at 65 °C, the bead-beating procedure was conducted at 60 m s^{-1} for 40 s using a FastPrep-24 Instrument (MP Biomedicals LLC, Santa Ana, CA, USA) to increase the effectiveness of DNA extraction. A NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, DE, USA) was used to assay DNA quality. To insure sample quality, the ratio of absorbance at 260 and 280 nm was required to be between 1.8 and 2.0. The extracted genomic DNA was used for PCR-DGGE (denaturing gradient gel electrophoresis).

2.2. Chemical analysis

Sulfide concentration was measured by the methylene blue method (NANOCOLOR Standard Experiment Sulphide, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Anions were measured by ion chromatography (LC-10AD, Shimadzu Corp.). Concentrations of CO components were measured using a gas chromatography system (GC-2014, Shimadzu Corp.) equipped with flame-ionization detector, using helium as the carrier gas. The column (50 mm × 0.2 mm, 0.5 μm film thickness) used in this experiment was HP-PONA (Agilent Technologies, Inc., Santa Clara, CA, USA). Column temperature was initially held at 40 °C for 20 min, then programmed to rise to 70 °C at a rate of 1.5 °C min^{-1}, then to 290 °C at 4 °C min^{-1}, and then to 320 °C at 2 °C min^{-1}, with a final hold time of 30 min. Trans-2-heptene was used as an internal standard. CO components dissolved in the water phase were extracted with 2.5 ml of hexane for 24 h. The organic phase was then dried with anhydrous sodium sulfate, and then analyzed by gas chromatography as described above.

2.3. Analysis of microbial consortia by PCR-DGGE

PCR amplification of the 16SrDNA (16S ribosomal RNA gene) of the samples was performed using universal primers: 341F (5′-CCTACGCCAGGCAGCAG-3′), with a GC clamp (CGCCCGCCGCGC-CCCGCGCCCGTCCCGCCGCCCCCGCCCG) attached to the 5′ end, and 907R (5′-CCGTCAATTCCTTT[A/G]AGTTT-3′). The 25-μl reaction mixtures contained 2.5 μl 10× ExTaq buffer, 6.25 nmol dNTP Mix, 7.5 pmol of each primer, 0.625U ExTaq polymerase (Takara Bio Inc., Japan), and 1 μl of the DNA extract samples. The program was performed as follows: an initial denaturation step at 94 °C for 2 min, followed by 10 cycles of two identical touchdown amplification (denaturation at 94 °C for 1 min; annealing at 65 °C for 1 min, with an decrement of 1 °C per cycle; and elongation at 72 °C for 3 min), another 10 amplification cycles (denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min;and elongation at 72 °C for 3 min), and a final elongation step at 72 °C for 7 min. PCR products were checked by electrophoresis with 2% (wt/vol) agarose gel, stained with ethidium bromide, and viewed on a UV transilluminator. PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using the DCode universal mutation detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 6% polyacrylamide gels with a 30–60% denaturing gradient. One hundred percent denaturation corresponded to 7 M urea and 40% (vol/vol) deionized formamide. The electrophoresis was conducted in 1× TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 14 h at 110 V and 60 °C. The gels were stained in SYBR Gold (0.01% vol/vol in 1× TAE) for 25–30 min. Bands of interest were excised using pipettes fixed with sterile flat tips, and then transferred into 1.5-ml Eppendorf tubes. DNA extraction from gel bands was performed by the freeze/thaw method, as described previously [10]. Nucleotide sequences were compared with sequences in GenBank.

2.4. Gene base analysis of SRB

Dissimilatory sulfite reductase (*dsrAB*) genes were amplified with the dsr-1F (5′-ACSCAYTGGAARCACG-3′, S = C or G, Y = C or T, R = A or G, D = A or G or T) and dsr-4R (5′-GTGTARCAGTTDCCRCA-3′) primers [11] (Table 2). PCR amplification was conducted as previously described, with the following modifications: initial denaturation for 2 min at 94 °C for; 30 cycles of 15 s at 94 °C, 30 s at 54 °C, and 2 min at 72 °C; and final extension for 7 min at 72 °C. PCR fragments were resolved by electrophoresis to confirm the expected size of the products (about 1.9 kb). The PCR products were purified by FastGene™Gel/PCR Extraction Kit (NIPPON Genetics, Japan). Purified DNA was cloned into pGEM-T Vector System I (Promega, Madison, WI) and directly transformed into *E. coli* JM109 (Promega Corporation). Clones containing recombinant plasmids were examined for the presence of appropriate inserts by PCR. The PCR products were classified by restriction fragment length polymorphism (RFLP). DNA fragments with representative restriction patterns were purified by phenol/chloroform extraction and used for conventional Sanger sequencing with Big Dye Terminator ver. 3.1 (Applied Biosystems) on an Applied Biosystems 3730xl DNA Analyzer. The sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) [12] and compared with sequences in the GenBank database.

Relative abundances of SRBs were estimated by qPCR. DNAs extracted *E. coli* K12 and *Desulfovibrio desulfuricans* (ATCC13699) were used as a standard. The 16SrDNA primers for all bacteria, 341F (see above), and 534R (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the bacterial 16S rDNA using the StepOne Real-Time PCR System (Applied Biosystems, Japan Ltd.). The *dsrA* gene primers for SRB, dsr-1F (see above) deg, and RH3-dsr-R (5′-GGTGGAGCCGTGCATGTT-3′) were used. PCR was performed in 20-μL volumes containing 10 μL Thunderbird™ SYBR® qPCR mix