Microbial diversity in the anaerobic tank of a full-scale produced water treatment plant

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ABSTRACT

Microbial characteristics in the anaerobic tank of a full-scale produced water treatment plant capable of anaerobic hydrocarbon removal were analyzed and compared to those in the influent produced water using cultivation-independent molecular methods. Clones related to methanogens including the methylotrophic *Methanomethylovorans thermophila* and hydrogen- and the formate-utilizing *Methanolinea tarda* were in abundance in both samples, but greater numbers of *M. tarda*-like clones were detected in the biofilm library. Both DGGE and cloning analysis results indicated that the archaea in the biofilm were derived from the influent produced water. Bacterial communities in the influent and biofilm samples were significantly different. *Epsilonproteobacteria* was the dominant bacterial group in the influent while *Nitrospira* and *Deltaproteobacteria* were the predominant groups in the biofilm. Many clones related to syntrophic bacteria were found among the *Deltaproteobacteria*. One *Deltaproteobacteria* clone was related to *Syntrophus*, which is commonly found in methanogenic hydrocarbon-degrading consortia. A number of *Deltaproteobacteria* clones were assigned to the clone cluster group TA, members of which predominate in various methanogenic consortia that degrade aromatic compounds. These results suggest that a microbial community associated with methanogenic hydrocarbon degradation may have been established in the biofilm.

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1. Introduction

Produced water, characterized by containing petroleum hydrocarbons, is concurrently recovered during crude oil and natural gas exploration, as well as production in oilfields. Biological processes have been used for the removal of contaminants from produced water [1–3]. In the Jidong Oilfield of China, a full-scale biological system, which consists of successive anaerobic and aerobic biofilm reactors, has been utilized to treat produced water. The anaerobic treatment tank was responsible for the removal of approximately 45% of the polycyclic aromatic hydrocarbons (PAHs) [4] and 25% of the total petroleum hydrocarbons (TPH) (this study) from the influent produced water. Although it has been assumed that the biomass in the anaerobic tank plays a key role in the removal of petroleum hydrocarbons, little is known about the microbial populations in the biofilm.

Microbes that utilize petroleum hydrocarbons under aerobic conditions are well known. In the late 1980s, bacteria were isolated

and shown to degrade hydrocarbons under anaerobic conditions. Hydrocarbons, including benzene, toluene, ethylbenzene, xylenes, alkanes, and PAHs, could be degraded by coupling them to sulfate reduction, nitrate reduction, or methanogenesis [5–7]. Currently, many anaerobic hydrocarbon-degrading bacteria, which are mainly sulfate-reducing (SRB) and denitrifying bacteria, have been isolated, and the steps in anaerobic metabolism that mediate hydrocarbon degradation have been elucidated in many representative microorganisms. Meanwhile, many library experiments have demonstrated that methanogenic archaea are involved in the anaerobic mineralization of petroleum hydrocarbons. Anderson and Lovley showed that rapid alkane decay via methanogenesis occurred in oil-bearing sediments [8], suggesting an important role for methanogenic hydrocarbon degrading organisms in petroleum-rich anaerobic environments. Other investigations have proved the existence of indigenous archaea and bacteria in reservoirs [9], and metabolites indicative of anaerobic hydrocarbon degradation have been isolated from many oil samples [10], suggesting that hydrocarbon-degrading microbial communities are present in oil reservoirs. Thus, whether the microbes from the oil reservoir play a role in the process of anaerobic hydrocarbon degradation in the anaerobic treatment tank in the produced water treatment system is an important question.

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In the present study, the archaeal and bacterial communities in the anaerobic biofilm of the full-scale produced water treatment plant in the Jidong Oilfield were analyzed and compared with those in the influent produced water. Several molecular techniques were used in this analysis, including denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene library construction, and fluorescence *in situ* hybridization (FISH).

2. Materials and methods

2.1. Sample collection

The biological treatment system was constructed and put into use in 2001 with a capacity of 5000 tons of wastewater per day. The system consists of two identical series-wound tanks (30 m × 18 m × 5.5 m) filled with fiber type biocarriers, with the second tank being aerated. The volumetric chemical oxygen demand (COD) load of the system was approximately 0.5 g l⁻¹ day⁻¹, and the oxidation–reduction potential (ORP) in the water, at a depth of 40 cm below the surface, was approximately –130 mV in the first reactor. The biofilm and influent produced water (1 l) samples were taken for DNA extraction in January 2009. The biomass on three biocarriers from the anaerobic tank was shaved and then mixed as the biofilm sample. For the characteristics of water quality, three samples of the influent and the effluents of the anaerobic tank were taken, respectively, over a period of 2 months. All of the samples were collected in sterile bottles and processed in the laboratory within 24 h after sampling.

2.2. Water quality analysis

The conventional water quality parameters of the influent and the effluents of the anaerobic tank were measured according to the standard methods [11]. The characteristics of water samples with standard deviations are listed in Table 1.

2.3. DNA extraction

The biofilm and influent samples were washed three times using phosphate-buffered saline (PBS, pH 7.0) and centrifugation at 4 °C, 10,000 rpm, for 15 min. The genomic DNA was then extracted using the method of Tsai and Olson [12]. Triplet genomic DNA were extracted from the biofilm and influent samples and then pooled.

2.4. FISH

Fresh biofilm and influent samples were fixed with 4% paraformaldehyde in PBS (0.13 M NaCl in 10 mM Na₂HPO₄, pH 7.2), and left for 4 h. The samples were dispersed with sonication and immobilized on glass slides. For hybridizations, probe ARCH 915 labeled with fluorescein isothiocyanate, specific for the domain *Archaea* was used [13]. Hybridization was performed at 46 °C for 3 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl, pH 7.2, 0.01% SDS) containing labeled probe (5 ng μl⁻¹). Hybridization stringency was adjusted by adding formamide to the hybridization buffer (35% for ARCH915). After hybridization, the slides were washed at 48 °C for 20 min with washing buffer containing the same components of the hybridization buffer except the probes. To enumerate the total cell number in the samples, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 5 μg ml⁻¹. Over 1000 DAPI-stained cells were counted to determine the ratio of the probe-labeled cells to the total cells.

2.5. PCR-DGGE

In order to minimize PCR bias in PCR-DGGE and subsequent cloning steps, three separate reactions were run for each sample and the results were pooled. The bacterial 16S rRNA gene fragments were amplified by PCR using primers 341F (5'-CCT ACG GGA GGC AGC AG-3') with the GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CCG GGG G-3') attached to the 5' end and 534R (5'-ATT ACC GCG GCT GCT GG-3') [14]. Touchdown PCR was employed as follows: 95 °C for 10 min, followed by 20 cycles of 95 °C for 1 min, 65 °C (reduced by 0.5 °C each cycle)

for 45 s, and 72 °C for 1 min, 10 cycles of 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Primers ARCH344f (5'-ACG GGG YGC AGG CGC GA-3') with the GC clamp and 519r (5'-GWA TTA CCG CGG CKG CTG-3') were used for the domain *Archaea* [15]. The reaction conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The resulting amplification products were separated using DGGE with the Dcode Universal Mutation System (Bio-Rad, Hercules, CA, USA). The PCR products (20–25 μl) were loaded onto 6% (w/v) polyacrylamide gels in Tris–acetate–EDTA buffer (pH 7.4). The gels had denaturing gradients of 35–57% (100% denaturant contains 7 M urea and 40% formamide) and were run at 150 V at 60 °C for 4.5 h. After electrophoresis, the gels were stained for 15 min with ethidium bromide and photographed on a UV transillumination table using a Gel Documentation System (Bio-Rad Laboratories-Segrate, Milan, Italy). DGGE gels were processed using Bio-Rad Quantity One 4.3.0 software.

2.6. Cloning and sequencing of 16S rDNA

The 16S rRNA gene fragments were amplified by PCR using primers A571F (5'-GCY TAA AGS RIC CGT AGC-3') and UA1406R (5'-ACG GGC GGT GWG TRC AA-3') [16] for archaea, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GAC GGG CCG TGT GTA C-3') [17] for bacteria. The conditions for the PCR amplification were: 95 °C for 10 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 30 min. PCR products were purified using TaKaRa purification kits. The amplified 16S rRNA gene fragments were cloned into the TOPO TA cloning vector pCR2.1, and then TOP10 chemically competent *E. coli* was transformed with the plasmids according to manufacture's instructions (Invitrogen). Transformants were selected by blue-white selection on Luria–Bertani (LB) agar plates containing ampicillin (100 μg ml⁻¹). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'). Clones were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

2.7. Phylogenetic analysis

Sequences were searched against the Ribosomal Database Project II (RDP II) release 9.49 and the GenBank database by using the BLASTn program to determine the nearest matches. Phylogenetic trees were constructed using MEGA, version 4.0 by the neighbor-joining algorithm, and the Jukes–Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed. The presence of chimerae in the obtained sequences was determined by using the CHECK_CHIMERA (RDP; <http://rdp.cme.msu.edu>) prior to phylogenetic analysis. The sequences sharing 98% or greater similarity were considered to represent the same operational taxonomic unit (OTU) using software DOTUR [18].

2.8. Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences that were determined have been deposited in the GenBank, nucleotide sequence databases under accession nos. GQ844316–GQ844394, and GQ923896–GQ923904.

3. Results

3.1. Performance of the anaerobic treatment system

The quality of the oilfield produced water before and after anaerobic biological treatment is shown in Table 1. Approximately 80 mg l⁻¹ COD (30%) and 7 mg l⁻¹ TPH (25%) were removed. Sulfate reduction occurred in the anaerobic tank, with the removal of more than 20 mg l⁻¹ sulfate from the influent produced water. Nitrate was not detected in the produced water.

3.2. DGGE profiles

DNA extracted from the influent and anaerobic biofilm samples gave the expected 180 bp archaeal and 200 bp bacterial PCR fragments. DGGE patterns of the PCR products were examined. As shown in Fig. 1A, the influent samples showed more abundant archaeal DGGE bands than the anaerobic tank samples (Coefficient of Dice, 64.4%). However, all of the archaeal bands in the biofilm samples had corresponding bands in the influent samples, showing that the archaeal representatives in the biofilm originated from the influent produced water. DGGE profiles of the bacterial PCR products, as shown in Fig. 1B, exhibited a lower similarity between the influent and biofilm samples (55.3%, Coefficient of Dice).

Table 1
Quality of the produced water before and after anaerobic treatment.

Parameter	Value	
	Before	After
pH	7.2 ± 0.3	7.3 ± 0.2
Temperature, °C	50 ± 2	50 ± 2.5
COD, mg l ⁻¹	270 ± 25	187 ± 20
TPH, mg l ⁻¹	28 ± 5	21 ± 3
Total dissolved solids, mg l ⁻¹	1400 ± 30	–
SO ₄ ²⁻ , mg l ⁻¹	25.5 ± 2.7	4.7 ± 1.2
NO ₃ ⁻ , mg l ⁻¹	Not detected	Not detected

Fig. 1. Denaturing gradient gel electrophoresis profiles for partial 16S rDNA fragments showing archaeal (A) and bacterial (B) diversities in the influent (lane 1) and biofilm (lane 2) samples.

Additionally, some bacterial biofilm bands did not have a corresponding band in the influent samples.

Some DGGE bands that were clearly separated from neighboring bands were isolated for sequencing (Table 2). Three archaeal sequences that were obtained were classified in the order *Methanosarcinales*. All bacterial sequences isolated from the influent were related to uncultured *Epsilonproteobacteria* clones, and those retrieved from the biofilm (b5 and b6) were affiliated with uncultured clones in the class *Betaproteobacteria* and *Thermotogae*, respectively.

3.3. Diversity and phylogenetic analysis

To obtain more detailed microbial community information, partial 16S rDNA fragments were PCR-amplified from the extracted DNA using bacterial and archaeal specific primer sets, and four clone libraries were constructed (AP, archaea in the influent produced water; AB, archaea in the biofilm; BP, bacteria in the influent produced water; BB, bacteria in the biofilm). Sequences with $\geq 98\%$ similarity were combined into one operational taxonomic unit (OTU) using the DOTUR software. Thus, 26 and 42 OTUs were obtained from 50 influent and 70 biofilm bacterial clones, respectively, and 6 and 5 OTUs were obtained from 48 influent and 44 biofilm archaeal clones, respectively.

Fluorescence *in situ* hybridization was also performed to quantify *Archaea* in both influent and biofilm samples using *Archaea* (ARCH915)-specific probes [19]. The *Archaea* cells in the biofilm and influent accounted for approximately 7% and 10%, respectively, of the total cells (determined by DNA staining with DAPI).

3.3.1. Analysis of the archaeal communities

Archaeal clones retrieved from the biofilm and influent samples showed low phylogenetic diversity (Fig. 2). The low archaeal diversity based on analysis of the 16S rRNA gene has also been observed in other high-temperature or hydrocarbon-rich environments, such as hot springs [20] and hydrocarbon-contaminated aquifers [21]. Methanogens were predominant in the archaeal clones, and the methanogen-like clones were distributed among the orders *Methanosarcinales* and *Methanomicrobiales* (Table 3). Clones closely related ($\geq 98\%$) to *Methanomethylovorans thermophila*, an obligate methylotroph, were predominant in the two archaeal clone libraries, but the percentage was higher in the influent (69% of AP clones, represented by OTU AP13) than in the biofilm (57% of AB clones, represented by OTU AB7). In contrast, the percentage of *Methanolinea tarda*-like clones was higher in the biofilm library (34% of AB clones, represented by OTU AB1) than in the influent library (10% of AP clones, represented by OTU AP3 and AP14). *M. tarda* utilizes H_2 and formate for growth and methane production. The remaining non-methanogen clones were affiliated with uncultured archaea clones. Among these, two influent OTUs (AP29 and AP30) were related to clone SCA76 which was retrieved from oil-contaminated soil in the Jidong Oilfield [22].

3.3.2. Analysis of the bacterial communities

Phylogenetic analysis showed that the majority of 16S rDNA clones in the BB library were distributed among two groups, *Nitrospira* (40% of BB clones) and *Deltaproteobacteria* (20% of BB clones), while *Epsilonproteobacteria* clones (74% of BP clones) predominated in the BP library (Table 4). Moreover, the BB clones were distributed among more diverse phylogenetic groups than the BP clones, suggesting that the bacterial diversity of the biofilm was higher than that of the influent. Taxa that were common to both libraries included *Betaproteobacteria*, *Sphingobacteria*, *Nitrospira*, and *Clostridia*. Taxa unique to the BB library included eight classes: *Deltaproteobacteria*, *Planctomycetacia*, *Chloroflexi*, *Spirochaetes*, *Actinobacteria*, *Fusobacteria*, *Thermotogae*, and *Verrucomicrobiae*. In contrast, *Gammaproteobacteria*, *Epsilonproteobacteria*, and *Deferribacteres* were only detected in the BP library.

In the influent community, the predominant bacterial clones were assigned to the order *Campylobacteriales* in the *Epsilonproteobacteria*. Seven OTUs representing 48% of the BP clones were related to *Sulfuricurvum* sp. (EU498374) in the family *Helicobacteraceae*, and affiliated with *S. kujiense*, a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium, isolated from an underground crude oil storage cavity. The remaining *Epsilonproteobacteria* clones were assigned to the family *Campylobacteraceae*, and most of those were related to clones retrieved from petroleum reservoirs.

Table 2
Nucleotide sequence similarity of sequenced DGGE bands.

DGGE band	Closest sequence match	Accession No.	Similarity %	Classification
a1	Uncultured archaeon clone WL-22 from reservoirs	FJ900708	98	<i>Methanosarcinales</i>
a2	Uncultured archaeon clone from anoxic soils	AJ556328	100	<i>Methanosarcinales</i>
a3	Uncultured archaeon clone from anoxic soils	AJ556328	100	<i>Methanosarcinales</i>
b1	Uncultured <i>Campylobacteraceae</i> bacterium	EU640992	98	<i>Epsilonproteobacteria</i>
b2	Uncultured bacterium clone DXH4-98	FJ558985	100	<i>Epsilonproteobacteria</i>
b3	Uncultured epsilon proteobacterium	AB478659	100	<i>Epsilonproteobacteria</i>
b4	Uncultured bacterium clone 724B10S32	EU560376	99	<i>Epsilonproteobacteria</i>
b5	Uncultured <i>Hydrogenobacter</i> sp.	FM957578	94	<i>Betaproteobacteria</i>
b6	Uncultured <i>Thermotogales</i> bacterium	AY862075	94	<i>Thermotogae</i>

Fig. 2. Phylogenetic analysis of archaeal OTUs retrieved from the influent and biofilm samples. Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.02 indicated changes per nucleotide (AP, archaeal clone from the influent; AB, archaeal clone from the biofilm).

Nitrospira was the most predominant group in the biofilm community, accounting for 40% of the BB clones, represented by nine OTUs. Among these, four OTUs representing 18% of the biofilm clones were affiliated with *Thermodesulfovibrio* which are known to use H_2 with sulfate as the electron acceptor. The other five OTUs clustered together, and were affiliated with uncultured bacteria derived from anaerobic sludge or oil-contaminated sediments.

Most *Deltaproteobacteria* clones were affiliated with syntrophic substrate-degrading bacteria. Six OTUs representing 12% of the BB clones were affiliated with a non-sulfate-reducing sub-cluster, group TA (Fig. 3). BB-B38 was affiliated with an uncultured *Syntrophorhabdaceae* bacterium (clone SJA-51) from a trichlorobenzene-transforming microbial consortium; BB-B40 with an uncultured bacterium (clone WCHB1-27) from a hydrocarbon-

contaminated aquifer; BB-HB117 with a clone from a geothermal spring mat with a low similarity of 92%; BB-B2, BB-B20, and BB-B93 with *Syntrophorhabdus aromaticivorans* which can utilize phenol, *p*-cresol, isophthalate, benzoate, and 4-hydroxybenzoate in obligatory syntrophic associations with methanogenic archaea. For OTU BB-LB39, the highest similarity was with the *Syntrophus* sp. clone B2 identified in a hydrocarbon-degrading methanogenic consortium. Other *Deltaproteobacteria* OTUs of interest were BB-HB110 which was related to the propionate-oxidizing *Syntrophobacter sulfatireducens* and *S. fumaroxidans*, and BB-B33 which was affiliated with an uncultured *Syntrophaceae* bacterium (clone D15_21) retrieved from tar-oil-contaminated aquifer sediments. BB-HB102 was closely related to a clone from a sulfate-reducing reactor and to clone W31 from a water injection well in the Dagang Oilfield, China.

Table 3
Distribution of archaeal OTU and related clone number in phylogenetic groups.

Group	AB library			AP library		
	No. of AB clones	No. of OTUs	Percentage of total archaeal clones	No. of AP clones	No. of OTUs	Percentage of total archaeal clones
<i>Methanosarcinales</i>	26	2	59	34	2	71
<i>Methanomicrobiales</i>	16	2	36	5	2	10
Unknown euryarchaeote	–	–	–	9	2	19
Unknown crenarchaeote	2	1	5	–	–	–

Table 4
Distribution of bacterial OTU and related clone number in phylogenetic groups.

Group	BB library			BP library		
	No. of BB clones	No. of OTUs	Percentage of total bacterial clones	No. of BP clones	No. of OTUs	Percentage of total bacterial clones
<i>Epsilonproteobacteria</i>	–	–	–	37	13	74
<i>Gammaproteobacteria</i>	–	–	–	1	1	2
<i>Deferribacteres</i>	–	–	–	2	2	4
<i>Betaproteobacteria</i>	4	2	6	5	5	10
<i>Nitrospira</i>	28	9	40	2	2	4
<i>Clostridia</i>	4	4	6	1	1	2
<i>Sphingobacteria</i>	1	1	1	2	2	4
<i>Deltaproteobacteria</i>	14	11	20	–	–	–
<i>Spirochaetes</i>	3	3	4	–	–	–
<i>Planctomycetacia</i>	6	2	9	–	–	–
<i>Verrucomicrobiae</i>	2	2	3	–	–	–
<i>Chloroflexi</i>	4	4	6	–	–	–
<i>Actinobacteria</i>	1	1	1	–	–	–
<i>Thermotogae</i>	2	2	3	–	–	–
<i>Fusobacteria</i>	1	1	1	–	–	–

Fig. 3. Phylogenetic analysis of bacterial OTUs retrieved from the biofilm (A) and influent (B) samples. Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.02 indicated changes per nucleotide (BB, bacterial clone from the biofilm; BP, bacterial clone from the influent).

Two biofilm OTUs were assigned to the *Betaproteobacteria*, and affiliated with *Azoarcus* spp. Members of the genus *Azoarcus* can degrade toluene and ethylbenzene, or even alkanes, coupled with nitrate reduction under anoxic conditions [5,23,24]. However, nitrate was not detected in the produced water.

Within the class *Clostridia*, two OTUs were assigned to the genus *Desulfotomaculum*. One (BB-HB131) was related to clone Nap2-2B

from a hydrocarbon-degrading methanogenic consortium, another (BB-LB52) was related to a bacterium isolated from North Sea oilfield, *D. thermocisternum*, which can degrade propionate in syntrophic association with methanogenic archaea [25]. In addition, OTU BB-HB48 was related to clone D10_24 from tar-oil-contaminated aquifer sediments, and OTU BB-B46 was similar to a thermophilic biofilm clone.

Fig. 3. (Continued).

4. Discussion

For archaeal communities, all biofilm DGGE bands had corresponding bands in the influent DGGE profile, and three of the clearly archaeal bands from the biofilm belonged to the order *Methanosarcinales*. Clones closely related ($\geq 98\%$) to the obligate methylotrophic *M. thermophila* in the order *Methanosarcinales* were predominant in both the biofilm and influent archaeal clone libraries, followed by *M. tarda*-like clones in the order *Methanomicrobiales*. Both the DGGE analysis and cloning analysis indicate that archaea in the biofilm were derived from the influent.

M. thermophila has been isolated from a thermophilic laboratory-scale upflow anaerobic sludge blanket reactor. *M. thermophila* can only use methanol and methylated amines as catabolic and methanogenic substrates, with an optimum temperature of 50 °C [26]. Species of the genus *Methanomethylovorans* are typical freshwater isolates (having low salt tolerance), whereas the other obligate methylotrophs are generally regarded as halophiles [27]. Many methylotrophic methanogens have been isolated from saline oilfields [9]. This is the first report of the dominant presence of *M. thermophila* in produced water. However, the role of *M. thermophila*-like archaea in the process of pollutant biodegradation will require further study.

The ratio of the hydrogen-utilizing *M. tarda* in archaeal clones increased considerably in the biofilm (from 10% in the influent to

34% in the biofilm). Methanogenic degradation of crude oil hydrocarbons is an important process in oil-contaminated anoxic environments, and methanogenic CO₂ reduction is thought to be the predominant methanogenic route [28]. This hypothesis is supported by modeling of the gas isotope composition during in-reservoir biodegradation and by data from methanogenic oil-degrading microcosms [29], suggesting that hydrogen-utilizing methanogens play an important role in methanogenic hydrocarbon degradation. The hydrogen-utilizing *M. tarda* relatives may play a similar role in the anaerobic reactor.

The bacterial communities in the biofilm and influent were significantly different. Both DGGE and phylogenetic analysis showed that *Epsilonproteobacteria* was the predominant group in the influent community, whereas no DGGE band or clone from this group was recovered from the biofilm. *Epsilonproteobacteria* are increasingly recognized in oilfield environments, mainly due to their role in the sulfur cycling in oilfield environments [30,31]. A large number of influent clones (48%) were affiliated with the chemolithoautotrophic *S. kujense*, which utilizes nitrate or oxygen as an electron acceptor. The disappearance of *Epsilonproteobacteria* bacteria in the biofilm is likely to be the result of the strictly anaerobic conditions and lack of nitrate in the anaerobic system (Table 1).

In contrast, *Nitrospira* was the dominant group in the biofilm clone library (40%), within which 18% of bacterial clones were

related to the sulfate-reducing *Thermodesulfovibrio*, which use molecular hydrogen as an energy source. This group of bacteria may be related to the removal of sulfate in the anaerobic reactor. *Thermodesulfovibrio* bacteria are commonly found in thermophilic methanogenic anaerobic sludges [32,33], which are suggested to function in reducing hydrogen partial pressure to ensure that anaerobic substrate degradation is thermodynamically feasible.

Deltaproteobacteria was the second largest bacterial group in the biofilm clone library, while there was no influent clone assigned to the *Deltaproteobacteria*. Many syntrophic bacteria relatives were found in this class. One *Syntrophus*-related OTU (BB-LB39) was found in the biofilm from the hydrocarbon-degrading anaerobic tank. In a study of methanogenic hexadecane metabolism, sequence analysis revealed that most bacterial clones from the consortium were closely related to the genus *Syntrophus* [34]. Similarly, Jones et al. found that an oil-degrading consortium derived from river sediment was dominated by *Syntrophus* sp. [29], suggesting that *Syntrophus*-related organisms may be responsible for the initial oxidation of hexadecane and oil. Meanwhile, it should be noted that a number of *Deltaproteobacteria* clones were assigned to a recently recognized clone cluster, the group TA (Fig. 3), which includes bacteria that are predominant in various methanogenic consortia degrading aromatic compounds, such as phenol, terephthalate, and phthalate isomers [35–37]. Thus, bacteria represented by the group TA-related sequences may be associated with anaerobic degradation of PAHs or other aromatic compounds.

In addition, two biofilm OTUs (BB-HB131 and BB-LB52) were affiliated with *Desulfotomaculum* cluster I in *Clostridia*. Some cluster I organisms, typically known as gram-positive SRB, have lost the capacity for sulfate respiration and adopt a syntrophic lifestyle in concert with methanogens [38]. Based on their phylogenetic position, these OTUs may play a comparable role in the biofilm. *Desulfotomaculum* cluster I bacteria have also been observed in an oil-degrading methanogenic consortium [39].

Based on the above phylogenetic analysis, a microbial community associated with methanogenic hydrocarbon degradation may have become established in the biofilm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2010.01.010.

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