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Bacterial Communities of Biofilms Sampled from Seepage Groundwater Contaminated with Petroleum Oil

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Abstract The diesel-degrading activities of biofilms sampled from petroleum-contaminated groundwaters in urban subway drainage systems were examined in liquid cultures, and the microbial populations of the biofilms were characterized by denaturing gel gradient electrophoresis (DGGE) and 16S rDNA sequence analysis. Biofilm samples derived from two sites (19 K and 20 K) at subway Station N and Station I could degrade around 80% of applied diesel within 20 and 40 days, respectively, at 15°C, and these results were strongly correlated with the growth patterns of the biofilms. The closest phylogenetic neighbor of a dominant component in the 19 K biofilm was *Thiothrix fructosivorans* strain Q (100% similarity). Four dominant strains in the 20 K biofilm were closely related to *Thiothrix fructosivorans* strain Q (100% similarity), *Thiothrix* sp. CC-5 (100% similarity), *Sphaerotilus* sp. IF14 (99% similarity), and *Cytophaga-Flexibacter-Bacterioides* (CFB) group bacterium RW262 (98% similarity). Three dominant members in the Station I biofilms were very similar to uncultured *Cytophagales* clone CRE-PA82 (91% similarity), *Pseudomonas* sp. WDL5 (97% similarity), and uncultured CFB group bacterium LCK-64 (94% similarity). The microbial components of the biofilms differed depending on the sampling site. This is the first report on the isolation of clones highly similar to *Thiothrix fructosivorans* and *Thiothrix* sp. from biofilms in petroleum-polluted groundwaters, and the first evidence that these organisms may play major roles in petroleum degradation and/or biofilm-development.

Key words: Diesel, seepage, groundwater, biofilms, *Thiothrix fructosivorans*, DGGE

During production, distribution, and consumption, petroleum hydrocarbons are commonly stored in tanks and transported through pipelines, where leakage can lead to contamination of both soil and groundwater. In addition, improper disposal of petroleum waste and accidental spills can form other major sources of contamination. Petroleum-contaminated groundwaters show little biodegradation because of lack of microbial sources, and this can result in serious consequences for the environment [41], particularly when contaminated groundwater seeps into subway drainage systems in urban areas.

Many studies have identified microbes capable of degrading a broad range of natural and xenobiotic organic compounds under a variety of environmental conditions [5, 7, 15, 28, 31, 35, 37, 38, 43]. Indeed, a great deal of field research suggests that many leaked petroleum hydrocarbons are naturally biodegraded by microorganisms [9, 32, 53]. Nevertheless, further work is necessary to expand our understanding of contaminant biodegradation and microbial action on petroleum-contaminated groundwater.

A biofilm is defined as a surface accumulation of cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin [14]. The appearances and locations of biofilms differ, depending on the supporting environment. For example, biofilms may be formed at the air-liquid interface of standing water as well as on the solid surface (streambed or pipe wall) of flowing water [25]. In addition, biofilms tend to develop filamentous projections at high-flow velocity, whereas no elongation of these shapes is observed at low-flow velocity [21]. The growth of hydrocarbon-degrading bacteria on hydrocarbons may cause ultrastructural changes such as the formation of a capsular polysaccharide (CPS), which favors the formation of a biofilm [6].

Development of microbial bioflims (mats) following oil pollution has been reported in seawater [17, 50] and coastal stream [1], but not in groundwater. *Pseudomonas* spp. is the most commonly found hydrocarbon-degrading bacteria in biofilms [25, 27], and *Thiothrix* spp., a sulfide-oxidizing

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filamentous bacterium, is the principal bacterial component of many aquatic biofilms [10, 11, 20, 26]. *Sphaerotilus* spp., characterized by filamentous rod-shaped cells, has been isolated from a broad range of natural and manmade environments, including rivers, sewage, and activated sludge [12, 45]. However, *Thiothrix* spp. and *Sphaerotilus* spp. have not previously been associated with biofilms from oil-contaminated groundwater.

Recently, microorganisms involved in the degradation of organic compounds under specific conditions have been investigated using molecular microbial techniques. Since only a small fraction of extant microorganisms are amenable to culture, conventional culture-based methods have often failed to detect or identify a significant portion of microorganisms present within tested samples. However, the relatively recent advent of microbial community analysis based on the amplification of the 16S rDNA gene and techniques such as denaturing gel gradient electrophoresis (DGGE) have provided researchers with a direct cultivation-independent means to examine microorganism populations [4, 34, 36]. In the present study, we employed these techniques to identify microorganisms in biofilms sampled from subway drainage systems containing petroleum-contaminated groundwater seepage, and compared the diesel-degrading activities of these biofilms and their component microbes.

MATERIALS AND METHODS

Site Description and Biofilm Sampling

Oil-contaminated groundwater had seeped into parts of the urban subway discharge systems of Subway Station N in Seoul, Korea and Subway Station I in Anyang, Korea. The groundwater near Station N was contaminated by petroleum spilled from more than 10 oil storage tanks located on the south side of the subway tunnel at a distance of 150–200 m. The source of contamination at Station I was an oil pipe (20.3 cm diameter) located south of the station at a distance of 230 m.

In both drainage systems, we observed free petroleum products and biofilms in the seeped groundwaters. To investigate the properties of the nearby biofilms, we collected four biofilm samples from the drainage canal at Station N, two from the left side of the station (19K-745 and 19K-775), and two from the right side (20K-185 and 20K-190). In addition, we obtained two samples (I-N and I-S) from the drainage canal at Station I. Groundwater and free petroleum products were also sampled for analysis of the petroleum hydrocarbons.

Growth Culture and GC Analysis

For investigation of the diesel-degrading abilities of the six biofilm samples (19K-745, 19K-775, 20K-185, 20K-190, I-N, and I-S), 1 ml of biofilm was mixed with 15 ml of distilled water (DW). The resulting biofilm suspension (0.5 ml) was then inoculated into a 20-ml screw-top test tube containing 5 ml of BMS medium (0.15 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2 \cdot 2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4NO_3, 9.0 g/l $Na_2HPO_4 \cdot 12H_2O$, and 0.08 g/l $(NH_4)_2SO_4 \cdot 12H_2O$) including 8,500 mg/l diesel as the sole carbon source. The samples were then incubated at 15°C with shaking at 180 rpm.

After 0, 6, 10, 13, 17, 20, and 32 days for N samples and 0, 6, 9, 13, 16, 28, and 34 days for I samples, the residual concentration of diesel in each culture broth was determined by gas chromatography. The samples were extracted with hexane, and the petroleum concentrations in the extracts were measured with a gas chromatograph (HP 5890 II plus, Hewlett-Packard, Wilmington, DE, U.S.A.) equipped with a flame ionization detector, using an HP-5 capillary column (30 m length, 0.25 mm internal diameter, and 0.25 mm film thickness). All temperature settings and operation were carried out as described previously [16] and all experiments were conducted in duplicate.

To analyze the growth profiles of the biofilm samples, the aqueous phase (the lower layer below the hexane phase) was used and intermittently monitored by optical density (OD) at 600 nm with a spectrophotometer (Spectronic 20, Milton-Roy Co., Rochester, NY, U.S.A.).

DNA Extraction and PCR Amplification

For extraction of microbial genomic DNA, each biofilm sample was centrifuged at 12,000 rpm for 5 min for removal of moisture (final wet weights were 0.2-0.3 g biofilm), and genomic DNA was extracted with the BIO101 FastDNA SPIN Kit for soil (Q-Biogene, Carlsbod, CA, U.S.A.) according to the manufacturer's instructions.

Fragments of the 16S rDNA were PCR amplified from the extracted genomic DNA using the bacterial universal primers, Bf341 (5'-CCT ACG GGA GGC AGC AG-3') and Br907 (5'-CCC CGT CAA TTC ATT TGA GTT-3'). The PCR reaction mixture contained 1 μl of template, 2.5 μl of 10×Buffer, 0.5 μg/μl BAS, 200 μM dNTP mixture, 20 pmol of primers (each), and 0.5 U/μl Taq polymerase (total volume: 25 μl). The reaction mixtures were preheated at 95°C for 5 min, followed by 16 cycles of 95°C for 1 min, annealing for 1 min (starting at 63°C and decreasing 1°C every second cycle), and 72°C for 1 min. This was followed by 14 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with an additional 5 min at 72°C as a final extension step. The amplification products were purified using the PCR purification kit (Qiagen, Hilden, Germany) and checked for expected product size (550 bp) by agarose gel electrophoresis (100 V, 25 min).

For nested PCR, these products were then reamplified with primers 341fGC (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') [40]

and 518r (5'-ATT ACC GCG GCT GCT GG-3') [42]. The second reaction was carried out in a volume of 50 μl with 1 μl of the PCR product as the template DNA. The samples were preheated at 95°C for 5 min, followed by 28 cycles of touchdown PCR consisting of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by 5 min at 72°C for a final extension. Prior to DGGE analysis, the presence of the expected 180 bp PCR product was confirmed by agarose gel electrophoresis and staining with ethidium bromide.

Denaturing Gradient Gel Electrophoresis (DGGE)

For genetic fingerprinting of the microbial communities present in the biofilm samples, DGGE was performed using a 16×16 cm 8% (w/v) polyacrylamide gel on the Dcode™ System (Bio-Rad, Hercules, CA, U.S.A.) in 7 l of 0.5× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA, pH 7.8). A gradient gel was prepared with 40 and 60% denaturant [100% denaturant contained 7 M urea and 40% (v/v) formamide]. The gel was run at 60°C and 50 V for 14 h, stained with ethidium bromide, documented with a Mupid-21 (Cosmo Bio Co., Tokyo, Japan), and analyzed with the GelCompar II vision 3.5 software (Applied Maths BVBA, Gent, Belgium), using Jaccard coefficient clustering.

DNA Sequencing and Phylogenic Analysis

Bands were excised from the gel, mixed with 20 μl of sterile deionized water, and DNA was extracted by freeze-thaw treatment (freezing at −20°C for 10 min and thawing at 65°C for 3 min, repeated three times). One microliter of the resulting DNA extract was used as the template for PCR amplification with the 341 forward primer (lacking the GC clamp) and the 518 reverse primer under the nested PCR conditions described above. The amplified products were purified with the QIAquick PCR purification kit (Qiagen, Germany), and 1 μl of the PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.), following the manufacturer's protocol. Plasmid clones were amplified in *E. coli* according to standard procedures, extracted from broth cultures using the Wizard® Plus SV Miniprep DNA Purification System (Promega), and the DNA insert was excised with *Eco*RI.

One-hundred ml of cloned PCR fragments using the T7 (5'-TAA TAC GAC TCA CTA CAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA AGA AT-3') primers was sequenced with an ABI Prism model 373A automated DNA sequencer (Perkin Elmer, Foster City, CA, U.S.A.). The obtained sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm feature of the National Center for Biotechnology Information (NCBI) website. Sequences were initially aligned with each other and with manual adjustment using proBiosys version 1.0 operating under the default parameters (proBionic Co., Daejon, Korea). Phylogenetic trees were constructed using maximum likelihood analysis, and maximum parsimony and neighbor joining analyses, as implemented by TreeCon version 1.3b operating under the default parameters (Dankook University, Seoul, Korea). For the neighbor joining analysis, the Kimura correction factor was implemented to account for the short sequence information. Bootstrap values were determined using 100 replicates. The 16S rDNA sequences obtained in this study were deposited at the NCBI databases under the following accession numbers: AY758243-AY758268; AY762984; AY856381.

RESULTS

Morphological Characterization of Biofilms

Photographs of the biofilms that we found in the tunnel drainage systems at Station N in Seoul and Station I in Anyang are shown in Fig. 1. At Station N, the biofilm layers (mats) were fully or partially developed along the drainage canals in two regions that we designated as 19 K and 20 K. Groundwater leaking through drain outlets in the tunnel walls of 19 K and 20 K regions ran in opposite directions toward separate collecting wells. The groundwater depths in the drainage systems at 19 K and 20 K were 5–20 cm and 20–40 cm, respectively. We found that the amount of spilled oil was lower in 19 K than in 20 K; therefore, the biofilms were correspondingly less developed in 19 K than in 20 K (data not shown). This is consistent with a previous report that the development of biofilms in drainage systems seems to be dependent on the amount of spilled petroleum oil [16]. The biofilms observed at regions 19 K and 20 K had similar physical properties, consisting of long, filamentous, milky white components with long loops; and some portions adhered to the floor while others floated and moved in response to the groundwater flow. However, as the distance from the seepage sites increased, the biofilms became dark brown and the filaments shortened. These morphological differences may also be due to the amount of spilled petroleum in the supporting water mass. As the amount of spilled oil gradually decreased in proportion to the distance from the seepage point (due to microbial consumption and diffusion), the biofilms seemed to darken in color, and the mats became smaller and composed of shorter filaments.

At Station I, groundwater seeped through collecting wells at the north and south, and was around 5 cm deep at both sides. The groundwater in the north canal ran faster than that in the south canal. Leaked oil was observed on both sides, and similarly conformed biofilms were evident at both leakage sites. In contrast to the mats from Station N, the biofilm layers at Station I adhered to the floor of the canal, probably due to the shallow water depth, and

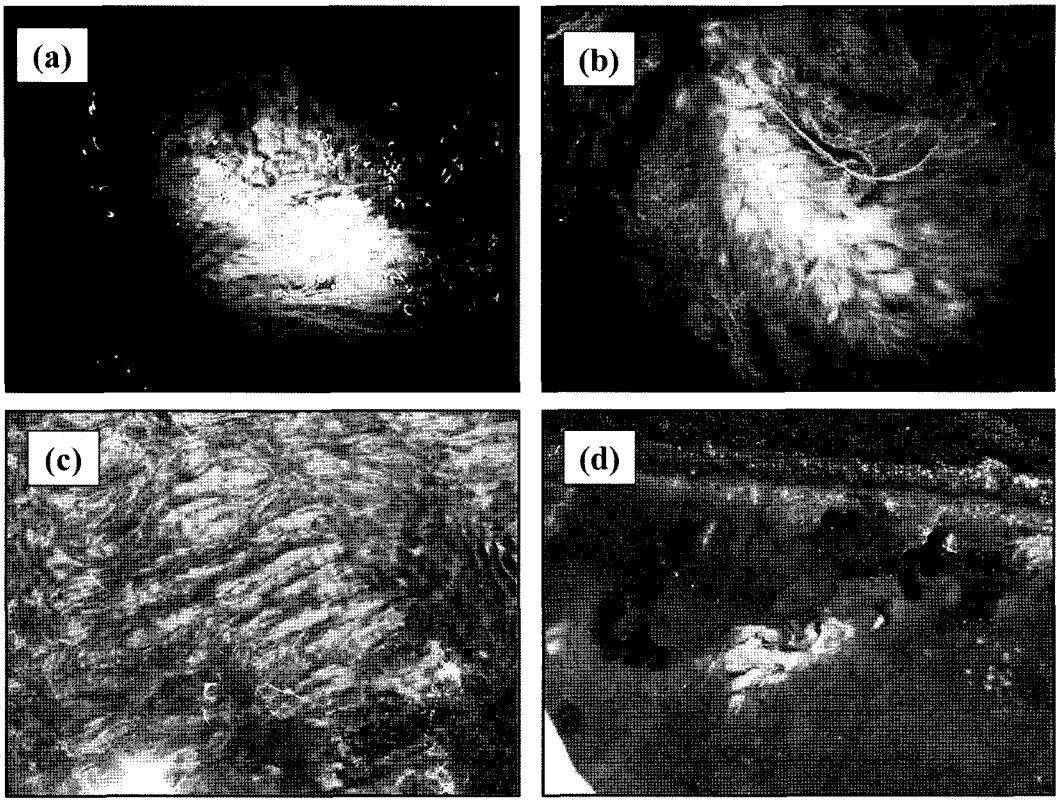


Fig. 1. Drainage canal biofilms sampled from 19 K (a) and 20 K (b) at Station N, and I-N (c) and I-S (d) at Station I.

showed mainly a short-filamentous morphology and brownish color, perhaps because the amount of spilled oil was comparably lower than at Station N.

Characterization of Diesel Degradation and Growth of Biofilm Samples

The diesel degrading activities and growth properties of biofilm samples from 19K-745, 19K-775, 20K-185, 20K-190, I-N, and I-S were examined at 15°C, which was the average temperature of the seepage groundwater. Residual diesel remaining in the biofilm samples was tested for 32 days for four N samples and for 36 days for two I samples [Figs. 2(a), 2(c), and 2(e)]. Biofilm samples from 19K-745, 19K-775, 20K-185, and 20K-190 were able to degrade about 80% of the applied diesel within 20 days, and those from I-N and I-S removed more than 80% within 40 days. The most rapid diesel degradation by samples from 19 K, 20 K, and I occurred between days 10 and 20, days 5 and 18, and days 15 and 40, respectively. Overall, the biofilms from 20 K showed the greatest ability to degrade diesel, followed by the biofilms from 19 K.

The growth patterns of all biofilm samples were spectrophotometrically monitored by optical density at 600 nm [Figs. 2(b), 2(d), and 2(f)]. While both 19 K and 20 K samples revealed a typical pattern of microbial growth showing lag phase (5 days), exponential phase (15 days), and stationary phase (12 days), Station I samples showed a continuous slow growth for 30 days and then rapid growth until the end of this experiment, which lasted for 36 days. These data were consistent with the results of the diesel degradation experiments; the periods of rapid degradation by Station N samples were mirrored by the exponential growth phase of the microbes in these samples. Interestingly, the Station I samples showed slow diesel degradation during the long period of slow growth, but the latter period of rapid growth was not reflected in increased diesel degradation, which may be a slightly delayed process.

For comparison among the growth curves, GC diagrams of different days [Figs. 3(a), 3(b), and 3(c)] indicated that all the biofilms could utilize a broad range of short or long chain alkanes (C_9 to C_{27}) at 15°C, existing in diesel.

Microbial Communities in the Biofilms

The microbial communities of the biofilms obtained in this study were characterized by DGGE analysis. The expected 180-bp PCR products specified by the bacterial 16S rDNA primers were successfully amplified in all of the biofilm samples. Separation of these fragments by DGGE produced unique patterns comprising several distinct bands for each of the samples (Fig. 4). Almost all of the observed bands appeared to be shared among samples obtained from the same location, but many were unique between samples

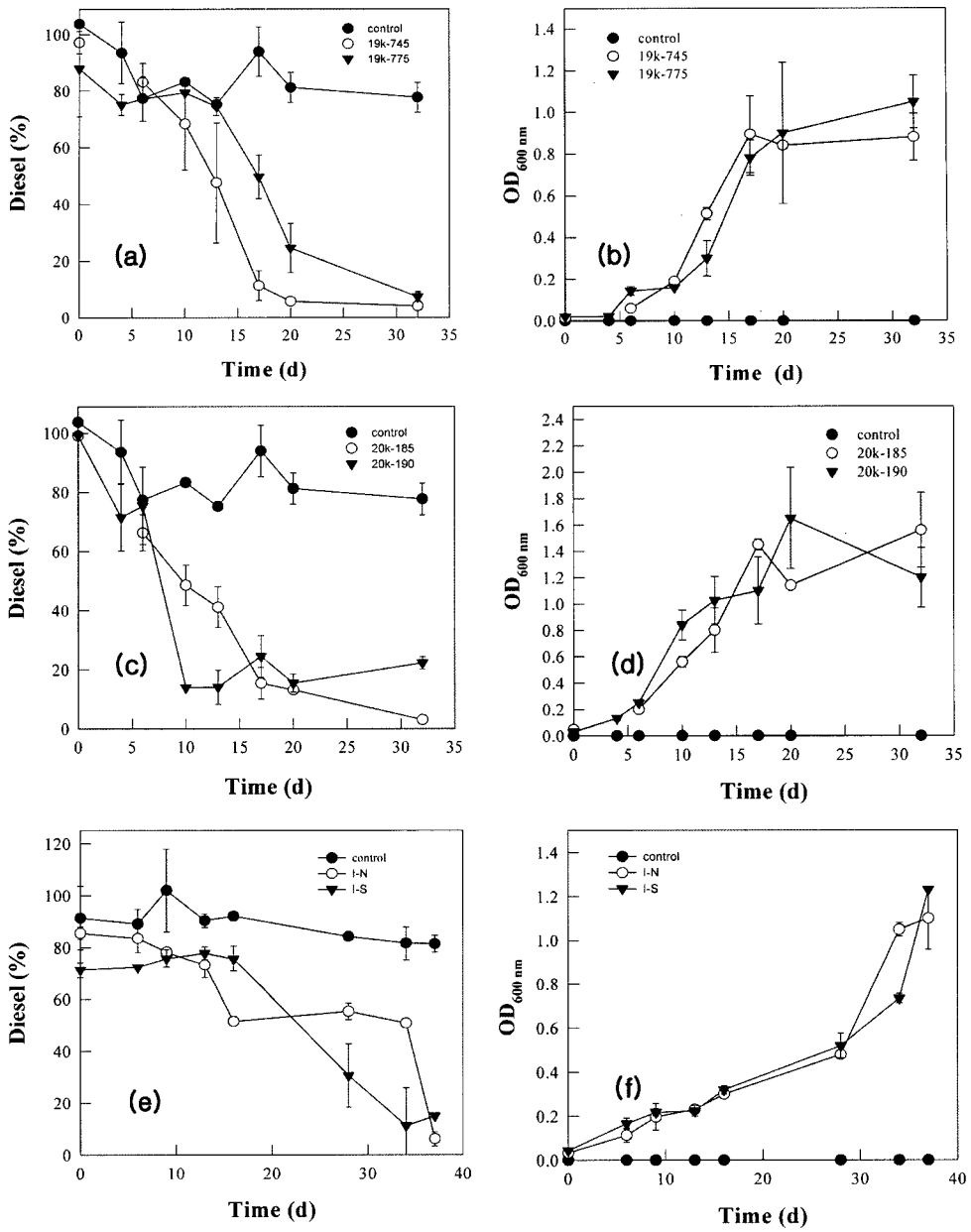


Fig. 2. Diesel degradation and bacterial growth in biofilm samples 19Ks [(a) and (b)], 20Ks [(c) and (d)], and Is [(e) and (f)].

obtained from different locations, especially between Station N and Station I (Fig. 5). Jaccard coefficient clustering was used to account for the presence or absence of individual bands in the original gels as 16S rDNA sequence patterns, and the results revealed 50–70% similarity between biofilms from the same site, approximately 40% within the same station, and 35% between the oil-contaminated sites.

Sequences of about 180 bp were obtained from all of the bands isolated. The highest similarities for each of the sequenced clones for all three locations (19K, 20K, and I) were obtained through BLAST comparisons, as shown in Tables 1, 2, and 3. Table 1 shows a phylogenetic analysis of the 19K biofilm clones based on 16S rDNA sequences. Clone 19K-1 was closely related (96% similarity) to uncultured bacterium clone LBF6, which was originally isolated from environmental samples. Clone 19K-2 was 99% identical to environmental clone CC-5, which was isolated from microbial mats of hydrogen sulfide-rich water in Cesspool Cave and belonged to the *Thiothrix* group of *γ-Proteobacteria* [23]. Clone 19K-3 had the highest similarity (87%) to uncultured CFB (*Cytophaga-Flexibacter-Bacterioides*) group bacterium clone GC1, originally identified in benthic cyanobacterial mats inhabiting a coastal stream site heavily polluted with petroleum

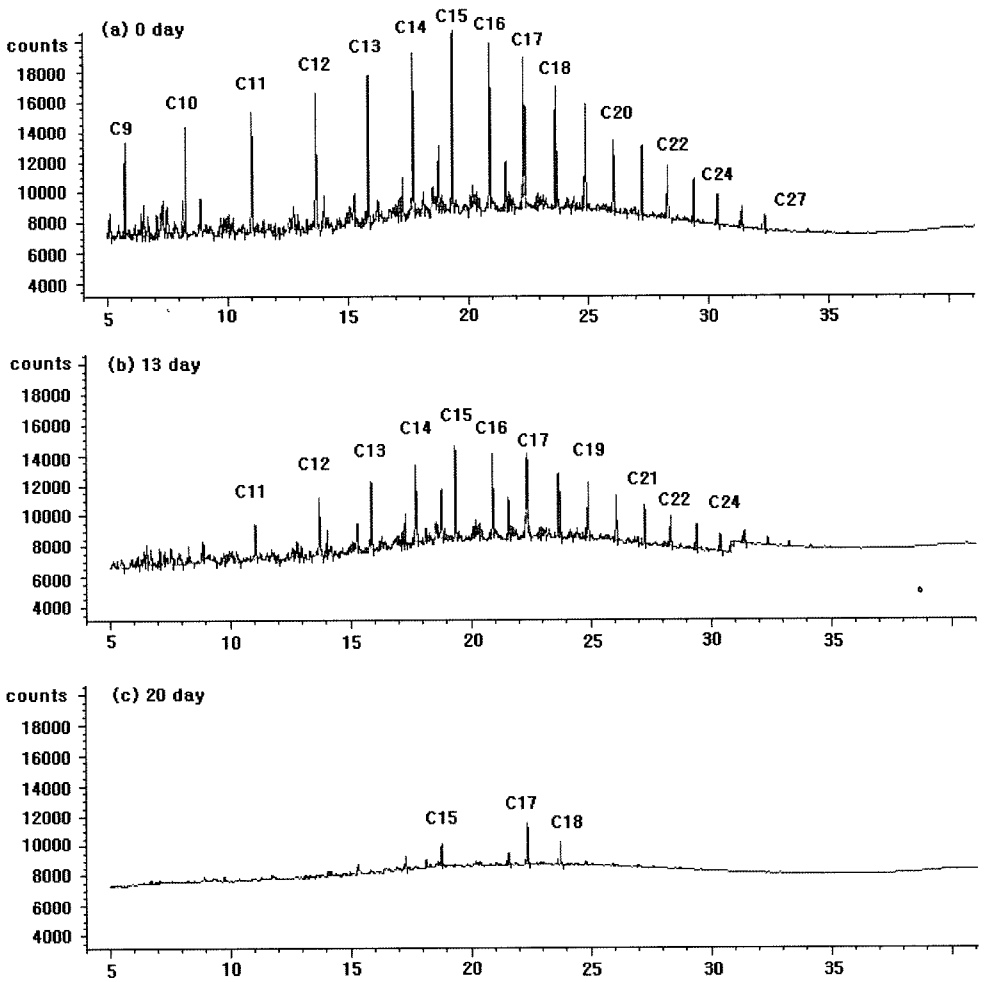
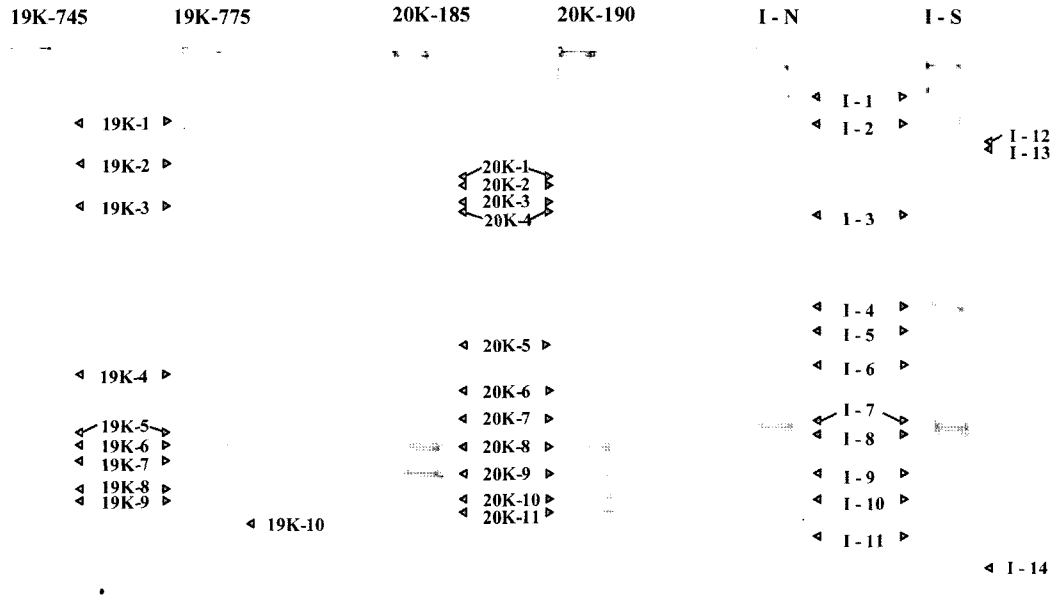


Fig. 3. Diagram of gas chromatograph of diesel content from sample 19K-775 at 0 (a), 13 (b), and 20 (c) days.

compounds and other contaminants [1]. Clone 19K-4 was 98% homologous to uncultured β-*Proteobacterium* clone LTUB09814, which was part of a microbial community in petroleum-contaminated soil. Interestingly, clones 19K-5, 19K-7, 19K-8, and 19K-9 were 100% similar to *Thiothrix fructosivorans* strain Q, which grows in wastewater treatment plants [29].

Clones 19K-5, 19K-8, and 19K-9 showed the most clear bands on DGGE, indicating that the filamentous microorganism *Thiothrix fructosivorans* is likely to be the most dominant species in the 19K biofilms. Clone 19K-6 also produced a clear band, and had 94% homology with uncultured γ-*Proteobacterium* clone CTD47B, previously identified in a diffuse flow hydrothermal vent habitat at Axial Volcano [30]. Clone 19K-10 was most similar to uncultured bacterium clone RB13C5, originating from the microbial community in an *in situ* reactor treatment system for monochlorobenzene-contaminated groundwater [3]; however, the band for this clone in the 19K-745 sample was almost undetectable on DGGE.

The phylogenetic analysis of the 20K biofilm clones is presented in Table 2. Many of the 20K clones were also present in the 19K samples, perhaps because the samples were taken from the same drainage system, although the corresponding groundwaters flowed in opposite directions. Clones 20K-1 and 20K-2 were highly similar (96%) to the filamentous microorganism *Sphaerotilus* sp. L19 y, a β-*Proteobacteria,* originally isolated from the sludge bulking process. Clones 20K-3, 20K-5, 20K-6, and 20K-11 were closely associated with *Thiothrix fructosivorans* strain Q, as were clones 19K-5, 19K-7, 19K-8, and 19K-9. In addition, the closest neighbors of clones 20K-4, 20K-7, and 20K-10 were the environmental clone CC-5 and clone 19K-2. Clones 20K-8 and 20K-9 were most closely related to the CFB group bacterium RW262, obtained from waters of the River Taff, and *Sphaerotilus* sp. IF14 (β-*Proteobacteria*) found in paper mill slimes, respectively [45]. The most prominent DGGE bands were observed for clones 20K-8, 20K-9, 20K-10, and 20K-11, indicating that the dominant species at site 20K were likely the *Cytophaga-Flexibacter-*



(a) (b) (c)

Fig. 4. DGGE profiles of 16S rDNA amplification fragments and corresponding bands for biofilm samples 19K-745 and 19K-775 (a), 20K-185 and 20K-190 (b), and I-N and I-S (c).

Bacterioides group bacterium, *Sphaerotilus* sp., *Thiothrix* sp. (clone CC-5), and *Thiothrix fructosivorans*.

The phylogenetic analysis of the Station I biofilm clones (shown in Table 3) revealed distinct differences from the clones identified in the Station N samples. Clones I-1 and I-2 were analogous with uncultured *Flavobacterium* sp. clone JG37-AG-16 and uncultured *Pseudomonas* sp. clone SR-DGGE, respectively. Clones I-3, I-4, I-12, and I-13 (one of three dominant strains) were 98%, 97%, 95%, and 98% homologous, respectively, to *Pseudomonas* sp.

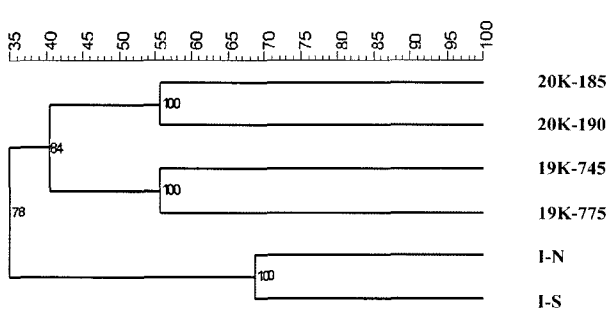


Fig. 5. Jaccard coefficient clustering of normalized DGGE gels showing the sequence patterns of the 19K-745, 19K-775, 20K-185, 20K-190, I-N, and I-S biofilm samples.
Numbers in the tree reveal the cophenetic correlation that is a measure of how faithfully the tree represents the dissimilarities among observations.

WDL5 of γ-*Proteobacteria*, which was originally isolated from linuron-degrading culture [19]. Clone I-5 was associated with uncultured *Cytophagales* bacterium strain KIN30, originally identified in Lake Kinneret (Israel) [47]. Clones I-6, I-10, and I-11 had 97% homology with unidentified bacterium clone Sai4P3-55, originally isolated from reservoir sediment samples [57]. One of the dominant clones, I-7, was closely related (91%) to uncultured *Cytophagales* clone CRE-PA82, a microbe belonging to *Sphingobacteria* found to dominate particle-attached bacterial communities in the Columbia River estuary [18]. Dominant clone I-8 was most closely related to uncultured freshwater bacterium LCK-64, a member of the *Cytophaga-Flexibacter-Bacterioides* group originally identified from the chemocline of meromictic alpine Lake Cadagno [8]. Clone I-9 was most closely related (87%) to uncultured ε-*Proteobacterium* clone 33-PA4B00, which was isolated from a subseafloor habitat following a deep-sea volcanic eruption; this was also the isolation source for the 19K-6-matching strain, uncultured γ-*Proteobacterium* clone CTD47B [30]. Clone I-14 was highly affiliated (100%) with uncultured β-*Proteobacterium* clone UCT N117, which was identified as one of the *Rhodocyclus*-related organisms involved in phosphorus removal in full-scale wastewater treatment plants [58].

Figure 6 shows a dendogram depicting the relationships among the microbial community members of the groundwater

Table 1. Summary of the phylogenetic diversity of biofilm clones (19K, Station N) based on 16S rDNA sequences.

Clone no.	Closest relative	Accession no.	Similarity (%)	Reference
19K-1	Uncultured bacterium clone LBF6	AF392757	164/170 (96%)	Unpublished
19K-2	Environmental clone CC-5	AF207531	169/170 (99%)	23
19K-3	Uncultured CFB (*Cytophaga-Flexibacter-Bacterioides*) group bacterium	AF423372	158/180 (87%)	1
19K-4	Uncultured beta proteobacterium clone LTUB09814	AY144230	171/173 (98%)	Unpublished
19K-5	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29
19K-6	Uncultured gamma proteobacterium clone CTD47B	AF469324	158/168 (94%)	30
19K-7	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29
19K-8	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29
19K-9	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29
19K-10	Uncultured bacterium clone RB13C5	AF407411	156/173 (90%)	3

biofilm samples, as revealed by comparative analysis of related 16S rDNA sequences stored in the GenBank database. Among 35 clones isolated in this study, 19 clones were grouped with 5 known clones within the γ-subgroup of *Proteobacteria*. Six of the clones were clustered with 5 known clones within the β-subgroup of *Proteobacteria*, and 8 were clustered within the *Cytophaga-Flexibacter-Bacterioides* group, *Sphingobacteria*, and *Flavobacteria*. The rest of the clones were classified within the ε-subgroup of *Proteobacteria* (1 strain) or remained unclustered (1 strain). The DGGE results and phylogenetic tree revealed that the dominant biofilm strains living in the petroleum-contaminated groundwaters sampled from Station N and Station I were close neighbors of *Thiothrix fructosivorans*, *Thiothrix* sp., *Pseudomonas* sp. (γ-*Proteobacteria*), *Sphaerotilus* sp. (β-*Proteobacteria*), *Cytophaga-Flexibacter-Bacterioides* group bacteria, and *Cytophagales* bacterium (*Sphingobacteria*).

DISCUSSION

We investigated and compared the diesel-degrading activities of biofilms sampled from subway drainage systems that are fed by petroleum-contaminated groundwater, and identified the bacterial biofilm components using DGGE and sequencing. The development and external appearance of the biofilms were found to differ between the sampling sites, likely due to differences in the amount of petroleum seepage, as described in a previous report [16]. A number of studies have shown that biofilms grown in turbulent flow tend to develop filamentous streamers for increased pressure drop [13, 21, 46, 51]. However, we observed filamentous structures even under a slow flow condition, and the length of the filaments seemed to depend on the amount of escaped petroleum in the supporting groundwater, suggesting that factors affecting filament development may differ according to the microbial species that make up the biofilm. We observed (Fig. 1) that the floating of biofilms in groundwater seemingly occurred in almost standing, relatively deep water (5–40 cm) [25] of Station N, as compared with the conditions at Station I, where attached biofilms were formed under flowing (slow, but faster than at Station N), shallow water conditions (5 cm). Apparently, this shallow depth was sufficient to supply oxygen to the organisms present in the resulting biofilms. It is, therefore, highly likely that they do not need to float for aerobic conditions. The microbial community makeup of each biofilm differed, depending on sampling sites, although all of the samples were collected from petroleum-

Table 2. Summary of the phylogenetic diversity of biofilm clones (20K, Station N) based on 16S rDNA sequences.

Clone no.	Closest relative	Accession no.	Similarity (%)	Reference
20K-1	*Sphaerotilus* sp. L19	AB087568	167/173 (96%)	Unpublished
20K-2	*Sphaerotilus* sp. L19	AB087568	167/173 (96%)	Unpublished
20K-3	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29
20K-4	Environmental clone CC-5	AF207531	165/170 (97%)	23
20K-5	*Thiothrix fructosivorans* strain Q	L79962	167/168 (99%)	29
20K-6	*Thiothrix fructosivorans* strain Q	L79962	167/168 (100%)	29
20K-7	Environmental clone CC-5	AF207531	170/170 (100%)	23
20K-8	CFB group bacterium RW262	AF493694	172/175 (98%)	Unpublished
20K-9	*Sphaerotilus* sp. IF14	AF072917	167/168 (99%)	45
20K-10	Environmental clone CC-5	AF207531	170/170 (100%)	23
20K-11	*Thiothrix fructosivorans* strain Q	L79962	168/168 (100%)	29

Table 3. Summary of the phylogenetic diversity of biofilm clones (Station I) based on 16S rDNA sequences.

Clone no.	Closest relative	Accession no.	Similarity (%)	Reference
I-1	Uncultured *Flavobacterium* sp. clone JG37-AG-16	AJ519403	162/167 (97%)	Unpublished
I-2	Uncultured *Pseudomonas* sp. clone SR-DGGE	AF548764	172/172 (100%)	Unpublished
I-3	*Pseudomonas* sp. WDL5	AF538932	168/171 (98%)	19
I-4	*Pseudomonas* sp. WDL5	AF538932	165/170 (97%)	19
I-5	Uncultured *Cytophagales* bacterium strain KIN30	AY136073	170/171 (99%)	47
I-6	Unidentified bacterium clone Sai4P3-55	AJ518732	168/173 (97%)	57
I-7	Uncultured *Cytophagales* clone CRE-PA82	AF141549	156/171 (91%)	18
I-8	Uncultured freshwater bacterium LCK-64	AF107332	161/171 (94%)	8
I-9	Uncultured *Epsilon-proteobacterium* clone 33-PA4B00	AF468740	149/170 (87%)	30
I-10	Unidentified bacterium clone Sai4P3-55	AJ518732	167/171 (97%)	57
I-11	Unidentified bacterium clone Sai4P3-55	AJ518732	168/173 (97%)	57
I-12	*Pseudomonas* sp. WDL5	AF538932	163/170 (95%)	19
I-13	*Pseudomonas* sp. WDL5	AF538932	168/171 (98%)	19
I-14	Uncultured *Beta-proteobacterium* clone UCT N117	AY062127	168/168 (100%)	58

polluted groundwaters. This diversity might be due to the presence of varying indigenous bacterial communities at the different locations (Fig. 5).

Our comparative analyses of degradation rates and growth rates showed that the 19K and 20K samples from Station N outperformed those from Station I (Fig. 2). The 19K and 20K biofilms, which developed in the presence of higher levels of contamination at Station N, appeared to more easily adjust to diesel hydrocarbons than did biofilms grown in lower levels of oil pollution at Station I. Likewise, the analysis of clones from each biofilm sample, using sequence data from the DGGE bands, showed that

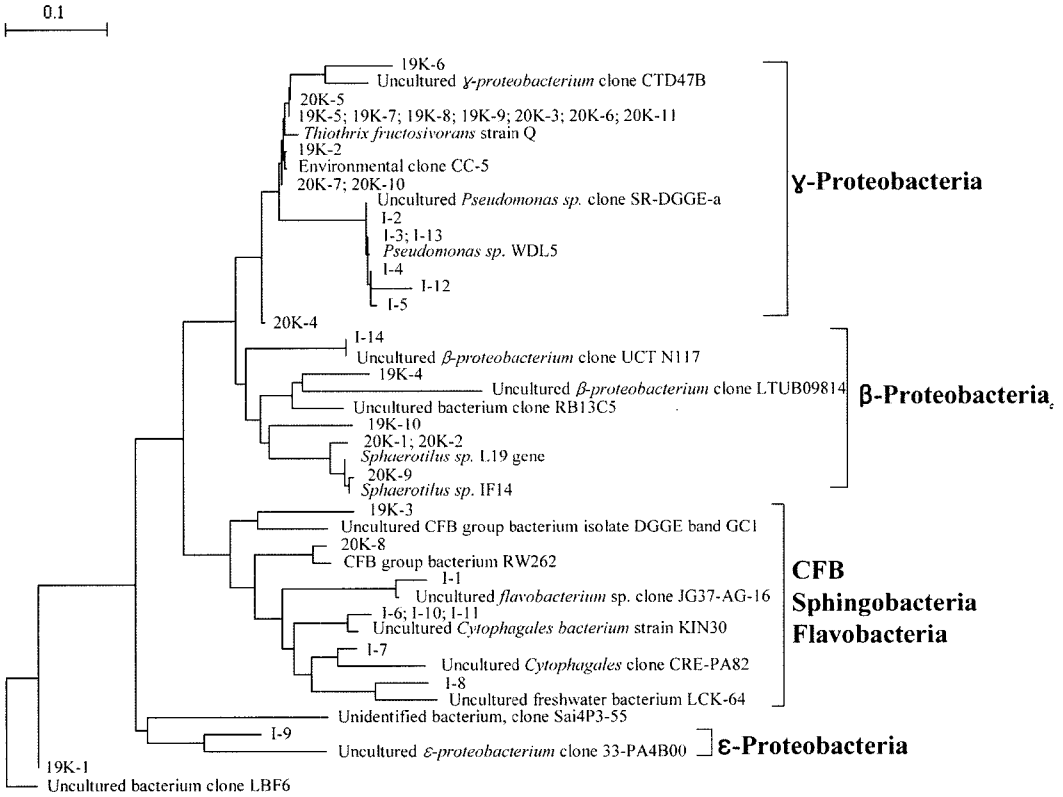


Fig. 6. Phylogenetic tree illustrating the relationships among the closest relatives in the RDP and GenBank databases and the biofilm clones.
The scale bar represents a 10% estimated sequence divergence.

two major strains were common among 19K and 20K samples, whereas none was shared with I samples. The closet neighbors of the two common major microbes are *Thiothrix fructosivorans* strain Q, and Environmental clone CC-5, which were phylogenetically affiliated with the *Thiothrix* group in a previous work [23]. These two strains likely play important roles in enhancing the diesel degradation, because band brightness revealed that the *Thiothrix fructosivorans* strain Q-like clones were the most dominant strain in 19K biofilm samples and one of four dominant strains in 20K biofilm samples, and environmental clone CC-5-like clones were also one of four dominant strains in 20K and one of seven members in 19K (Fig. 4). The members of the *Thiothrix* group are known as filamentous sulfur-oxidizing bacteria [55], and have been described as components of biofilms formed in water wells [10], irrigation systems [24], drainage systems [33], and wastewater treatment plants [56]. However, to the best of our knowledge, this report is the first to show that *Thiothrix* sp. was isolated from oil-polluted groundwater.

A high concentration of sulfate (SO_4^{2-}) in the drainage system at Station N has recently been reported [13], reflecting the presence of sulfur-oxidizing microbes such as *Thiothrix* sp., which can oxidize hydrogen sulfide (H_2S) or thiosulfate ($S_2O_3^{2-}$), both components of petroleum oil [2, 48]. Furthermore, many reduced sulfur compounds can be used as energy sources for sulfur-oxidizing bacteria [23]. Brigmon *et al.* [11] reported that *Thiothrix* spp., a principal component of aquatic biofilms, caused biofouling in various water systems and had a white, filamentous appearance. Their observations are consistent with our finding of a white, filamentous morphology of mats at 19K and 20K, where *Thiothrix* sp. was a dominant species. Although *Thiothrix fructosivorans* was the first identified [29], and has since been isolated from activated sludge [29, 48], further work is required to determine its degrading capacity for various organic contaminants including petroleum hydrocarbons.

Sphaerotilus sp. IF14 and L19 were closely related to one of four major components and to a minor member, respectively, of the 20K biofilms. *Sphaerotilus* sp. is characterized by a tubular sheath enclosing rod-shaped cells, and can be mainly found in flowing water [52], sewage [45], and activated sludge [22]. Even though *Sphaerotilus* sp. does not directly degrade petroleum as a carbon source [16], this species plays a vital role as a biocontainer; it traps free petroleum products by means of extracellular polymetric substances, allowing further removal by other biofilm components [45]. In addition, *Sphaerotilus* sp. contributes to the formation of biofilms by stabilizing colonies of other organisms [49].

Pseudomonas sp. WDL5 was most clustered with a dominant strain in Station I biofilms. Although this *Pseudomonas* sp. strain was isolated from linuron-degrading cultures and was very dominant in the DGGE patterns, it has previously been reported not to use linuron as an N or C source [19]. Although this strain showed no direct degradation of linuron, it has a synergistic effect with another linuron-degrading bacterium [19]. In addition, *Pseudomonas* sp. can generally degrade many petroleum hydrocarbons and can exist widely in nature as a component of biofilms [25, 27]. Therefore, this strain appears to play some role in direct degradation of petroleum hydrocarbons or a synergistic effect.

Among the dominant strains, three were closely related to unknown strains at the genus level. Strain I-7, which was the most dominant strain in the Station I biofilms, was most similar to uncultured *Cytophagales* clone CRE-PA82 [18]. This strain was originally isolated from the particle-attached community responsible for most of the degradation of detrital organic matter in an estuary, so it likely plays a key role in forming biofilms and degrading petroleum hydrocarbons in the groundwater at Station I. One of the other dominant strains (20K-8) was associated with CFB group bacterium RW262, originally isolated from a river. Dominant strain I-8 was closely related to uncultured freshwater bacterium LCK-64, a member of the *Cytophaga-Flexibacter-Bacterioides* group found in a dense microbial community at the redox transition zone of a meromictic alpine lake. Even though the two identified dominant strains were closer to unknown species, they could likely be classified into the *Cytophaga-Flexibacter-Bacterioides* group. CFBs may be major contributors to the formation of epilithic biofilms, and may play a critical role in the turnover of organic pollutants, because of their heterotrophic activity on various organic carbons [44].

Uncultured CFB group bacterium GC1 and uncultured bacterium clone RB13C5 were phylogenetically related to minor biofilm components 19K-3 and 19K-10, respectively. These 19K-3 and 19K-10 components were isolated from a site heavily polluted with diesel, petroleum, and many other pollutants, and from monochlorobenzene-contaminated groundwater, respectively. Thus, these two strains likely degrade or aid in the degradation of petroleum in the biofilms. Because uncultured γ-*Proteobacterium* clone CTD47B and uncultured ε-*Proteobacterium* clone 33-PA4B00, nearest neighbors to minor components 19K-6 and I-9, respectively, were isolated from an aquatic habitat where hydrogen sulfur was plentiful from volcanic eruption, these two strains likely function to oxidize hydrogen sulfide, as does the major dominant strain of 19 K, *Thiothrix fructosivorans*. Other minor members in the biofilms have yet unknown roles that will require further study for their elucidation.

As shown in the phylogenetic tree (Fig. 6), most of the isolated clones fell within the proteobacterial (γ and β) and *Cytophaga-Flavobacterium* groups. This result is concordant

to previous reports that these groups are present in oil-polluted sites [39, 54]. Whereas almost all of the clones in samples from 19K and 20K were clustered within these three groups, around half of the isolated clones from Station I samples fell within the ε-*Proteobacteria*, *Flavobacteria*, and *Sphingobacteria* groups (Table 3). These results indicate that the biofilms at Station I contain a broader range of microbes than those from Station N. However, the Station N biofilms were more effective at degrading applied diesel, indicating that it is not always true that the higher microbial variation in a biofilm reflects more effectiveness for biodegradation of contaminants. Therefore, our results indicate that the identities of the microbial components composing a biofilm may be more important than the absolute microbial diversity.

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