Characterization of Active Microbes in a Full-Scale Anaerobic Fluidized Bed Reactor Treating Phenolic Wastewater

CHIA-LUNG CHEN1,2, JER-HORNG WU3, I-CHENG TSENG4, TEH-MING LIANG5, and WEN-TSO LIU6,7,*

1Department of Civil Engineering, National University of Singapore, Block E1A #07–03, Engineering Drive 2, Singapore 117576; 2Centre of Innovation in Environmental and Water Technology, Ngee Ann Polytechnic, Block 34 #01–01, 535 Clementi Road, Singapore 599489; 3Department of Environmental Engineering, National Cheng Kung University, Ta-Hsueh Road, Tainan 701, Taiwan; 4Department of Life Sciences, National Cheng Kung University, Ta-Hsueh Road, Tainan 701, Taiwan; 5Energy and Environment Research Laboratories, Industrial Technology Research Institute, 321 Kuang Fu Road, Hsin Chu 300, Taiwan; 6Division of Environmental Science and Engineering, National University of Singapore, Block EA #03–12, Engineering Drive 1, Singapore 117576; and 7Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, 205 North Mathews Ave, IL 61801, USA

(Received January 29, 2009—Accepted April 2, 2009—Published online, April 25, 2009)

This study investigated the active microbial community in a full-scale granular activated carbon-anaerobic fluidized bed (GAC-AFB) reactor treating wastewater from the manufacturing of phenolic resin, using 16S rRNA-based molecular analyses. The results of cDNA from 16S rRNA revealed that Methanosaeata-related (83.9% of archaeal clones) and Syntrophorhabdaceae (formerly named Deltaproteobacteria group TA)-related (68.9% of bacterial clones) microorganisms were as the most predominant populations in the phenol-degrading GAC-AFB reactor. The high abundance of Syntrophorhabdaceae was supported by a terminal restriction fragment length polymorphism (T-RFLP) analysis, which showed that a Syntrophorhabdaceae-like fragment of 119 bp (~80% of total fragments) was the most predominant phylotype. Furthermore, fluorescence in situ hybridization (FISH) analyses suggested that Syntrophus and Chloroflexi-like cells were also in high abundance in the GAC biofilm. A non-layered structure of microorganisms was found in the GAC biofilm, where Methanosaeta (thick filamentous), Syntrophorhabdaceae (oval-shaped), Syntrophus (small rods) and Chloroflexi (thin-filamentous) were randomly distributed with high abundance. These findings greatly improve our understanding of the diversity and distribution of microbial populations in a full-scale mesophilic bioreactor treating an actual phenol-containing waste stream.

Key words: phenol, anaerobic, microbial, phylogenetic, FISH

Syntrophic association is an important process in anaerobic catabolism, whereby two or more microorganisms cooperate closely to completely degrade a substance neither can degrade alone. This process generally requires interaction between fermentative bacteria and methanogenic archaea (methanogens) to overcome the thermodynamic barrier in the initial step of degradation (e.g., fatty acids, solvents and aromatic substrates), and leads to the production of methane. Biodegradation of phenol under methanogenic conditions has been known for more than two decades (42, 48), and a number of different anaerobic processes (23, 37, 40, 42) have been developed to successfully treat industrial waste streams containing phenol. A bottleneck to the starting up of these anaerobic processes was considered to be the cultivation/acclimation of unique microbial specialists. A few reports have characterized these syntrophic phenol-degrading microorganisms. Fang and co-workers (16, 49) suggested that Desulfotomaculum, Clostridium and Syntrophus, together with Methanosaeataceae, Methanomicrobiales and Methanobacteriaceae, were the predominant microbial populations in a laboratory-scale upflow anaerobic sludge bed (UASB) reactor that degraded phenol under ambient temperature (26°C). Last year, we reported that Deltaproteobacteria group TA- and Pelotomaculum-related populations were the most important fermentative bacteria, in phenol-degrading enrichments under mesophilic (37°C) and thermophilic (55°C) conditions, respectively (9). More recently, a syntrophic phenol-degrading bacterium (Syntrophorhabdus aromaticivorans) was isolated, and a novel family, Syntrophorhabdaceae (formerly known as Deltaproteobacteria group TA), was proposed (30). Although efforts have been made to identify the specific microbial populations important for the degradation of phenol under anaerobic conditions (9, 16, 49), microorganisms that grow under well-controlled conditions in the laboratory may not fully represent those in full-scale bioreactors receiving actual wastewater.

In our previous study, a Syntrophorhabdaceae-like bacterial population was also found abundantly by fluorescence in situ hybridization (FISH) in a full-scale mesophilic granular activated carbon anaerobic fluidized bed (GAC-AFB) reactor treating phenol-containing wastewater (9). Though the dominance of Syntrophorhabdaceae-like populations in a real wastewater treatment system may provide a lead to improving the startup strategy, an in-depth understanding of the exact microbial community in a full-scale plant is necessary to facilitate the development of full-scale processes.

In this study, a sludge sample from a full-scale GAC-AFB reactor treating industrial wastewater from the manufacturing of phenolic resin under mesophilic conditions was used
to characterize the diversity and spatial distribution of microorganisms. To investigate the composition of active microbial populations, archaeal and bacterial community structures were analyzed using cDNA reverse-transcribed from 16S r RNA. The spatial distribution of important phylogenetic groups of microorganisms was determined by FISH.

Materials and Methods

Biological GAC sample

The biological GAC sample was obtained from a full-scale AFB reactor treating wastewater from the manufacturing of phenolic resins. The reactor had a total volume of 275 m³ (5 m in diameter and 16 m in height) about 25% of which was filled with GAC at a size of 1–3 mm as a carrier for biomass growth. It was started-up in 1999 with swine manure as the inoculum and had been operating continuously for more than six years when the GAC sludge was sampled for analysis. The influent wastewater contained 2,500–5,200 mg/L COD, with 60% contributed by phenol and formaldehyde, respectively.

During the sampling period, the reactor had a COD removal efficiency of about 85% under a loading rate of 3–5 kg COD m⁻³ day⁻¹. GAC particles were randomly sampled from the reactor and transported on ice to the laboratory. Samples were preserved at −80°C prior to the extraction of total RNA, and at −20°C after fixation for FISH.

Batch assay for determining microbial activity

Phenol degradation and the specific methanogenic activity (SMA) of the biological GAC sludge from the full-scale AFB reactor were examined in 300-mL serum bottles to determine levels of microbial activity. The biofilm-attached GAC particles were first homogenized in an oxygen-free flask purged with nitrogen gas. After homogenization, the sludge solution containing GAC pellets was kept static for 3–5 min, allowing a separation of GAC pellets from the sludge (biofilm biomass). The sludge supernatant was then used as the inoculum for the batch assay.

A 300-mL serum bottle was filled with 95 mL of anaerobic culture medium (3, 8) and 5 mL of biofilm biomass under an atmosphere of 80% N₂–20% CO₂ (v/v). The quantity of sludge used in the degradation experiment ranged from 162 to 223 mg volatile suspended solids (VSS) L⁻¹, measured after the experiments. A concentrated phenol solution was added as the sole carbon source to a phenol solution at concentrations of 80 to 634 mg L⁻¹ using a disposable syringe. Duplicate bottles were prepared for testing the degradation of phenol at various concentrations, and incubated at 35°C in an orbital shaker. Biogas composition (methane, hydrogen and carbon dioxide) in the headspace of serum bottles was measured using a gas chromatograph equipped with a GC-17A thermal conductivity detector (Shimadzu, Kyoto, Japan). Soluble short-chain volatile fatty acids (i.e., acetate, propionate and butyrate) and phenol in the solution were determined by using a gas chromatograph equipped with a GC-14B flame-ionization detector (Shimadzu), and HPLC detectors equipped with an Eclipse XDB-C18 column (Agilent, Palo Alto, CA, USA) and an SP-D10A UV-detector respectively, according to methods described previously (8). The SMA was obtained by calculating the initial methane production rates (mL CH₄ day⁻¹) per gram sludge (g VSS) in each serum bottle.

SEM

The GAC sludge sample was fixed in 0.1 M phosphate buffer with 4% glutaraldehyde at 4°C for 8 h. Dehydration was carried out in a series of ethanol solutions (50, 75, 85, 95 and 100% [v/v]), followed by critical point drying using a HCP-2 critical point dryer (Hitachi, Tokyo, Japan), and subsequently coated with gold by a JFC-1100 ion sputter (JEOL, Tokyo, Japan). The specimen was examined using a S-2500 scanning electron microscope (Hitachi).

RNA extraction, clone library, and phylogeny analysis

Total RNA of the GAC sludge was extracted using a combination of physical and chemical lysis. Briefly, the biomass pellets were lysed in a low pH buffer (10 mM sodium acetate, 10 mM NaCl, 3 mM EDTA [pH 5.1]) and 0.5 g of sterile glass beads (0.1 mm in diameter) by a bead-beater (BioSpec Products, Bartlesville, OK, USA) at 4,800 oscillation s⁻¹ for 4 min. Subsequently, chemical-lysis (0.5 mg mL⁻¹ lysozyme) and phenol-chlorform-isoamyl alcohol (25:24:1, v:v:v; pH 5.1) purification were performed. RNA was harvested by ethanol precipitation with a 1/10 volume of 3 M sodium acetate solution (pH 5.2) and resuspended in diethyl pyrocarbonate (DEPC)-treated water. DNA was eliminated by RQ1 RNase-free DNase (Promega, Madison, WI, USA) treatment according to the manufacturer’s protocol. The quality of the total RNA was checked by 1% agarose electrophoresis and absorbance was measured at 260 nm and 280 nm using a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Purified total RNA (1 μg) was then reverse-transcribed into ribosomal cDNA by M-MuLV reverse transcriptase (New England Biolabs, Hertfordshire, UK) with the prokaryotic 16S rRNA primer 1490R (43), and incubated at 37°C for 1 h. The cDNA was used as a template in PCR amplification for further cloning and terminal fragment length polymorphism (T-RFLP) analyses.

For cloning, microbial communities from the domains Archaea and Bacteria were amplified with the archaeal primer set A1F and A1100R (13), and bacterial primer set EUB8F and 1490R (43), respectively. A TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) was used to construct the clone libraries according to the manufacturer’s directions. Restriction fragment length polymorphism (RFLP) and denaturing gradient gel electrophoresis (DGGE) were used to screen archaeal and bacterial clone libraries, respectively (25, 28). Partial 16S r RNA sequences of representative archaeal (>1,000 bp) and bacterial (>1,400 bp) clones were obtained by sequencing on a CEQ 8000 (Beckman Coulter) and compared to available rRNA gene sequences in GenBank using the NCBI BLAST program (1). Chimeric artifacts were determined using Pintail software (4) and excluded from further phylogenetic analysis. The MEGA3 program was used to align the sequences (by provided ClustalW function) and construct a neighbor-joining tree with the Juke-Cantor correction and bootstrapping for 1,000 replicates used to estimate the confidence of the tree’s topology (22).

T-RFLP

T-RFLP analysis was performed as described previously (24). cDNA from the GAC sludge was amplified by PCR using the domain Bacteria-specific primer set 9F (with cytosine 5 [C]₅ [fluorescently labeled at the 5’ end] and 927R (8). Single-stranded pseudo-terminal restriction fragments were removed by digestion with a mung bean nuclease (New England Biolabs) (12). The PCR products were subsequently purified using the QiAquick PCR purification kit (Qiagen, Valencia, CA, USA), and digested with a tetramer restriction enzyme, MspI (New England Biolabs) at 37°C for 3 h. The MspI-digested terminal restriction fragments (T-RF) were separated and detected using a CEQ 8000 automatic DNA sequencer (Beckman Coulter). Fragment sizes were determined based on internal standards (DNA size standard kit-600, Beckman Coulter) using the CEQ 8000’s software (Beckman Coulter). Peak area was used to assess the relative abundance of the major peaks, and only those T-RFs with an abundance of greater than 1% of total intensity were used. T-RFLP fingerprints were obtained based on the average of two duplicates, and reproduced with Microcal™ Origin® Version 6.0 (Microcal Software, Northampton, MA, USA).

The phylogenetic affiliation of the major peaks observed in the T-RFLP analysis was theoretically identified based on an in-silico T-RFLP analysis of the bacterial clones.

FISH

GAC sludge particles were pretreated according to a protocol described previously for FISH analyses (2). The sample was fixed

Phenol-Degrading Methanogenic Consortium
in a 4% paraformaldehyde solution for 8 h at 4°C. The GAC particles were embedded in Jung Tissue Freezing Medium™ (Leica, Nussloch, Germany), and subsequently cut into 20 µm-thick sections with a CM3050S cryotome (Leica). Hybridization was carried out at 46°C for 3 h with hybridization buffer containing 5 ng µL⁻¹ of a specific fluorescent probe. The oligonucleotide probes used in this study included EUBmix (i.e., EUB338, EUB338-II, EUB338-III), targeting most of the members in the domain Bacteria (2, 10); ARCH915, targeting most of the members in the domain Archaea (2); Delta-TA664, targeting members of Syntrophorhabdaceae (Deltaproteobacteria group TA) (9); MX825, targeting members of Methanosaetaceae (31); Syn424, targeting members of Syntrophus (5); and GNSB941, targeting members of Chloroflexi (18). FISH hybridization was performed under stringent conditions appropriate for each probe using different formamide concentrations in the hybridization buffer (20% [v/v] for MX825 and GNSB941; 25% for Delta-TA664 and Syn424; and 35% for EUBmix and ARCH915). When dual-staining FISH was performed, probes were labeled with cyanine (Cy3) and Cy5, respectively. FISH-stained images were captured by a LSM 5 Pascal confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with two helium/neon lasers (543 nm and 633 nm) under X100 objective lens. More than 30 biofilm sections from at least five GAC particles were examined to determine the general spatial microbial structures.

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences reported in this paper are EU399654 to EU399680.

Results

Batch degradation

Fig. 1A illustrates the degradation of phenol in the batch test with initial phenol concentrations ranging from 80 to 634 mg L⁻¹. The time taken for complete degradation increased with the concentration. Complete degradation of phenol after 15 days was only achieved at a starting concentration of between 80 and 259 mg L⁻¹. At a starting concentration of 431 mg L⁻¹ and 634 mg L⁻¹, approximately 61.7% and 23.2% of the initial phenol concentrations were degraded, respectively, after 15 days. No lag phase was observed for the serum bottles with starting concentrations of 80 mg L⁻¹, whereas about a two-day lag was observed for the serum bottles with higher initial phenol concentrations. Fig. 1B shows that the SMA decreased with an increase in the phenol concentration. The highest SMA, 130.1 (mL-CH₄ g-VSS⁻¹ day⁻¹), was achieved at a starting concentration of 80 mg L⁻¹. When the phenol concentration exceeded 80 mg L⁻¹, the microbial activity was gradually inhibited and the SMA decreased to 88.4 (mL-CH₄ g-VSS⁻¹ day⁻¹) at a starting concentration of 634 mg L⁻¹. Phenol can be converted to methane at a rate of 0.20–0.26 mL-CH₄ mg-phenol⁻¹.

SEM of GAC sludge

The GAC particles appeared to be plate-like with a thick biofilm attached to the surface of GAC. Fig. 2 shows a SEM image of a typical GAC particle from the full-scale AFB reactor. The surface of the spherical particle was porous with numerous cavities (Fig. 2A). Filamentous microorganisms (bamboo-shaped cells) (0.7–0.8×5–15 µm) (arrow 1) were predominantly observed on the surface of the GAC sludge (Fig. 2B). These cells morphologically resembled members of acetotrophic Methanosaeta-like methanogens, which were

![Fig. 2. SEM images of (A) a whole GAC particle and (B) the surface of a GAC particle from the full-scale phenol-degrading GAC-AFB reactor.](image-url)
reported to be the predominant population in the mesophilic phenol-degrading granules (7, 15). Although no other predominant morphotypes were observed in Fig. 2B, at least two morphotypes were noticeable, including oval-shaped cells (0.6–0.8×1 μm) (arrow 2) and small rods (0.3×1.2 μm) (arrows 3). The oval-shaped cells morphologically resembled the dominant phenol-degrading bacterial population (i.e., Syntrophorhabdaceae) in the mesophilic enrichment process described previously (9).

**Microbial compositions as revealed using a 16S rRNA clone library**

Clonal libraries were individually constructed for active members in the domains Archaea and Bacteria. A total of 56 and 106 clones with the 16S rRNA inserts were randomly selected from the archaean and bacterial libraries, respectively, and screened for distinct phylotypes using RFLP and DGGE. Seven and 20 unique sequence types or operational taxonomy units (OTUs) were obtained from the archaean and bacterial libraries, respectively, and subjected to further sequencing and phylogenetic analysis. For the members of the domain Archaea, all seven OTUs were affiliated with methanogens in the Eucaryarchaea (Fig. 3). Among them, *Methanosaeta*-related populations were most predominant in the phenol-degrading methanogenic community, as five OTUs, representing 83.9% of all archaean clones, were closely related to mesophilic acetotrophic *Methanosaeta concilii*. *Methanosaeta*-related populations could be phylogenetically assigned to two subclusters. One OTU (PS-Ar5, 37.5%) was closely related to the clones found in the MP enrichment system (9), and the other four (PS-Ar14, PS-Ar19, PS-Ar24 and PS-Ar56; 46.4%) were closely related to clones from reactors treating municipal wastewater. Additionally, PS-Ar61 (5.4%) was closely related to *Methanomethylovorans hollandica*, an obligately methylotrophic methanogen, which can utilize methanol, methylamines, methanethiol and dimethyl sulfide (26) in the family Methanosarcinaceae. The remaining OTU (PS-Ar78, 10.7%) was closely related to the mesophilic phenol-degrading clone MP126 (9) and clustered with the environmental clones and one novel hydrogenotrophic *Methanolinea tarda* (21) in Methanomicrobiales. The detection of acetotrophic and hydrogenotrophic methanogen-related sequences was consistent with the principle of anaerobic phenol degradation, in which methanogens played a role in converting the intermediates acetate and H₂/CO₂ (formate) into methane and carbon dioxide in the subsequent step.

For the members of the domain Bacteria, 20 OTUs were identified and classified into five different taxonomic groups (Fig. 4). Among them, 14 OTUs, accounting for 88.7% of...

![Phylogenetic tree of archaeal 16S rRNA sequences obtained from the full-scale phenol-degrading GAC-AFB reactor.](image)

**Fig. 3.** Phylogenetic tree of archaeal 16S rRNA sequences obtained from the full-scale phenol-degrading GAC-AFB reactor. The MEGA3 package was used to align relevant sequences (ClustalW program), and to construct a neighbor-joining tree with the Jukes-Cantor model (bootstrapping number=1,000). The 16S rRNA sequence of *Methanopyrus kandleri* (M59932) was used as the outgroup. Only bootstrap values greater than 50% are indicated at branch points. Percentages of total archaeal clones are shown in parentheses. The scale bar represents the estimated number of nucleotide changes per sequence position.
Fig. 4. Phylogenetic tree of bacterial 16S rRNA sequences obtained from the full-scale phenol-degrading GAC-AFB reactor. The MEGA3 package was used to align relevant sequences (ClustalW program), and to construct a neighbor-joining tree with the Jukes-Cantor model (bootstrapping number=1,000). The 16S rRNA sequence of Aquifex pyrophilus (M93548) was used as the outgroup. Only bootstrap values greater than 50% are indicated at branch points. Percentages of total bacterial clones are shown in parentheses. The scale bar represents the estimated number of nucleotide changes per sequence position.
all the bacterial clones, were affiliated with the *Deltaproteobacteria*. In *Deltaproteobacteria*, 11 OTUs (68.9% of bacterial clones) were affiliated with a recently proposed family *Syntrophorhabdaceae* (30), which was a clone cluster formerly known as “group TA” (44). Within *Syntrophorhabdaceae*, six OTUs (50.0%) were closely related to the clones retrieved from mesophilic terephthalate- and phenol-degrading consortia (9, 44) and a recent phenol-degrading isolate *Syntrophorhabdus aromaticivorans* (29, 30), while the remaining five OTUs (PS-Ba100, PS-Ba90, PS-Ba101, PS-Ba151 and PS-Ba160; 18.9%) were affiliated with 16S rRNA clones mainly obtained from soil and contaminated aquifer (6, 11). In addition, PS-Ba100 (2.8%) was very similar to the environmental clone WB03 retrieved from freshwater sediment with 99.9% sequence similarity, forming a cluster with *Desulfonema magnun* as a close neighbor. Moreover, PS-Ba83 (5.7%) was clustered with environmental clones and branched from the *Syntrophus* group in which clone PS-Ba14 (11.3%) was placed with *Syntrophus gentianae* as the closest relatives. For the non-*Deltaproteobacteria* OTUs, two were affiliated with green nonsulfur bacteria (*Chloroflexi*) (4.7%), one with the candidate division OP8 (3.8%), two with the *Acidobacteria* (1.8%) and one with *Bacteroidetes* (0.9%).

**Microbial composition as revealed by T-RFLP analysis**

T-RFLP analysis revealed that T-RFs with a length of 119 bp (~80% of total fragment intensity) and 469 bp (~15%) were the predominant phylotypes in the phenol-degrading community (Fig. 5). Four additional minor T-RFs (*i.e.*, 102, 504, 650 and 693 bp) (1–2%) were also observed. The T-RFLP fingerprint obtained in this study was similar to that of the mesophilic phenol-degrading enrichment (MP) system described previously (9). Based on the sequences obtained from the bacterial clone library, the 119 bp T-RF and 469 bp T-RF probably represented members of *Syntrophorhabdaceae* and *Syntrophus*-like populations, respectively.

**Predominant microbial populations in the GAC biofilm revealed by FISH**

As shown in Fig. 6 (A and B), the FISH analysis firstly revealed the spatial distribution of *Bacteria* and *Archaea* populations in the biofilms approximately 150 μm in thickness. A mixed structure was observed based on the random distribution of bacterial (green) and archaeal (*i.e.*, methanogens) (red) populations with an approximately equal abundance in the thin-sectioned biofilms (Fig. 6A and B). Yellow signals represented clusters where bacterial and archaeal cells were so closely associated that the individual fluorescent signals could not be clearly distinguished. This non-layered structure was repeatedly observed in all of the biofilm sections analyzed.

Further, both domain- and group-specific oligonucleotide probes individually labeled with Cy3 and Cy5 were used to uncover the distribution of different major microbial populations inside the GAC biofilm. Technically, the cells hybridized with both oligonucleotide probes appeared yellow/orange in FISH micrographs (Fig. 6C–F). As the cells lighted up in yellow, the results clearly revealed that members of the *Methanosetaea* (thick filamentous or rod with flat-ends, Fig. 6C), *Syntrophorhabdaceae*- (oval-shaped, Fig. 6D), *Syntrophus*- (small rods, Fig. 6E), and *Chloroflexi*- (thin filaments, Fig. 6F) related groups were present abundantly in the biofilm. The presence of *Methanosetaea*-like populations (Fig. 6C) in high abundance was well consistent with the results of SEM and cloning analyses. *Methanosetaea*-like cells were randomly distributed in the GAC biofilm. In addition, *Methanosarcinaceae*-like cocoid cell clusters were observed (inset of Fig. 6C). This finding coincided with retrieval of the 16S rRNA clone (PS-Ar61) related to *Methanomethylcovars hollandica* (26) in the clone library (Fig. 3).

FISH analysis further revealed that bacterial populations related to the *Syntrophorhabdaceae* were randomly distributed in the thin-sectioned biofilm (Fig. 6D). A close-up FISH image (inset of Fig. 6D) revealed that the cells detected by the Delta-TA664 probe had an oval-shaped morphology, similar to those observed by SEM (arrow 2, Fig. 2), those found in the mesophilic phenol-degrading enrichment process (9), and *Syntrophorhabdus aromaticivorans* grown on phenol (30). Moreover, *Syntrophus*-like microorganisms were abundant in the GAC biofilm (Fig. 6E). As the 11–15% fraction was detected in the clone library and T-RFLP analyses, the high abundance of *Syntrophus*-like populations agreed with the previous findings (16, 49), suggesting that *Syntrophus*-like populations likely played an important role in the methanogenic communities associated with phenol degradation. Interestingly, the FISH analysis revealed the presence of thin-filamentous *Chloroflexi*-like populations throughout the biofilm sections (Fig. 6F). Nevertheless, the abundance of *Chloroflexi*-like populations in the biofilm is in contradiction to the relatively low clone frequency (4.7% of bacterial clones) obtained in the clone library. This difference was likely due to insufficient cell lysis during RNA extraction or imperfect primer specificity in PCR amplification for these bacteria.

**Discussion**

The full-scale AFB reactor examined here had been treating wastewater from the manufacturing of phenolic resin for more than six years. During its long-term operation, the GAC serving as the carrier for microbial growth had developed a thick biofilm, in which microbial populations exhibited strong activity to degrade wastewater whose major component is phenol (~60% of total COD), into the final products methane and carbon dioxide. Microbial activity for the degradation of phenol reached a SMA value of 130.1 mL·CH₄·g-VSS⁻¹·day⁻¹ with a conversion rate of 0.23 mL·CH₄·mg-
phenol$^{-1}$ (Fig. 1). This superior microbial performance associated with phenol degradation and methane production was observed when a low concentration of phenol was used, implying that the microbial populations adapted to the low phenol environments, and had good affinity for the phenol substrate. Due to the high recirculation ratio (i.e., $\geq 100$) normally applied in AFB reactors to maintain a high upflow velocity (e.g., $20 \text{ m h}^{-1}$), the actual phenol concentration inside the GAC reactor for microorganisms is relatively low (i.e., 25–30 mg L$^{-1}$). Since phenol is toxic to microorganisms, increasing the phenol concentration could result in inhibitory effects on these microbial activities.

To identify the populations that are ecologically important, the community structure of the GAC biofilm was characterized using clone library and T-RFLP analyses of reverse transcribed cDNA from native RNA as well as the FISH technique. Consistent with the results from cloning, T-RFLP and FISH analyses suggested that members of the Methanosaeta and Syntrophorhabdaceae groups were the most predominant Archaea and Bacteria populations in the biofilm community. These thick filamentous Methanosaeta-like cells were randomly distributed in the biofilm sections, and have been found to be one of the predominant populations in phenol-degrading systems from laboratorial studies and an important population for the granulation of sludge (7, 9, 15). Some members of the Syntrophorhabdaceae have been suggested to be effective in the anaerobic degradation of organics such as terephthalate (44), isophthalate (29), trichlorobenzene (41), 1,2-dichloropropane (33) and phenol (9, 16, 17) in engineered ecosystems. Other members of this family are closely related to clones retrieved from natural anoxic/anaerobic environments, including lake sediment, forested

**Fig. 6.** FISH analyses of the full-scale phenol-degrading GAC-AFB sludge. In panels (A) and (B), samples were hybridized with the Cy3-labeled EUBmix probe specific for the domain Bacteria (green), and the Cy5-labeled ARC915 probe specific for the domain Archaea (red). Samples were hybridized with the Cy3-labeled ARC915 probe (green) and Cy5-labeled MX825 probe (red) specific for Methanosaetaeae (C); and Cy3-labeled EUBmix probe (green) and Cy5-labeled probes (red) Delta-TA664, Syn424 and GNSB941 specific for Syntrophorhabdaceae (D), Syntrophus (E) and Chloroflexi (F), respectively.
wetland, soil and a contaminated aquifer (Fig. 4). Also, a recent isolate *Syntrophorhabdus aromaticivorans* in the family *Syntrophorhabdaceae* showed expertise in the utilization of multiple aromatic compounds such as phenol, *p*-cresol, 4-hydroxybenzoate, isophthalate and benzoate (30). These findings suggest the *Syntrophorhabdaceae* population to be widely distributed in anoxic/aerobic environments, and an ecological success in consortia associated with the degradation of aromatic compounds.

In our previous study, it was observed that *Syntrophus*-like populations were relatively low in abundance in batch cultures enriched with phenol (9). Nevertheless, according to the results obtained in that study and others (16, 49), the populations were considerably abundant in the GAC biofilm and granular sludge. This difference could be due to the difference in the physical association among microbial populations (granulated vs. suspended). It is suggested that a granulated microbial consortium is usually more effective in the transfer of intermediates (e.g., benzoate) to neighboring microbial populations (e.g., *Syntrophus* spp.) than suspended counterparts, which are relatively far apart from each other. Therefore, *Syntrophus*-related populations could be more important for the degradation of the intermediate benzoate in a granular bioreactor than in a suspended enrichment system. This was further supported by a recent finding that a phenol-degrading isolate (*Syntrophorhabdus aromaticivorans*) degraded phenol to acetate in association with a hydrogenotrophic methanogen, with no *Syntrophus* involved (30). Overall findings further suggested that *Syntrophorhabdaceae* can work together with *Syntrophus* to degrade phenol to acetate in granular bioreactors, and can also work alone when little or no *Syntrophus* is available in suspended cultures. The degradation pathways (benzoate/acetate/hydrogen or acetate/hydrogen) and the abundance of *Syntrophus*-like populations may be regulated by the optimal hydrogen partial pressure in these two conditions (i.e., granulated and suspended).

Despite the possibility of different degradation pathways, acetate is one of the most important intermediates in the anaerobic degradation of phenol. In principle, 1 mol of phenol can produce 3 mol of acetate. The high concentration of acetate obtained from the degradation of phenol can be further converted to methane by either acetotrophic methanogens or acetate-oxidizing bacteria with hydrogenotrophic methanogens (20). In this study, there was no known syntrophic acetate-oxidizing bacteria (e.g., strain AOR, *Clostridium acetobutylicum*, Thermacetogenium phaeum and *Thermotoga lettinghiae*) (20) found in the GAC-AFB reactor. Due to the low abundance of hydrogenotrophic methanogens (Fig. 3) and lack of competition from syntrophic acetate-oxidizing bacteria, *Methanoseta* was the most predominant acetate-utilizing population in the GAC-AFB reactor during phenol degradation. In addition, the random distribution of *Methanoseta* in the GAC biofilm suggested that acetate from phenol degradation was available throughout the biofilm. Apart from the phenol and formaldehyde, about ~35% of the COD in wastewater discharged from plants manufacturing phenolic resin remains unknown. The wastewater’s complexity can greatly diversify the structure of the microbial community such as microbial populations found in another four bacterial phyla (i.e., *Chloroflexi*, *Acidobacteria*, *Bacteroidetes*, and OP8). The FISH analysis clearly revealed a large *Chloroflexi*-like population in the biofilm sections (Fig. 6F). For the first time, *Chloroflexi* populations were observed in high abundance in an anaerobic bioreactor treating inhibitory or hazardous-type phenol-containing wastewater from resin-manufacturing processes. Regardless of its low frequency in the cloning analysis, the *Chloroflexi*-like population may play an important role in the biofilm. So far, at least six species in *Chloroflexi* subphylum-I were obtained in pure cultures and all were long and thin filamentous (46, 47). These microorganisms have been found to be commonly distributed within granular sludge in methanogenic environments with high genetic diversity of 16S rRNA (34, 36, 45). In addition, based on their filamentous morphology and significant abundance, their eco-physiological functions included important roles in granulation and the maintenance of the granular structure (34–36, 38, 39, 45) and the anaerobic degradation of carbohydrates and some amino acids (36, 45). As to their niches in metabolizing wastewater substrates, more studies are required to further interpret the predominance of *Chloroflexi*-like populations in unique ecosystems.

The topology of structured microbial consortia can reflect the kinetics of the degradation of substrates as a result of the flux of the food web associated with various microbial populations. A number of studies have reported a layered spatial distribution of microorganisms in granular sludge from reactors treating easily hydrolyzed substrates (i.e., glucose, hydrolysed proteins, sucrose, brewery wastes, powdered skim milk and a mixture of sucrose/acetate/propionate) (14, 19, 27, 32, 34), while a non-layered structure of granular sludge was generally obtained with slowly degrading substrates such as phenol (15, 49) and terephthalate (44). As shown in Fig. 6, all the predominant microbial populations examined (i.e., *Methanoseta*, *Syntrophorhabdaceae*, *Syntrophus* and *Chloroflexi*) were randomly distributed in the biofilm sections. This chaotic spatial distribution can be attributed to (i) the slow degradation of phenol, (ii) a porous and loose biofilm structure, (iii) the influence of the GAC adsorption/desorption dynamic process, (iv) the degradable nature of unknown substrates in wastewater, and (v) the difference of flow regime in the reactors (e.g., high upflow velocity for AFB).

In conclusion, based on the investigation using full-cycle 16S rRNA approaches in the present study, it was found that the GAC biofilm from a full-scale AFB reactor treating phenol-rich wastewater had a similar community structure to microbial consortia from laboratory systems for the anaerobic degradation of phenol and terephthalate under mesophilic methanogenic conditions. This clearly highlights the crucial influence of substrate type and incubation temperature on microbial diversity. More significantly, the results obtained in the present study validated the numerical predominance of the syntrophic phenol degrader, *Syntrophorhabdaceae*, in a real wastewater treatment system, likely elucidating syntrophic association for phenol degradation with methanogens. This together with previous findings have implications for engineering such as the use of *Syntrophorhabdaceae* populations as an indicator of the anaerobic degradation of phenol and terephthalate. In addition, the abundance of *Syntrophorhabdaceae* can be further investigated by quanti-
tative molecular techniques to provide useful information for selecting the most appropriate seeding sludge. By taking advantage of the capability of *Syntrophorhabdaceae* groups to utilize aromatics, phenol can be effectively used for microbial growth to possibly speed up the startup of terephthalate-containing wastewater treatment.

Acknowledgements

The authors thank Siew Lok Toh and Yee Wei Lee at the Division of Bioengineering, National University of Singapore for technical support with the operation of the cryotome.

References

20. Hattori, S. 2008. Syntrophic acetate-oxidizing microbes in methano-
ization of microbial diversity by determining terminal restriction frag-
32. Satoh, H., Y. Miura, I. Tsushima, and S. Okabe. 2007. Layered struc-


