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Microbial Community Analysis of a Methane-Oxidizing Biofilm Using Ribosomal Tag Pyrosequencing

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Current ecological knowledge of methanotrophic biofilms is incomplete, although they have been broadly studied in biotechnological processes. Four individual DNA samples were prepared from a methanotrophic biofilm, and a multiplex 16S rDNA pyrosequencing was performed. A complete library (before being de-multiplexed) contained 33,639 sequences (average length, 415 nt). Interestingly, methanotrophs were not dominant, only making up 23% of the community. *Methylosinus*, *Methylomonas*, and *Methylosarcina* were the dominant methanotrophs. Type II methanotrophs were more abundant than type I (56 *vs.* 44%), but less richer and diverse. Dominant non-methanotrophic genera included *Hydrogenophaga*, *Flavobacterium*, and *Hyphomicrobium*. The library was de-multiplexed into four libraries, with different sequencing efforts (3,915 – 20,133 sequences). Sørrenson abundance similarity results showed that the four libraries were almost identical (indices > 0.97), and phylogenetic comparisons using UniFrac test and P-test revealed the same results. It was demonstrated that the pyrosequencing was highly reproducible. These survey results can provide an insight into the management and/or manipulation of methanotrophic biofilms.

Keywords: Pyrosequencing, community analysis, methanotrophs, biofilm, quantitative real-time PCR

Aerobic methane-oxidizing bacteria, methanotrophs, utilize the greenhouse gas methane as a sole carbon and energy source. A total number of 18 genera have been identified as methanotrophs [18, 35]. They are classified into two groups (*i.e.*, types I and II) by their physiological characteristics and phylogenetic affiliation. Hanson and Hanson [15] grouped methanotrophs into three groups: Types I, II, and X. Type X is commonly considered a subset of type I [35]. Methanotrophs have methane monooxygenase, which presents in two forms, a soluble form (sMMO) and a particulate form (pMMO). Both types I and II members express pMMO, but certain type II members and a type I member (=type X) can express sMMO [15, 35]. Both MMOs have been found to degrade chemical pollutants, such as halogenated alkanes and alkenes, owing to their nonspecificity, but sMMO shows activity over a broader range of substrates [15, 35].

Biofilms are an aggregate of microorganisms, which is a common feature of microbial communities that appear in environments and engineering systems [14, 38]. Methanotrophic biofilms (*i.e.*, biofilms capable of removing methane) have been broadly utilized in biotechnological processes for the removal of methane and diverse chemical pollutants, such as trichloroethylene and nitrate [6, 15, 28, 35, 44]. The complexity of a variety of microorganisms may be the fundamental nature of methanotrophic biofilms, which consist of methanotrophs and co-inhabitants. For successful manipulation and management of a biofilm to enhance the performance, a better understanding of its microbial ecology is needed [34, 38]. However, the microbial ecology of methanotrophic biofilms remains unknown, although they have been utilized for several decades.

There are many molecular ecological techniques for examining microbial communities, such as DGGE, microarray, and high-throughput sequencing techniques. Pyrosequencing, one of the high-throughput sequencing techniques, has become popular for microbial community studies in microbial ecology and biotechnology [10, 33, 39, 41, 45]. The Genome Sequencer 454 FLX system produces more than 400,000 sequence reads, with 200–300 nucleotides [9]. The read length produced by the system is shorter than the traditional cloning/Sanger sequencing approach. Recent technological advancement on the Genome Sequencer 454 FLX Titanium system allows a sequence read length of more than 400 nucleotides [30]. This technique is perhaps

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the most powerful tool for microbial compositional studies to date. However, experimental replication and repetition have been neglected in studies employing pyrosequencing [33, 39, 45], although the reproducibility of pyrosequencing cannot be guaranteed. For the correct interpretation of results from high-throughput genetic technologies, replication and repetition are generally recommended owing to experimental errors and variation within a sample or system [23, 29, 31]. Pan *et al*. [29] recently reported that variation in the yields and purities of nucleic acid extracted from an environmental sample leads to significant bias in downstream molecular ecological techniques.

The main objective of this study was to elucidate the microbial ecology of a methanotrophic biofilm. A methane-oxidizing biofilm community was concurrently analyzed using pyrosequencing and quantitative real-time PCR. In addition, the reproducibility of pyrosequencing was assessed, with different sampling efforts. Pyrosequencing is a quantitative tool for assessing microbial community compositions, but does not reflect the population density of a microbial community. It was expected that the concurrent use of quantitative real-time PCR (qRT-PCR) with pyrosequencing provides more comprehensive information of the microbial community owing to their ability to accurately quantify the population of a phylogenetic marker gene used in pyrosequencing.

Materials and Methods

Inoculum Sources

A methane-oxidizing consortium, earthworm cast, and landfill soil were used as inocula. A methane-oxidizing consortium originating from a landfill soil (Gong-ju, Chungchungnam-do, South Korea) was maintained in nitrate mineral salt (NMS) medium, with methane, before use [22]. For a large-scale cultivation of the consortium, a 5 L fermentor was used. About 100 ml of the consortium was added to the fermentor and filled with 1.9 L of NMS medium, which contained 1 g of $MgSO_4 \cdot 7H_2O$, 0.295 g of $CaCl_2 \cdot 2H_2O$, 1 g of KNO_3, 0.26 g of KH_2PO_4, and 0.41 g of $Na_2HPO_4 \cdot 2H_2O$ in 1 L. $CuSO_4$ was added to a final concentration of 30 μM. Methane (99.9%; Seoul Gas, Seoul Korea) and air were continuously fed to the fermentor from a gas bag [methane:air = 1:4 (v/v) 3 L/day]. The fermentor was maintained at 30°C, with a 20 day solid retention time and hydraulic retention time, with agitation (250 rpm) for 70 days. The out-gas was collected using a 10 L gasbag and periodically sampled using a gas-tight syringe for measurement of the methane concentration. The earthworm cast was obtained from the Nanji municipal sewage treatment plant (Goyang, Kyungido, Korea). Municipal sewage sludge, treated with a consortium of mixed earthworms for more than 2 years, was air-dried for 6 months. Soil was collected from a landfill cover (Gapyeong-gun, Kyungido, Korea). One hundred g of the earthworm cast and 100 g of the landfill soil were completely mixed in 3 L of NMS. After mixing, a static condition was maintained for 30 min. About 2 L of the supernatant and 500 ml of the methane-oxidizing consortium were used as an inoculation source.

A Biofilm Reactor for Methane Removal

A biofilm reactor for methane removal (*i.e*., biofilter) was made of acryl, and consisted of two parts: a packing column and a medium container. The height and inner diameter of the packing column and medium container were 100 and 8 cm (approximately 5 L), and 20 and 15 cm (approximately 3.5 L), respectively. The packing column had a perforated plate at the bottom in order to allow the methane to evenly spread. After setting up, the gas tightness of the reactor was verified with water and compressed air. Hyuga pumice (Japan), with a diameter of 5–10 mm, and granular activated carbon (Jaeil Carbon, Yong-in, Korea), with a diameter of 4–8 mm, were completely mixed [10:1 (w/w)] and filled into the packing column. The inoculum, 2.5 L as described above, was added into the medium container and circulated 4 times per day for 7 days to allow cell attachment to the packing material. During the operation, the NMS medium was circulated 4 times per day, and replaced every week to provide moisture and nutrients for cell growth. Methane (99.9%) and compressed air were introduced into the bottom of the filter through a 1-m-long humidifier filled with water, at a flow rate of 250–375 ml/min (a space velocity of 3–4.5 h^{-1}). Methane was continuously fed at concentrations between 1% and 10%. The biofilter was operated at 20 ± 5°C for 130 days. The methane concentration was monitored at the inlet and outlet using gas chromatography (GC 6850N; Agilent Technologies Inc., Santa Clara, USA), equipped with a flame ionization detector and a wax column (30 m×0.32 mm×0.25 μm; Supelco, Bellefonte, USA).

DNA Extraction

The packing material was collected from the reactor at the end of the experimental period. The biofilm was detached from the packing material by sonication and agitation. Then, 20 g of the sample was added to each flask (n=2) containing 20 ml of sterile saline solution (0.9%). The flasks were sonicated using a commercial ultrasonic bath (model 8510; Branson, Danbury, USA) for 30 min, and then agitated at 250 rpm for 30 min. About 1.5 ml of the suspensions was transferred to 1.5 ml microtubes, and these were centrifuged at 16,000 ×*g* for 10 min. The supernatant was discarded from the tubes. A total number of 10 microtubes (five of each) were used for DNA extraction. DNA was extracted individually using the NucleoSpin Soil kit (Macherey-Nagel GmbH, Düren, Germany), with a modification that samples were disrupted using a BeadBeater-8 system (BioSpec, Bartlesville, USA) at 5,000 rpm for 30 s. DNA was eluted in 100 μl of the elution buffer and stored at 20°C prior to use. DNA was quantified using an ASP-2680 spectrophotometer (ACTGene, Piscataway, USA). DNA concentrations were 55–65 ng/μl.

Polymerase Chain Reaction (PCR)

Two individual DNAs were randomly selected from each replicate, resulting in four DNA samples. For PCR, the primer set 340F (5'-TCCTACGGGAGGCAGCAG-3') and 805R (5'-GACTACHVGGGT ATCTAATCC-3') was used to amplify the 340–805 region of the 16S rRNA gene (positions based on *Escherichia coli*) containing the V3 and V4 regions. The 340F and 805R primers cover approximately 90% of the sequences with a 300–850 region (based on the 16S rRNA gene of *E. coli*) in the RDP database (http://rdp.cme.msu.edu). Four composite primer sets were made based on the 340F–805R set for multiplex pyrosequencing; the composite forward primer 5'-CCATCTCATCCCTGCGTGTCTCCGAC-*TCAG*-NNNNNNNNNN-TCCTACGGGAGGCAGCAG-3' and the composite reverse primer

5'-CCTATCCCCTGTGTGCCTTGGCAGTC-*TCAG*-GACTACHVG GGTATCTAATCC-3', where the underlined sequences are the Genome Sequencer FLX Titanium adaptors A and B, the sequences in italics are the four-base library key, NNNNNNNNNN is the 10 bp multiplex identifier tag, and the last is the primer targeting the 16S rRNA gene. There were different identifier tags for four DNA samples. For each DNA sample, four independent PCR mixtures were prepared in parallel to avoid PCR bias. The 50 μl mixtures contained 5 μl of 10× PCR buffer (Genenmed, Seoul, Korea), 10 μg of bovine serum albumin, 2 μl of each composite primer (10 μM), 1 U of Ace *Taq* polymerase (Genenmed), 4 μl of 2.5 mM dNTPs, and 2.5–3 μl of template DNA (approximately 150 ng). The reactions were performed in a 2700 GeneAmp PCR system (Applied Biosystems, Foster City, USA). An initial denaturation step at 95°C for 3 min and 25 cycles of denaturation at 94°C for 45 s, primer annealing at 50°C for 45 s, and elongation at 72°C for 45 s were performed, followed by a final elongation step at 72°C for 5 min. Four PCR products were combined, and electrophoresis of the PCR products was done on 2% low melting agarose gels. The expected size of the PCR products was about 530 bp. The agarose gels containing the PCR products were excised using a scalpel and then purified using the QIAquick Gel extraction kit (Qiagen, Valencia, USA), as specified by the manufacturer.

Pyrosequencing

The concentrations of the purified DNAs were measured using an ASP-2680 spectrophotometer (ACTgene). Different amounts of the barcoded PCR amplicons (340, 565, 882, and 1,304 ng) were combined in a single tube to produce pyrosequencing libraries with different read numbers (*i.e.*, sequencing efforts). It was sent to Macrogen Incorporation (Seoul, Korea) to be run on a Genome Sequencer 454 FLX Titanium system (Roche Diagnostics, Mannheim, Germany).

Pyrosequencing Data Analysis for Microbial Community

First, the composite pyrosequencing primer regions were trimmed, and DNA sequences with lengths <350 bp and an ambiguous sequence were removed using the quality trimming tool of the RDP pyrosequencing pipeline [7]. Sequences with average quality scores below 25 were also removed. Pyrosequencing reads not assigned to the domain Bacteria (the RDP classifier of the pipeline with confidence levels >80%) were excluded. The RDP pyrosequencing pipeline was used for analyzing the pyrosequencing data in this study, unless otherwise stated. Operational taxonomic units (OTU) were determined at 0–10% dissimilarity levels for bacterial community analysis. A rarefaction analysis between reads and OTUs was performed. Chao1 richness estimates and Shannon–Weaver diversity indices were calculated. Pyrosequencing reads were taxonomically assigned using the RDP classifier of the pipeline, with confidence levels more than 80%. The RDP classifier provides rapid and independent taxonomic classifications from domain to genus [40]. The classification result was imported into MEGAN software version 4.40 for further analysis and result presentation [17]. A rarefaction analysis between reads and observed genera was performed using the MEGAN software. For result presentation, bacterial taxa with a number of assigned reads less than 10 were excluded.

Reads assigned to methanotrophic genera [18, 35] belonging to the type I or II group were retrieved from the pyrosequencing library using the MEGAN software. OTUs were determined at 0–3% dissimilarity for methanotrophic community analysis. Chao1 richness estimates and Shannon–Weaver diversity indices were calculated. For further classification from genera to species or strains of interest, the pyrosequencing reads of a bacterial genus were retrieved and grouped at 3% dissimilarity. Sequences representing OTUs were compared with those of the bacterial type strains using the EzTaxon server version 2.1 (http://www.eztaxon.org) [5].

Comparison of the Four Sub-Libraries

The complete library was de-multiplexed, and OTUs were determined. The four libraries were compared at the 3% dissimilarity level using EstimateS software version 8.0 (http://viceroy.eeb.uconn.edu/EstimateS) with Sørrenson incidence and abundance similarity tests [3, 4]. For phylogenetic comparison, representative sequences for OTUs were retrieved from the four libraries. Multiple comparisons of the sequences were performed, with *Methanobacterium kanagiense* 16S rRNA gene sequence (Accession No. AB368917) as an outgroup, using Clustal X software version 2.1 [21]. A P-test and weighted UniFrac test were performed to evaluate the phylogenetic divergences among the four libraries using the UniFrac server (http://bmf.colorado.edu/unifrac) [25]. Unifrac and P-test significance values were corrected using the Bonferroni correction for multiple comparisons.

Pyrosequencing reads of each library were taxonomically assigned using the RDP classifier. The classification results were imported into the MEGAN software for comparison of the microbial community compositions of the four libraries. The pyrosequencing reads obtained in this study were deposited to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra) under the accession number DRA000415.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed with the primer sets A189f (5'-GGBGAC TGGGACTTCTGG-3') and mb661r (5'-CCGGMGCAACGTCYT TACC-3'), targeting the *pmoA* gene for methanotrophs [26], and 340F and 805R. *Methylobacter luteus* (NCIMB11914) was used to prepare standard curves for quantitative detection. The DNA standards ranged from 1×10^3 to 1×10^7 gene copies. DNA quantification was performed using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems). The 25 μl reaction mixture for the *pmoA* gene consisted of 2.5 μl of 10× PCR buffer, 0.125 μl of Ace *Taq* polymerase (Genenmed), 0.5 μl of 50× SYBR green I (Invitrogen, Carlsbad, USA), 0.5 μl of each primer (10 μM), 0.5 μl of 50× ROX (Invitrogen) as a reference dye, and 2 μl of template DNA. Control reactions contained the same mixtures, but with 2 μl of sterile water replacing the DNA template. PCR was initiated at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 30 s, with an additional 30 s reading step at 83°C added at the end of each cycle. The reaction mixture for the 16S rRNA gene consisted of 2.5 μl of 10× PCR buffer, 0.125 μl of *Taq* polymerase (Qiagen), 0.5 μl of 50× SYBR green I (Invitrogen), 0.5 μl of 340F primer (10 μM) and 1 μl of 805R primer (10 μM), 0.5 μl of 50× ROX (Invitrogen) as a reference dye, and 2 μl of template DNA. Control reactions contained the same mixtures, but with 2 μl of sterile water replacing the DNA template. PCR was initiated at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 50°C for 40 s, and 72°C for 30 s. The correlation coefficients of the standard curves constructed from both primer sets were more than 0.99, with PCR efficiencies greater than 90%.

Table 1. Summary of the bacterial 16S rRNA gene pyrosequencing libraries from a methanotrophic biofilm reactor.

Sample	Number of reads	Average read length	16S rRNA gene copy[b]	*pmoA* gene copy[b]
Library 1	5,329	416	5.50×10^5	5.14×10^4
Library 2	20,133	416	1.24×10^5	3.22×10^4
Library 3	3,915	415	6.60×10^5	2.94×10^4
Library 4	4,282	415	6.55×10^5	6.98×10^4
Complete library[a]	33,659	415	4.97×10^{5c}	4.57×10^{4c}

[a]Sum of the four libraries (before being de-multiplexed).
[b]qRT-PCR quantification. The units were expressed as copies/µl (extracted DNA from samples).
[c]Mean values of the four sublibraries.

RESULTS

Biofilm Reactor Performance

The methane removal bioreactor was continuously operated for 130 days. The removal performance improved with time (data not shown). At the end of the operation, the methane elimination capacity was 694.8 g-$CH_4 \cdot m^{-3} \cdot day^{-1}$ at an inlet load of 824.3 g-$CH_4 \cdot m^{-3} \cdot day^{-1}$, with a removal efficiency of 84.2%.

Microbial Ecology of Methanotrophic Biofilm

The complete library (before being de-multiplexed) contained a total number of 33,639 pyrosequencing reads, with an average length of 415 bp after trimming (Table 1). The numbers of observed operational taxonomic units (OTU), Chao1 richness estimates, and Shannon–Weaver diversity (H') indices are listed at sequence divergences of 0–10% in Table 2. There were 8,521 OTUs, from which Chao1 and H' were 31,637 and 6.288, respectively, at the 0% dissimilarity level. The most abundant OTU was represented by 3,011 sequences, which made up 8.9% of the library. When grouped at the 3% dissimilarity level, 973 OTUs (Chao1, 1,581, and H', 4.057) were observed. The most abundant OTU was represented by 4,200 sequences, which

Table 2. Operational taxonomic units, Chao1 richness estimates, and Shannon–Weaver diversity indices of the bacterial 16S rRNA gene libraries.

	Dissimilarity	OTU[a]	Chao1[b] (95% CI[c])	H'[d]
Library 1	3%	306	503 (431–616)	3.700
	5%	236	354 (305–438)	3.390
	10%	132	180 (154–234)	2.957
Library 2	3%	756	1,188 (1,078–1,335)	4.041
	5%	540	742 (679–835)	3.773
	10%	270	325 (299–376)	3.263
Library 3	3%	305	511 (435–629)	3.915
	5%	238	359 (308–449)	3.653
	10%	132	171 (149–223)	3.168
Library 4	3%	284	521 (430–667)	3.779
	5%	229	366 (308–465)	3.505
	10%	129	150 (138–177)	3.125
Complete library	0%	8,521	31,637 (29,854–33,570)	6.288
	1%	2,622	5,113 (4,770–5,511)	5.006
	3%	973	1,581 (1,441–1,764)	4.057
	5%	672	901 (835–994)	3.785
	10%	328	393 (364–444)	3.293

[a]Observed operational taxonomic units.
[b]Chao1 is a nonparametric richness estimator.
[c]Confidential interval.
[d]Shannon–Weaver index of diversity.

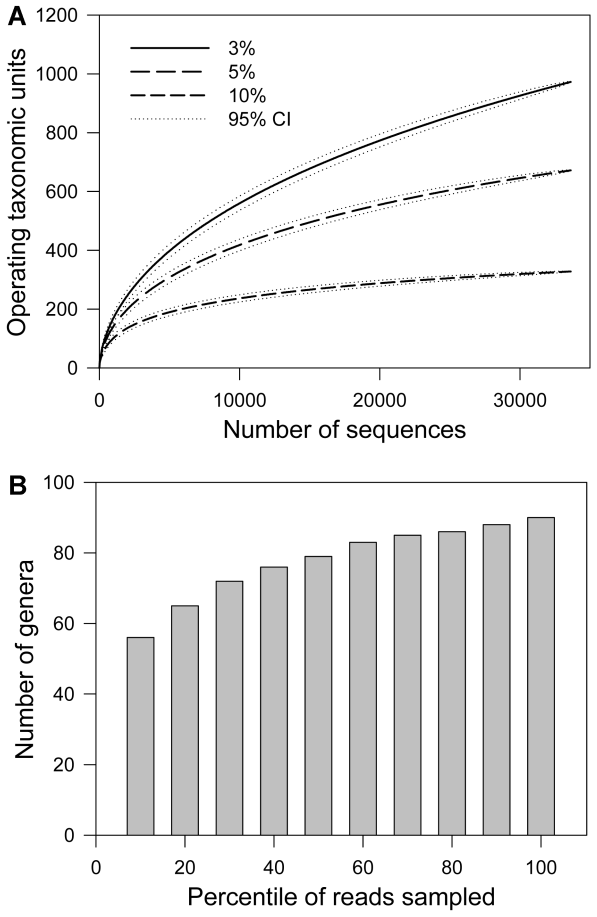

Fig. 1. Rarefaction curves of the operational taxonomic units (**A**) and bacterial genera (**B**) in the complete library.

was 12.4% of the library. The 10 greatest OTUs had 19,600 sequences, which made up 58.2% of the library, with 419 OTUs represented by a single sequence (singleton). Rarefaction curves of the observed OTUs at the 3%, 5%, and 10% dissimilarity levels were constructed (Fig. 1A). None of the curves showed saturation.

All 33,639 sequences were individually classified from domain to genus. A rarefaction curve between the observed genera and sequencing effort showed no saturation (Fig. 1B). At the phylum level, Proteobacteria (79.7% of the library) dominated the community, followed by Bacteridetes (11.5%), unassigned bacteria (3.5%), Verrucomicrobia (2.6%), and Acidobacteria (1.1%) (Fig. 2). Dominant orders were Rhizobiales (31.7%), Burkholderiales (27.6%), Methylococcales (12.2%), Flavobacteriales (7.2%), Xanthomonadales (5.1%), Caulobacterales (4.5%), Verrucomicrobiales (2.5%), Sphingobacteriales (2%), Rhodobacterales (1.7%), and Acidobacteria (1.2%). The genus *Hydrogenophaga* (26.9%) was dominant, followed by *Methylosinus* (17.5%), *Flavobacterium* (8.5%), *Methylomonas* (7.6%), *Hyphomicrobium* (6.1%), *Methylosarcina* (5.8%), *Caulobacter* (4.4%), *Pseudoxanthomonas* (3.8%), *Luteolibacter* (2.8), and *Rhodobacter* (1.9%) (Fig. 3).

Methanotrophic Community

Seven methanotrophic genera that included *Methylocaldum*, *Methylococcus*, *Methylomonas*, *Methylosarcina*, *Methylosoma*, *Methylocystis*, and *Methylosinus* were observed. When these reads were retrieved from the complete library, a total number of 7,796 reads were obtained, which made up approximately 23% of the library. Methanotrophs were grouped into type I (γ-Proteobacteria) and type II (α-Proteobacteria) (Table 3 and Fig. 4). Population levels of types I and II were quite comparable (44% *vs*. 56%, respectively).

The numbers of observed OTUs, Chao 1 estimates, and H' indices at the 0–3% dissimilarity levels are listed in Table 3. There were 780 *vs*. 633 OTUs, 2,643 *vs*. 1,400 Chao1 estimates, and 3.721 *vs*. 2.429 H' indices at the 0% dissimilarity level for types I and II, respectively. When grouped at 3% dissimilarity, 46 *vs*. 30 OTUs, 71 *vs*. 48 Chao1 estimates, and 1.606 *vs*. 0.247 H' indices were observed for types I and II, respectively. The estimated richness and diversity of type I members were greater at the listed dissimilarity levels, although the abundance of type II members was slightly greater. *Methylomonas* and *Methylosarcina* dominated the type I community, and *Methylosinus* was predominant in the type II community (Fig. 4).

Comparison of the Four Libraries

For comparison, four libraries were constructed by de-multiplexing from the complete library. Libraries 1–4 contained 5,329, 20,133, 3,915, and 4,282 reads, respectively,

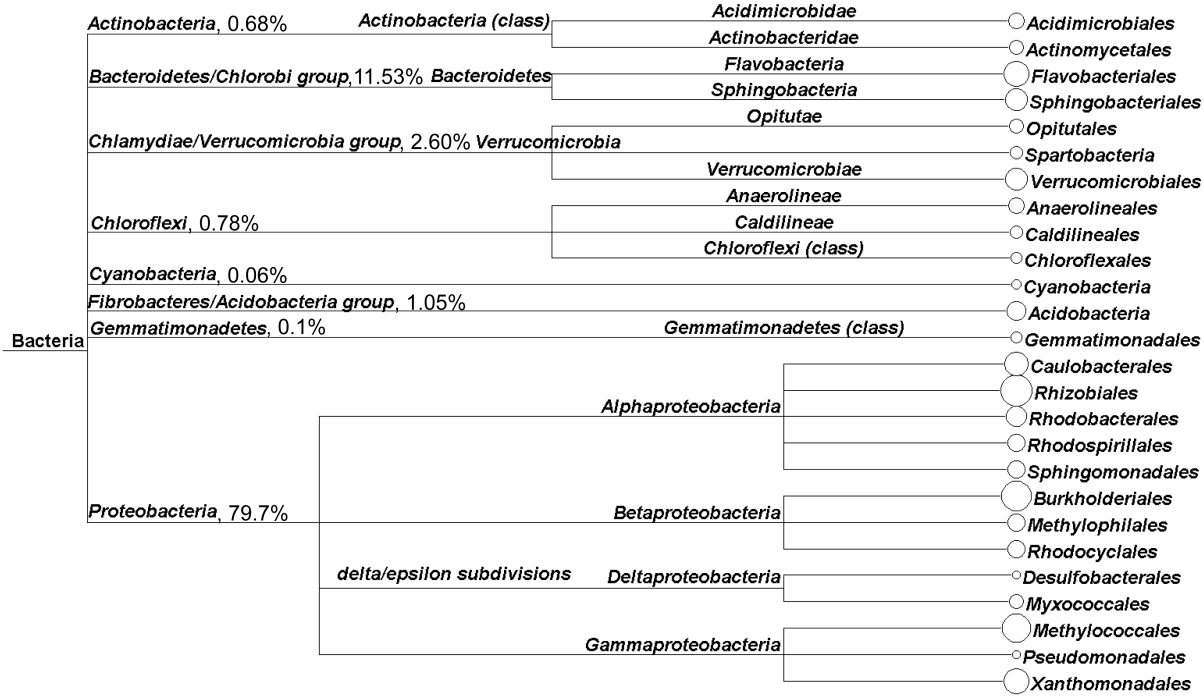

Fig. 2. Bacterial phyla, classes, and orders in the complete library.
Phylotypes with reads (n) <10 were excluded. Percentile indicates the percentage of bacterial phyla to total bacteria. The sizes of the circles reflect the abundance of the order.

with about the same read lengths (Table 1). Library 2 was more than 5-fold greater than the smallest one. The OTU abundances and Chao1 estimates of library 2 were 2-fold greater than the other libraries, with diversity levels also greater at the 3–10% dissimilarity levels (Table 2). The OTU abundances, Chao1 estimates, and H' indices were comparable among libraries 1, 3, and 4. Sørrenson similarity indices were calculated for comparing the four microbial communities based on the incidence/absence and relative abundance (Table 4). The Sørrenson indices of the compositional similarity were more than 0.459 among the four libraries, and libraries 1, 3, and 4 were more similar. The Sørrenson indices of relative abundance were more than 0.971 among the four libraries. A phylogenetic comparison among the four libraries was performed using statistical significance tests, such as a P-test and weighted

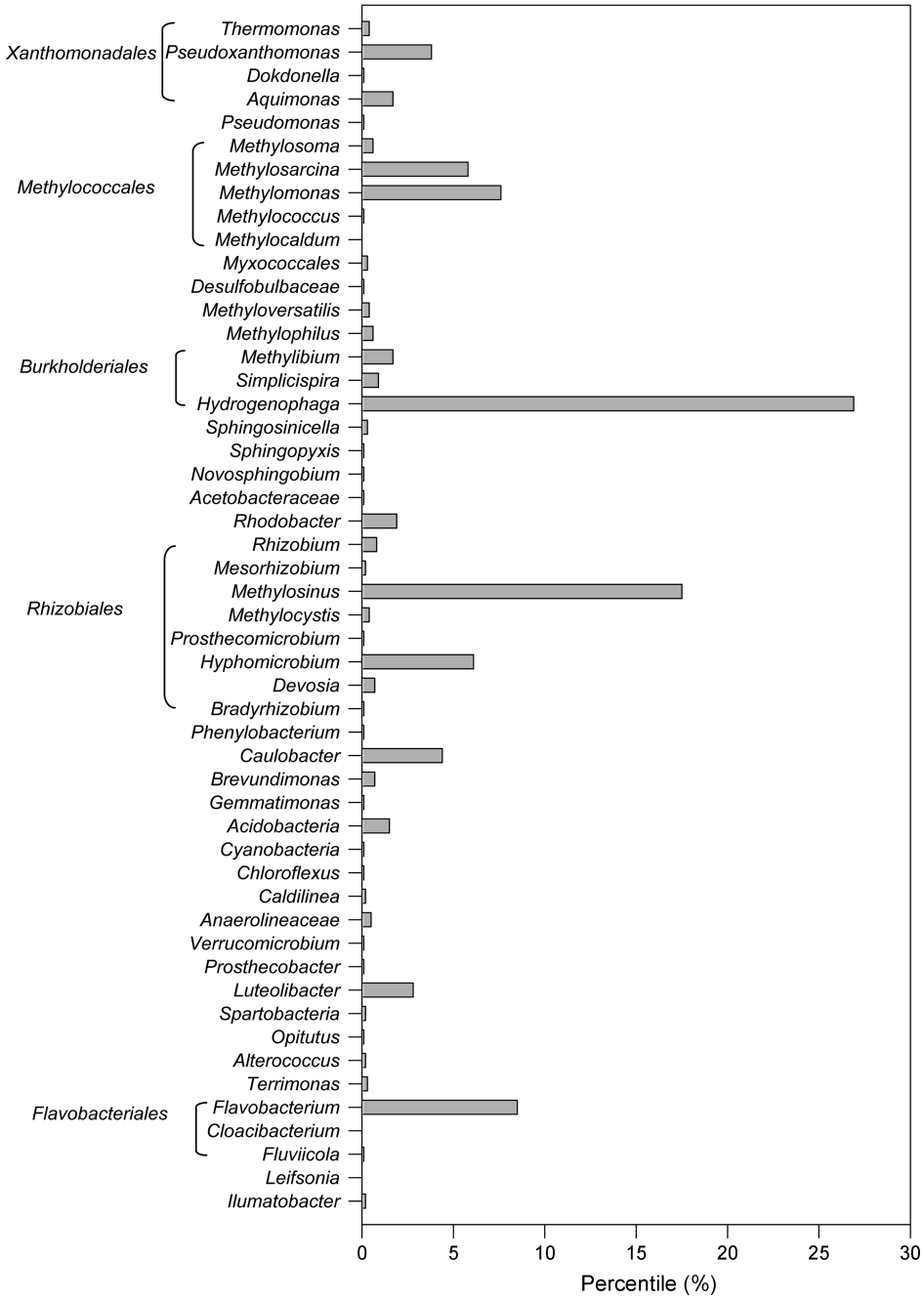

Fig. 3. Bacterial genera in the complete library.
Genera with reads (n) < 10 were excluded.

Table 3. Chao1 richness estimates and Shannon–Weaver diversity indices of types I and II methanotrophs.

	N	Dissimilarity	OTU[a]	Chao1[b] (95% CI[c])	H'[d]
γ-Proteobacteria; Type I	3,442	0%	780	2,643 (2,227–3,178)	3.721
		1%	178	333 (268–446)	2.324
		2%	59	125 (86–221)	1.714
		3%	46	71 (55–116)	1.606
α-Proteobacteria; Type II	4,354	0%	633	1,400 (1,210–1,652)	2.429
		1%	137	186 (161–237)	0.997
		2%	43	58 (48–90)	0.314
		3%	30	48 (35–95)	0.247

[a]Observed operational taxonomic units.
[b]Chao1 is a nonparametric richness estimator.
[c]Confidential interval.
[d]Shannon–Weaver index of diversity.

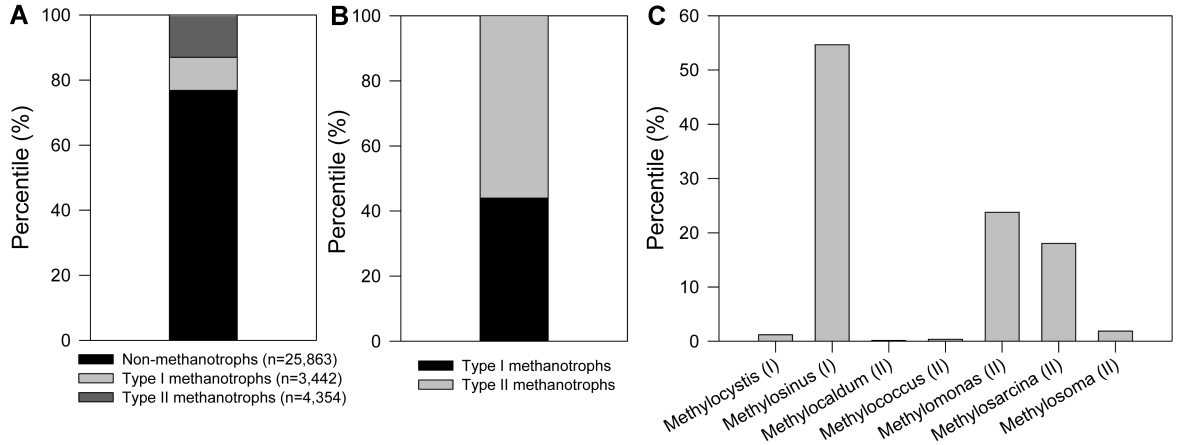

Fig. 4. Methanotroph community analysis using pyrosequencing.
Relative abundances of methanotrophs and non-methanotrophs (**A**), types I and II methanotrophs (**B**), and methanotrophic genera (**C**). Methanotrophs were grouped: type I methanotrophs (γ-*Proteobacteria*) and type II methanotrophs (α-*Proteobacteria*).

UniFrac test (Table 5). The results from both tests indicated that the phylogenetic trees of the four libraries did not differ. When the taxonomical compositions of the four libraries at the genus level were further compared (Fig. 5), the four libraries had the same taxonomic members, with similar relative abundances.

DISCUSSION

The microbial community of a methane-oxidizing biofilm was described, using 16S rDNA pyrosequencing for community composition analysis and quantitative real-time PCR for population analysis, that has been remained elucidated.

Table 4. Shared operational taxonomic units and Sørrenson similarity indices of the bacterial 16S rRNA gene libraries at 3% dissimilarity.

	Library 1 (354)	Library 2 (791)	Library 3 (342)	Library 4 (319)
Library 1 (354)	354[a] (1[bc])	284[a] (0.496[b])	200[a] (0.576[b])	198[a] (0.588[b])
Library 2 (791)	0.983[c]	791[a] (1[bc])	276[a] (0.487[b])	255[a] (0.459[b])
Library 3 (342)	0.975[c]	0.978[c]	342[a] (1[bc])	188[a] (0.568[b])
Library 4 (319)	0.982[c]	0.983[c]	0.971[c]	319[a] (1[bc])

[a]Shared OTU.
[b]Sørrenson incidence similarity index.
[c]Sørrenson abundance similarity index.

Table 5. Phylogenetic comparison of the bacterial 16S rRNA gene libraries using a P-test and weighted UniFrac test.

	Library 1	Library 2	Library 3	Library 4
Library 1	1[ab]	1[a]	0.42[a]	1[a]
Library 2	1[b]	1[ab]	1[a]	1[a]
Library 3	1[b]	1[b]	1[ab]	1[a]
Library 4	1[b]	1[b]	1[b]	1[ab]

[a]Weighted UniFrac P-value.
[b]P-test P-value.

There were diverse members in the biofilm, and interestingly, methanotrophs were not a dominant member, making up 23% of the community. These comprehensive survey results can provide a deep insight into the manipulation and/or management of methanotrophic biofilms. It was also determined that pyrosequencing analysis was useful for analyzing the microbial community in the biotechnological process, as it provided comprehensive information of the community and was highly reproducible irrespective of sequencing efforts.

A comparison of the numbers of OTUs with the Chao1 richness estimates revealed that this sequencing effort covered 62–83% of the estimated taxonomic richness at the 3–10% dissimilarity levels. qRT-PCR results showed that 1 µl of extracted DNA contained 7.75×10^5 16S rRNA gene copies (Table 1). It was extrapolated that a couple of million 16S rRNA gene copies were used for the pyrosequencing analysis, because the PCR reactions for the pyrosequencing contained 2.5–3 µl of sample DNAs. The results indicated that this sequencing effort retrieved 2–3% of the 16S rRNA gene population. Rarefaction curves of the observed OTUs were not saturated, suggesting that the library was not big enough to cover the entire diversity of the OTUs at those genetic distances, although 33,639 sequences were identified. Moreover, a rarefaction curve of the observed genera was similar with those of the OTUs. More than 60% of the total number of bacterial genera was observed, with only a 10% sampling size. The fact that there were many singletons making up 43% of the library should be noted.

There were a diverse number of genera in the biofilm, confirming the contention that there are a complex of microorganisms in methanotrophic biofilms. It was very interesting that bacteria other than methanotrophs were the main component of the biofilm. *Hydrogenophaga*, generally considered as chemolithoautotrophic bacteria (H_2 as an energy source and CO_2 as a carbon source) [42], are found in soil and water [24, 42]. However, some members are also able to grow heterotrophically [1]. About 60%, 28%, and 11% of the *Hydrogenophaga* members are closely related with heterotrophic *H. palleronii* (99.5% similarity, Accession number: AF019073), *H. intermedia* (98.6%, AF019037), and *H. bisanensis* (98.6%, EF532793), respectively.

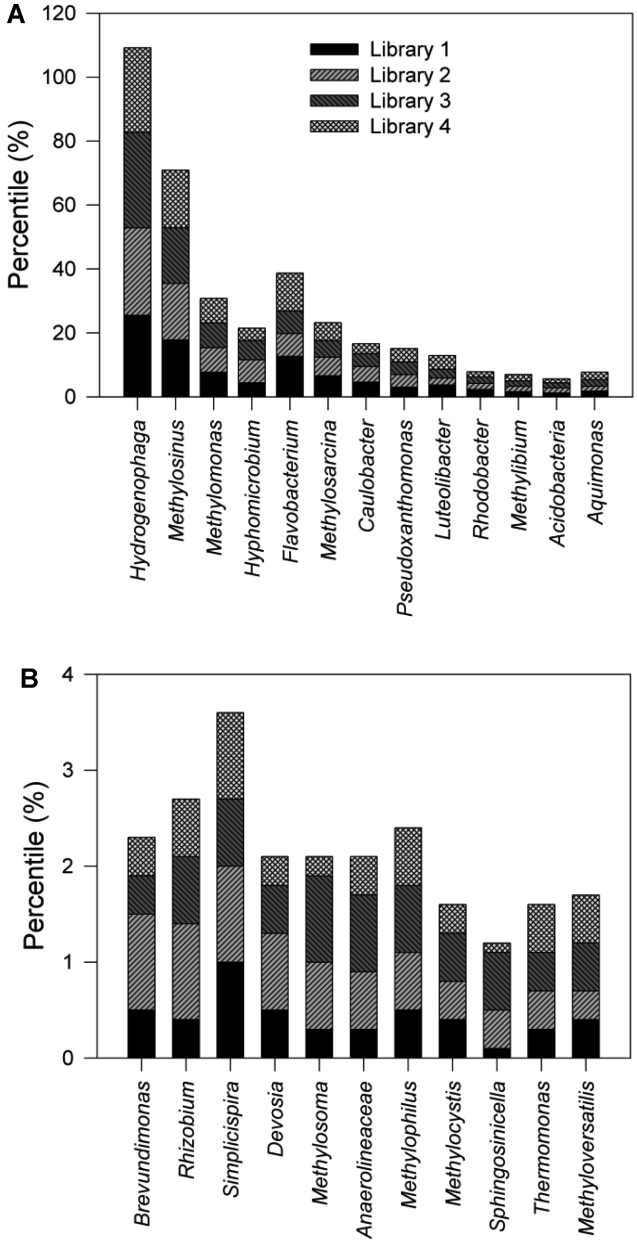

Fig. 5. Relative abundance of bacterial genera from each 16S rRNA gene library.
More and less than 5% (**A** and **B**, respectively). Genera with the assigned read number <0.5% of the sequencing efforts were excluded. Only genera observed in at least one library are shown.

Some of the *Hydrogenophaga* members, including the first two strains, are known as degraders of pollutants [1, 20, 43]. *Flavobacterium* spp. are aerobic chemoorganotrophs, and 98% of the observed *Flavobacterium* was closely related with *F. macrobrachii* (98.6%, FJ593904). *Hyphomicrobium* spp. are heterotrophic and restrictedly methylotrophic, and about 64%, 14%, and 10% of the observed *Hyphomicrobium* were *H. aestuarii* (99.3%, Y14304), *H. vulgare* (99.8%, Y14302), and *H. zavarzinii* (100%, Y14305), respectively.

About 55% and 44% of *Pseudoxanthomonas* were *P. japonensis* (98.8%, AB008507) and *P. mexicana* (99.7%, AF273082), respectively, which are heterotrophic [37]. Based on abundance, the listed non-methanotrophic genera might play an important role in the biofilm since community members influence the development and function of the biofilm, either directly or indirectly [38]. However, it is uncertain if these non-methanotrophs are common co-inhabitants with methanotrophs, and there is an association between them in the methanotrophic biofilm. It is also unknown if the relative abundance of methanotrophs is consistent in a biofilm, and the abundance ranking of non-methanotrophs is stable. It is possible that co-inhabitants are capable of degrading chemical pollutants, which is one of the purposes of methanotrophic biofilms, as well as methanotrophs, since some are known as degraders of chemical pollutants. To our knowledge, this is the first report to comprehensively survey the microbial community of a methanotrophic biofilm. It is still necessary to gather further information of the methanotrophic biofilm ecology to address those questions.

Methanotrophs comprised approximately 23% of the microbial community. The qRT-PCR results showed that 1 μl of extracted DNA contained 4.57×10^4 *pmoA* gene copies. Compared with the abundance of the 16S rRNA gene copies, the *pmoA* gene constituted approximately 9.2%. The *pmoA* gene is probably the most frequent target in molecular ecological techniques for methanotrophs, such as DGGE, qRT-PCR, and microarray [2, 12, 19, 36]. The presence of multiple copies of 16S rDNA and the less coverage of the *pmoA* primer set compared with that targeting rDNA could overestimate the value [19, 27, 32]. These results suggest that the number of methanotrophs may be 2.5 times greater than estimates of the *pmoA* gene abundance. Population levels of types I and II were quite comparable (44 *vs*. 56%, respectively), which differed from the previous observations that type II methanotrophs dominated the communities of landfill biofilters with clay minerals, as determined from a microarray assay [12] and phospholipid fatty acid analysis [11]. In contrast, the similar abundance of types I and II has been commonly observed in the environments [13, 16], as well as the predominance of type I members [8]. It is generally accepted that a type of methanotrophs can dominate a particular habitat, depending on its characteristics. It should be pointed out that all of the previous observations were made in soil environments. It is doubtful that an even distribution of types I and II is a common feature of methanotrophic biofilms. However, it was conclusive that the methanotrophic biofilm produced in the system had an almost equal distribution of types I and II (Fig. 4).

The complete library consisted of four sublibraries (*i.e*., pools of four individuals with different amounts of the 16S rRNA gene). The complete library was de-multiplexed into four libraries with different sequencing efforts (3,915–20,133 sequences). Those sequencing efforts covered 55–64% of the estimated taxonomic richness at 3% dissimilarity. The estimated richness and diversity levels depended on the size of the pyrosequencing libraries (*i.e*., library 2 *vs*. libraries 1, 3, and 4) despite the resampling. In practice, fewer sequences may result in lower numbers of observed and predicted OTUs. The Sørrenson incidence indices were more than 0.459 among the four libraries, whereas the Sørrenson abundance indices were greater than 0.971. Singletons constituted 46–59% of the observed OTUs in the four libraries, resulting in the difference between the incidence and abundance results. In addition, the Sørrenson incidence index is sensitive to the sample size [3]. The P-test and UniFrac results indicated that the four libraries were phylogenetically similar. The quantitative comparison of taxonomic members showed that the four libraries had the same genera, with similar relative abundance (Fig. 5). The relative abundance of genera with assigned reads greater than 1% of the sequencing efforts was very similar among the four libraries, although they were also comparable below 1%. The smallest library, 3, could mirror the microbial community, although only about 0.1–0.2% of the 16S rRNA gene population was examined. Collectively, the comparative results clearly indicated that pyrosequencing was highly reproducible, as it produced comprehensive information of the microbial community. A sequencing effort was not an important concern for the microbial community analysis. The results suggest that experimental replication and repetition are not necessary, at least if a biotechnological system is homogenized, although replication is necessary for microbial community studies [31].

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