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Bacterial Community Composition and Diversity of a Full-Scale Integrated Fixed-Film Activated Sludge System as Investigated by Pyrosequencing

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The integrated fixed-film activated sludge (IFAS) system is a variation of the activated sludge wastewater treatment process, in which hybrid suspended and attached biomass is used to treat wastewater. Although the function and performance of the IFAS system are well studied, little is known about its microbial community structure. In this study, the composition and diversity of the bacterial community of suspended and attached biomass samples were investigated in a full-scale IFAS system using a high-throughput pyrosequencing technology. Distinct bacterial community compositions were examined for each sample and appeared to be important for its features different from conventional activated sludge processes. The abundant bacterial groups were *Betaproteobacteria* (59.3%), *Gammaproteobacteria* (8.1%), *Bacteroidetes* (5.2%), *Alphaproteobacteria* (3.9%), and *Actinobacteria* (3.2%) in the suspended sample, whereas *Actinobacteria* (14.6%), *Firmicutes* (13.6%), *Bacteroidetes* (11.6%), *Betaproteobacteria* (9.9%), *Gammaproteobacteria* (9.25%), and *Alphaproteobacteria* (7.4%) were major bacterial groups in the attached sample. Regarding the diversity, totals of 3,034 and 1,451 operational taxonomic units were identified at the 3% cutoff for the suspended and attached samples, respectively. Rank abundance and community analyses demonstrated that most of the diversity was originated from rare species in the samples. Taken together, the information obtained in this study will be a base for further studies relating to the microbial community structure and function of the IFAS system.

Keywords: IFAS, pyrosequencing, activated sludge, wastewater, diversity, community

Wastewater treatment using activated sludge processes has been widely practiced, mainly because of their high treatment efficiency and low operating cost [12]. Activated sludge processes play important roles in the biodegradation of organic materials, transformation of toxic matters into harmless products, and the removal of nutrients. The integrated fixed-film activated sludge (IFAS) system is a variation of the activated sludge process in which biomass support materials or media are incorporated into suspended growth bioreactors [2, 7, 12, 14]. The biomass support materials are typically suspended plastic pieces or fixed synthetic mesh, which provide a large surface area for the attachment of microorganisms [7]. The IFAS system takes advantage of attached as well as suspended microorganisms for the treatment of wastewater. Attached microorganisms are relatively resistant to being washed out of the system on organic and hydraulic shock loadings [12]. Moreover, the IFAS system increases the sludge retention time, and enhances nitrification compared with conventional suspended growth systems [14]. Other advantages of the IFAS system include improved process stability, reduced excess biomass production, and better microorganisms settling characteristics [2]. Thus, the IFAS system is frequently used to retrofit or upgrade wastewater treatment plants (WWTPs) without increasing the bioreactors and settling tanks volumes [21].

Researchers have been trying to correlate the microbial community structures with the performance of wastewater treatment, and reported the link between the microbial community structures and the efficiencies of nitrification [25], phosphorus removal [11], and anaerobic digestion [10]. Moreover, the compositions, diversity, and dynamics of a microbial community are believed to affect the efficiency, robustness, and stability of WWTPs [1, 24]. Thus, the study of the microorganisms in WWTPs is crucial to better understand the functions and performance of wastewater treatment. Moreover, a thorough knowledge of microbial information is essential to develop operating strategies and improve process performance [23].

The study of microbial community structures has been regarded as the key to better understand the IFAS system.

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To date, several microbial studies have focused on specific groups of microorganisms in the system, including the relationship between viscous bulking and ammonia oxidizing bacteria [22], the effect of nitrifying community compositions on the performance of nitrification [8], and the identification of polyphosphate-accumulating organisms [13]; however, little attention has been paid to the total microorganisms. Furthermore, it is important to elucidate the microorganisms attached onto media and the suspended microorganisms simultaneously, because the IFAS system operates *via* the two groups of microorganisms.

The objective of this study was to investigate the compositions and diversities of the bacterial community of attached and suspended microorganisms, respectively, in an IFAS system. To this end, a high-throughput pyrosequencing technology was used for an extensive analysis of microorganisms for a full-scale IFAS system. Unlike the Sanger dideoxy chain termination technology, pyrosequencing detects released pyrophosphate during DNA synthesis [16]. This technique can perform around 400,000 simultaneous reactions [4], and has the advantages of accuracy, flexibility, parallel processing, and simplicity over the Sanger method [3], which is a suitable tool for studying microbial ecology. This study investigated similarities and differences of the microbial community structure of the IFAS system as evaluated by the two different methods (*i.e.*, pyrosequencing technique versus Sanger sequencing technique, which was another objective of this study).

Materials and Methods

WWTP, Sampling, and DNA Extraction

Biomass samples were collected in June 2009 from the Guri WWTP (Guri, South Korea). The WWTP employs an IFAS system, named the CNR process (H2L Co. Ltd., Anyang, South Korea), which treats 160,000 m^3/d of domestic wastewater. The bioreactor of the CNR process consists of anaerobic, anoxic, and aerobic basins for enhanced removals of nitrogen and phosphorus as well as organic matter. In the aerobic basin, a fixed synthetic mesh made from acryl and polyether was incorporated. The detailed operational conditions and characteristics of the wastewater used are summarized in Table 1. Grab samples were collected from the aerobic basin. A bucket-type sampler was used for the suspended microorganisms, whereas a sterilized scissor was used to cut off a part of the fixed synthetic mesh, after lifting the mesh, for the attached microorganisms. Samples were immediately frozen before being transported to the laboratory. Genomic DNA was extracted from 1.5 ml of suspended microorganisms and 1.5 g of fixed synthetic mesh, in duplicate, using the UltraClean soil extraction kit (Mobio Laboratories, Solana Beach, USA) following the manufacturer's protocol.

PCR Amplifications

Bacterial 16S rRNA gene fragments were PCR-amplified using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3'). Each 50-μl PCR reaction included 1× EF-*Taq* buffer (Solgent, Daejeon, South Korea), 2.5 units of EF-*Taq* polymerase (Solgent), 0.2 mM dNTP mix, 0.1 μM of each primer, and 100 ng of template DNA. The PCR temperatures were as follows: 95°C for 10 min; 35 cycles consisting of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The duplicate PCR products were pooled and purified using the QIAquick gel extraction kit (Qiagen, Seoul, South Korea). The purified PCR products were used for the following cloning and pyrosequencing.

Table 1. Wastewater characteristics and operational data for the Guri wastewater treatment plant.

Parameter	Value*
Temperature (°C)	23.3
pH	7.0
Dissolved oxygen (mg/l)	3.5
Hydraulic retention time (h)	5.9
Mixed liquor volatile suspended solids (mg/l)	3072
Solids retention time (day)	8.5
Biochemical oxygen demands (mg/l)	120.5, 8.0†
Suspended solids (mg/l)	124.4, 8.0†
Total nitrogen (mg N/L)	32.2, 15.0†
Total phosphorus (mg P/L)	2.9, 1.5†

*All values are average of a month data before sampling.
†Values of influent and effluent.

Cloning and Sanger Sequencing

The purified PCR products were cloned using the pGEM-T Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions. Randomly picked transformed *Escherichia coli* clones were transferred to 96-well plates and sequenced using the T-7 primer on ABI 3730XL sequencers (Applied Biosystems, Foster City, USA) by Macrogen (Seoul, South Korea).

Pyrosequencing

The ends of the purified PCR products (~1 μg) were blunted, with short adaptors (14-bp long), and then ligated onto both ends. The adaptors play roles in priming sequences for both the amplification and sequencing reactions, and provide the sequencing key, which is recognized by the system software for base calling. The modified PCR products to be sequenced were attached onto DNA capture beads, as one fragment per bead, and then amplified using emulsion-based clonal amplification. The beads were set into the wells of a PicoTiterPlate device (1 of 8 lanes), with appropriate chemicals and enzymes, and inserted into the Genome Sequencer FLX Titanium system (Roche, Mannheim, Germany) for pyrosequencing. All of the procedures followed the manufacturer's protocols (Roche) and were conducted at Macrogen.

Data Analysis

Initially, the raw sequences obtained from pyrosequencing were processed using the GL FLX software (Roche) for sorting by the key (*i.e.*, sequences from the suspended and attached samples), with low quality sequences discarded, and the primer sequences trimmed. In addition, sequences less than 250-bp long were discarded. The processed sequences were subjected to taxonomicall classification

using the Classifier in the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) following the developer's instructions. A rarefaction curve, diversity indices (*i.e.*, observed OTUs and the Chao1 estimator), Venn diagram, and rank abundance curve were generated using the Mothur utility [19], after aligning the sequences and constructing a distance matrix using the RDP's Pyrosequencing Aligner and RDP's Column Formatted Distance Matrix, respectively, according to the developer's introduction.

Sequence Deposition

The sequences determined in this study have been deposited in GenBank under accession numbers from GU481685 to GU549391.

RESULTS

Bacterial Community Compositions

Pyrosequencing yielded a total of 23,536 (average length=434.5 bp) and 44,003 bacterial 16S rDNA sequences (average length=455.6 bp) for the suspended and attached biomass samples, respectively. An RDP classifier, with an 80% cutoff value, assigned taxa to the sequences, and identified 19 and 15 phyla for the suspended and attached biomass samples, respectively. Fig. 1A summarizes the taxonomic compositions by phylum for each sample, demonstrating distinct bacterial community compositions.

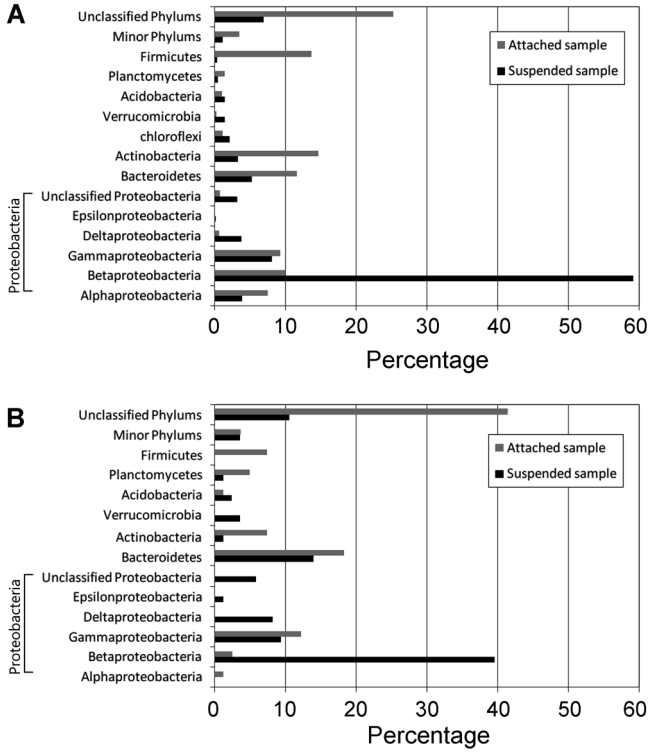

Fig. 1. Taxonomic composition by phylum for the sequences retrieved from the suspended and attached samples of a full-scale IFAS system using (**A**) pyrosequencing and (**B**) cloning and Sanger sequencing technologies.

Proteobacteria dominated the suspended sample (78.1% of the total sequences), followed by *Bacteroidetes* (5.2%), *Actinobacteria* (3.2%), and *Chloroflexi* (2.0%). *Betaproteobacteria* was the principal member of the *Proteobacteria* (59.3%). *Gamma-*, *Alpha-*, and *Deltaproteobacteria* accounted for 8.1%, 3.9%, and 3.7%, respectively. As for the suspended sample, *Proteobacteria* was the major member of the attached sample, but constituted only 27.9% of the total sequences, followed by *Actinobacteria* (14.6%), *Firmicutes* (13.6%), and *Bacteroidetes* (11.6%). Within the phylum *Proteobacteria*, *Beta-*, *Gamma-*, *Alpha-*, and *Deltaproteobacteria* constituted 9.9%, 9.2%, 7.4% and 0.6%, respectively. The difference in the compositions of the bacterial community between the two samples was obvious when analyzing at the lower taxonomic levels. Table 2 shows the 15 dominant taxa found in each sample at the genus level. *Propionivibrio* (13.2%) and *Dechloromonas* (5.1%) were the two most dominant genera in the suspended sample, whereas *Thermomonas* (5.5%) and *Lactobacillus* (5.2%) were those in the attached sample.

Cloning and Sanger sequencing retrieved a total of 86 and 82 bacterial 16S rDNA sequences for the suspended and attached biomass samples, respectively. Taxonomic assignment of the sequences by phylum showed similar trends with that obtained from pyrosequencing (Fig. 1B), but the relative ratio among phyla was significantly different (P>99%). In addition, the dominant genera of the two samples were identified to be quite different. For instance, as shown in Table 2, cloning and Sanger sequencing identified *Methylobacter* as the most dominant genus in the attached sample (4.9%), but pyrosequencing demonstrated *Thermomonas* (5.5%) as the dominant genus.

Bacterial Diversities

For the sequences determined from pyrosequencing, 3,034 and 1,451 operational taxonomic units (OTUs) were identified at the 3% cutoff for the suspended and attached samples, respectively. For comparison of the species richness between the two samples, rarefaction curves were generated using 3% and 5% cutoffs, respectively, as shown in Fig. 2. The suspended sample showed a steeper slope than the attached sample with the two cutoffs, demonstrating the higher richness of the suspended sample than the attached sample. In addition, the slope of the suspended sample did not become flat, indicating the number of samples (*i.e.*, the total number of sequences obtained in this study) was not big enough to explain the large fraction in the OTUs. The Chao1 richness estimated 4,482 and 1,847 OTUs at the 3% cutoff for the suspended and attached samples, respectively, also demonstrating the higher diversity within the suspended sample.

Similar analyses were conducted for the sequences obtained from cloning and Sanger sequencing. A total of

Table 2. The 15 most dominant genera identified from the suspended and attached samples of a full-scale IFAS system based on pyrosequencing and cloning–Sanger sequencing technologies.

Genus	Percentage of sequences			
	Suspended sample (Pyrosequencing)	Suspended sample (Cloning)	Attached sample (Pyrosequencing)	Attached sample (Cloning)
Nocardioides	0.13	0.00	2.07	2.44
Mycobacterium	0.63	1.16	2.52	1.22
Haliscomenobacter	1.27	2.33	0.06	0.00
Terrimonas	0.05	0.00	1.39	3.66
Cloacibacterium	0.00	0.00	1.07	2.44
TM7	0.08	0.00	2.38	2.44
Lactobacillus	0.01	0.00	5.19	1.22
Weissella	0.00	0.00	2.71	1.22
Peptostreptococcaceae	0.05	0.00	1.14	0.00
Novosphingobium	0.52	0.00	1.81	0.00
Dechloromonas	5.06	2.33	2.12	0.00
Propionivibrio	13.18	3.49	1.88	0.00
Methylobacter	0.65	0.00	0.52	4.88
Thermomonas	0.23	0.00	5.53	3.66
Planctomyces	0.17	0.00	0.41	2.44
Total	22.03	9.31	30.8	25.62

76 and 58 OTUs were observed for the suspended and attached samples, respectively (3% cutoff). The Chao1 richness indices were significantly lower than those for the sequences obtained from the pyrosequencing method, these being 545 and 231 OTUs at the same cutoff (*i.e.*, 3%) for the suspended and attached samples, respectively. These results strongly support that conventional cloning and Sanger sequencing underestimated the bacterial diversity of the samples compared with the pyrosequencing technology.

DISCUSSION

The composition of the bacterial community from the conventional suspended growth-activated sludge process has been extensively studied owing to its importance in the performance and function of wastewater treatment. According to a review article by Wagner and Loy [23], which surveyed eight different WWTPs, *Beta*-, *Alpha*-, and *Gammaproteobacteria*, and *Bacteroidetes* were frequently found, in that order, in WWTPs, although the relative ratios were somewhat variable depending on the feed wastewater, treatment process, size of bioreactor, *etc*. The composition of the bacterial community of the suspended sample identified in this study showed a similar trend with that report (Fig. 1), whereas the bacterial assemblage of the attached sample was quite different. Pyrosequencing demonstrated that sequences within the *Actinobacteria* (14.6%), *Firmicutes* (13.6%), and *Bacteroidetes* (11.6%) were more dominant than those within the *Beta*- (9.9%), *Alpha*- (7.4%), and *Gammaproteobacteria* (9.2%). It is speculated that the synthetic mesh incorporated into the bioreactor provided an environment where some microorganisms (*i.e.*, some members within the *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*) could grow preferentially; otherwise, the microorganisms would have been washed out of the system. In addition, oxygen stress appeared to affect the distinct bacterial community structure of the attached sample. Typically, the oxygen level reaches almost 0 mg/l below the depth of a 200 μm fixed biofilm [18]. It was expected that oxygen would not be available for the microorganisms under that depth. It is noted that *Lactobacillus*, an anaerobic fermentive bacteria, was mostly

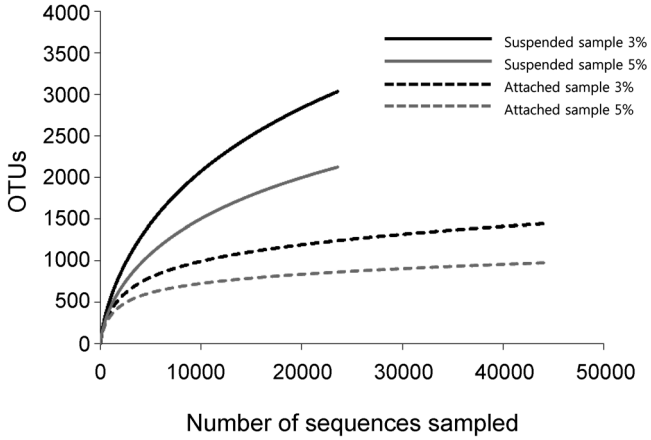

Fig. 2. Rarefaction curves of OTUs defined by 3% and 5% sequence variations in the suspended and attached samples of a full-scale IFAS system.

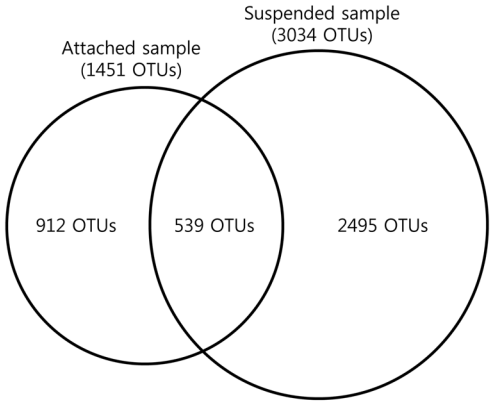

Fig. 3. Venn diagram of shared OTUs between the suspended and attached samples of a full-scale IFAS system.

found in the attached sample (Table 2). Taken together, the distinct bacterial community compositions between the suspended and attached biomass samples might contribute to the features of the IFAS system that are different from the conventional suspended systems.

The surface of the attached biomass contacts with the suspended biomass in the IFAS system. Thus, it would be reasonable to expect that a portion of the bacterial consortium was shared between the two samples. This hypothesis was evaluated by identifying the numbers of shared OTUs between the two samples using a Mothur's Venn diagram analysis (Fig. 3). The suspended and attached samples had 539 OTUs in common (37.2% and 17.8% of the total OTUs identified in the attached and suspended samples, respectively). An RDP classifier, with an 80% cutoff value, assigned taxa at the genus level to the shared OTUs. Genera *Dechloromonas*, *Mycobacterium*, *Propionivibrio*, and *Thermomonas* were the major members shared by the two samples.

Unassigned bacterial sequences at the phylum level were significant in our results, these being 6.9% and 25.2% of the total sequences for the suspended and attached samples, respectively (Fig. 1A). It is unlikely that the sequences were generated from unspecific PCR amplification or had never been observed. Rather, the accumulation of 16S rDNA sequences in the RDP database is outpacing our ability to classify these sequences, as inferred by Sanapareddy *et al*. [17]. The high identity of the unclassified sequences with previously observed sequences in the GenBank database (96.8% and 97.2% average identify for the unclassified suspended and attached sequences, respectively) confirms our reasoning.

Compared with the bacterial richness (*i.e*., the Chao1 value) estimated by conventional cloning and Sanger sequencing, those estimated *via* pyrosequencing were about eight times higher. This was more evident for the differences in the bacterial richness from the rank

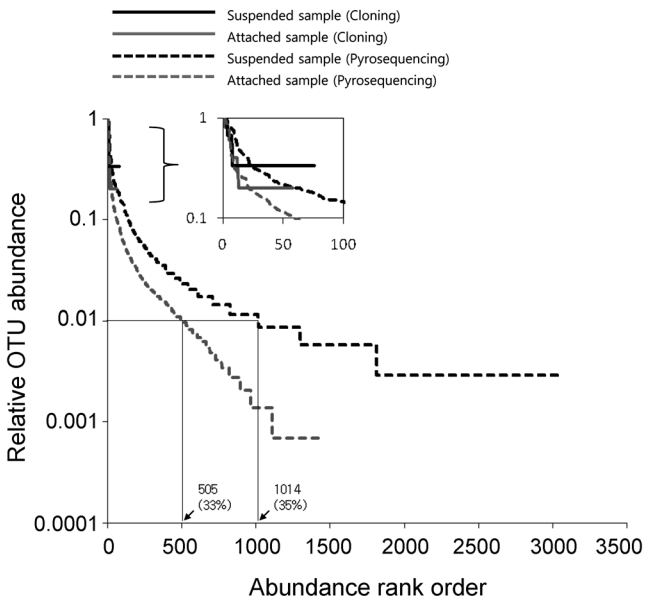

Fig. 4. Rank abundance curves of OTUs defined by a 3% sequence variation in the suspended and attached samples of a full-scale IFAS system.

abundance curve for OTUs shown in Fig. 4. Samples analyzed by pyrosequencing had longer tails (*i.e*., higher abundance rank) than those analyzed by cloning and Sanger sequencing. The increased numbers of sequences found in the pyrosequencing might affect the high diversity, which is similar to that found in other reports [6, 15]. Interestingly, 1% of the total sequences (*i.e*., relative OTU abundance=0.01 in Fig. 4) constituted 67% and 65% of the total bacterial diversity (*i.e*., observed OTUs) for the suspended and attached biomass samples, respectively, demonstrating that most of the diversity in the IFAS system originated from rare species in the samples. This finding raises a question that the low abundance of the majority of diversity might not contribute to the process performance. However, the diversity in a wastewater treatment plant appears to be important to maintain the bioreactor stability to the change of wastewater loading and the input of toxic matters [1]. In addition, pyrosequencing observed very rare bacterial signatures that have not been reported previously, which might also have affected the high diversity. For instance, sequences belonging to the phylum *Spirochaetes*, *TM7*, *Gemmatimonadetes*, *OP10*, *OD1*, and *SR1* were found in the suspended sample, although the quantity was less than 1.0% of the total observed sequences. The diversity estimated in this study was around one-order of magnitude higher than that found in previous reports. Wagner and Loy [23] reported 17 to 268 bacterial OTUs for activated samples, using a cloning approach for the estimation. It is also worthwhile noting the higher diversity of the suspended sample compared with that of the attached

sample, which was supported by the observed OTUs, the Chao1 richness index, the rarefaction curve (Fig. 2), and the rank abundance curve (Fig. 4). A possible cause for the difference in bacterial diversity of the two samples has not been proved in this study. Nevertheless, the difference appears to affect functional differences of the two samples based on several evidences reporting the relationship between the microbial diversity and some functions of WWTPs such as stability [1, 9, 24] . The results obtained in this study focused on the microbial diversity rather than the quantitative distribution of specific bacteria, which is more important for determining the function of WWTPs. Although the percentages of some specific groups of microorganisms provided in this study (Fig. 1 and Table 2) could not represent precise quantitative information owing to the bias introduced by DNA extraction, PCR amplification, and cloning, the information may be worthwhile to identify dominant groups of microorganisms in the IFAS system, considering the reports of the comparative community analyses demonstrating general agreement between clone library data and quantitative fluorescent *in situ* hybridization results in activated sludge samples [5, 20].

Interestingly, pyrosequencing demonstrated quite different results for the composition of the bacterial community as well as for the diversity compared with those obtained from cloning and Sanger sequencing, as shown in Fig. 1 and Table 2. The estimated relative ratios of the bacterial composition and richness were different depending on the sequencing method used. A cloning step after the PCR amplification of 16S rDNA fragments appeared to affect the difference between the two methods.

To our knowledge, this study is the most comprehensive examination to date to identify the composition and diversity of the bacterial community in a full-scale IFAS system. The bacterial assemblages of the suspended and attached samples were investigated using a high-throughput pyrosequencing and conventional cloning and Sanger sequencing technologies, which revealed the members of bacteria affiliated to each sample. Distinct bacterial community compositions appeared to affect the characteristics of the IFAS system. In addition, the bacterial diversity of a WWTP was about one-order of magnitude larger than those found in previous reports. Taken together, the information obtained in this study could be used as a base for future studies including isolation of specific microorganisms, phylogenetic analysis of some important groups of microorganisms in wastewater treatment (*e.g.*, nitrifying bacteria), and targeting novel microorganisms in the IFAS system. For a practical application of the bacterial community information to the operation and design strategy of the IFAS system, the relationship between the bacterial community structure and environmental or operational factors of the IFAS system should be determined.

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