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Distribution and abundance of *Spirochaetes* in full-scale anaerobic digesters

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HIGHLIGHTS

- Ecology of *Spirochaetes* was studied by pyrosequencing of 16S rRNA gene.
- Local effects were important in shaping the structure of *Spirochaetes*.
- The abundance of *Spirochaetes* was influenced by environmental variables.
- Acetate addition increased the activity of *Spirochaetes*.

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ABSTRACT

To investigate the distribution and abundance of spirochaetal communities within anaerobic digesters, pyrosequencing of the 16S rRNA gene was conducted. Phylogenetic analysis identified a cluster which included the majority of core spirochaetal operational taxonomic units (OTUs) and environmental clones but no pure-culture strains. Distribution of the core OTUs demonstrated an importance of local factors in shaping the structure of *Spirochaetes*. Spirochaetal to bacterial 16S rRNA gene copy numbers varied from 1.3% to 30.0% depending on digester samples. Environmental variables were found to influence the relative abundance of *Spirochaetes*. In a batch anaerobic digestion experiment testing the response to different substrates, acetate most stimulated the activity of *Spirochaetes*, suggesting possible acetate oxidation by syntrophic acetate oxidation process. Taken together, the results obtained in this study provides an insight into the ecology of *Spirochaetes* in anaerobic digesters and a basis for future studies examining ecological function of these bacteria.

1. Introduction

Anaerobic digestion is a microbial process that transforms complex organic matters into biogas consisting mainly of methane and carbon dioxide. Because methane has an energy value of 35 kJ/L at standard conditions (Rittmann and McCarty, 2001), anaerobic digestion has received attention as an efficient way of providing a decentralized energy source. For example, wastewater treatment plants frequently exploit anaerobic digestion and biogas production for beneficial purposes such as heating and electricity generation as well as reducing the volume of sludge produced during aerobic activated sludge process.

Anaerobic digestion is achieved by the interplay between microorganisms within *Bacteria* and *Archaea* domains (Raskin et al., 1995; Rittmann and McCarty, 2001). Initially the complex organic matters are sequentially disintegrated, hydrolyzed, and fermented into acetate and hydrogen which are the two main intermediates of anaerobic digestion produced by anaerobic bacteria. Acetate and hydrogen are then transformed into methane and carbon dioxide by anaerobic methanogenic archaea (i.e., aceticlastic and hydrogenotrophic methanogens). Generally methanogenic archaea grow slower than anaerobic bacteria, therefore a great deal of microbial research on anaerobic digestion has been focused on methanogenic archaea.

Diverse studies have been attempted to identify key microbial taxa to better understand their ecophysiology in relation to anaerobic digestion (Chouari et al., 2005; Delbes et al., 2000; Godon et al., 1997; Kim et al., 2013; Li et al., 2013; Mao et al., 2013; Patil et al., 2010; Raskin et al., 1995; Zhilina et al., 1996) and to ultimately improve anaerobic digestion efficiency and process stability. Bacteria within the phylum *Spirochaetes* are frequently

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detected in anaerobic digestion systems that treat municipal sludge (Chouari et al., 2005; Zhilina et al., 1996), industrial wastewater (Ben-Dov et al., 2008), livestock wastewater (Metcalf & Eddy, 2003; Rittmann and McCarty, 2001), and even synthetic organic matters (Lima et al., 2005). Although they are ubiquitous in anaerobic digesters, their proportions are reported to be quite varied. For instance spirochaetal clones constituted 4.1% of total bacterial clones in a digester treating distillation waste (Godon et al., 1997); whereas 47.2% were attributed to an OTU belonging to *Spirochaetes* in a laboratory-scale anaerobic digester fed with glucose (Fernandez et al., 1999). However, the distribution and abundance of *Spirochaetes* in anaerobic digesters have rarely been studied because of a limited understanding about the possible importance of *Spirochetes* in anaerobic digestion. *Spirochaetes* are Gram-negative bacteria that live chemoheterotrophically in anaerobic environments and are generally known to have helically coiled morphology. Five families and 13 genera are reported (Ludwig et al., 2008); however, no information on pure-culture strains isolated from anaerobic digesters is available. In anaerobic digesters, *Spirochaetes* are assumed to be glucose fermenters based on a finding of increased *Spirochaetes* activity in response to the addition of glucose into an anaerobic digestion reactor (Delbes et al., 2000). However, the ecophysiological function of *Spirochaetes* found in anaerobic digesters is virtually unknown (Delbes et al., 2000).

The main objective of this study was to investigate distribution and abundance of *Spirochaetes* and their correlation with environmental variables in anaerobic digesters. The distribution was analyzed based on spirochaetal 16S rRNA gene sequences obtained from 454 pyrosequencing in seven full-scale anaerobic digesters. Furthermore, the abundance was inferred using a qPCR assay developed for this study. In addition, batch experiments were also performed to identify the ecophysiological function of *Spirochaetes* found in the anaerobic digesters.

2. Methods

2.1. Anaerobic digesters, sludge sampling, and pyrosequencing

Distribution and abundance of *Spirochaetes* were analyzed for the seven full-scale anaerobic digesters (J2, J3, N1, N2, S1, S2, and T1 digesters) located in Seoul, South Korea. Sludge samples were collected monthly from a sludge recycling pump from January 2011 until June 2011. For pyrosequencing of the bacterial 16S rRNA gene, total DNA was extracted from the sludge samples and the 16S rRNA gene was amplified via PCR using universal primers (27F forward primer and 518R reverse primer, Table 1). Using the PCR amplicons, single-stranded DNA library construction, emulsion PCR, and pyrosequencing were conducted at Macrogen (Seoul, South Korea). After removing poor quality sequences, adapter sequences, non-bacterial sequences, and chimeric sequences, quality DNA sequences were obtained for data analyses. Detailed information about characteristics of the digesters, sampling, pyrosequencing, and purification of raw sequences were described in a previous study (Lee et al., 2012).

2.2. Design of primers for Spirochaetes

A specific primer for the 16S rRNA gene belonging to the Cluster 2 *Spirochaetes* (Spiro-21F, Table 1) was designed based on the consensus sequences (majority rule) of the Cluster 2 *Spirochaetes* using the Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/). Furthermore, the specificity of Spiro-21F was evaluated using the Probe Match program in the RDP website (http://rdp.cme.msu.edu/) by assessing the extent to which the primer binds to both the target group and non-target sequences (Lee and Cho, 2011). Using the RDP database (release 10.29), a total of 199,604 sequences that were over 1.2 kb in size were used for the *in silico* evaluation of primer specificity. The specificity of the Spiro-21F primer was also tested empirically. Total DNA was extracted from a sludge sample obtained from the S1 digester. The total DNA was used as a template for the PCR primer set consisting of the Spiro-21F forward primer and universal reverse primer 342R (Lane, 1991). The reaction mixture included 25 μl of TaKaRa Ex Taq premix (Takara, Shiga, Japan), 1 μl of each forward and reverse primers (20 μM), 200 ng of template DNA, and sterilized distilled water for a final reaction mixture volume of 50-μl. The PCR thermal profile was as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 60 °C for 1 min, and extension at 72 °C for 2 min; the final elongation step was extended to 20 min. PCR amplification was performed with a Mastercycler Pro (Eppendorf, Hamburg, Germany). The PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and cloned into TOPO cloning vectors with a TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) in order to construct a clone library according to the manufacturer's protocol. A total of 50 clones were randomly selected from the clone library and sequenced using ABI3700 (Applied Biosystems) at Solgent (Daejeon, South Korea). Lastly, phylogenetic affiliations of the clones were determined using RDP's Bayesian Classifier (Wang et al., 2007) to test for clone specificity.

2.3. qPCR of Bacteria and Spirochaetes

The bacterial 16S rRNA gene was quantified using universal primers 27F and 342R, while the Cluster 2 *Spirochaetes* were quantified using a primer set designed in this study (i.e., Spiro-21F and 342-R primers). The sequence information of these primers is shown in Table 1. qPCR was performed using the Bio-Rad CFX-96 real time system (Bio-Rad, Hercules, CA, USA). Each reaction mixture included 10 μl of SYBR Premix Ex Taq™ (Takara, Shiga, Japan), 0.4 μl each of the forward and the reverse primers (10 μM), 0.4 μl of 50 × ROX™ Reference DyeII, 2 μl of template DNA, and sterilized distilled water to make a final volume of 20-μl. The thermal profile of qPCR for the bacterial and spirochaetal 16S rRNA gene was as follows: initial denaturation at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 34 s. Fluorescent signal intensity was collected at the end of the annealing/extension step. At the end of each run, a dissociation protocol (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s) was performed to ensure that nonspecific amplicons were absent. A

Table 1
Primers used in this study for pyrosequencing and real-time qPCR.

Name	Target	Sequences (5′ → 3′)	Position[a]	GC %	Reference
27F	Bacteria	AGAGTTTGATCCTGGCTCAG	8–27	50	Lane (1991)
342R	Bacteria	CATGCAAGTCGAACGGTAGG	342–357	55	Lane (1991)
518R	Bacteria	CCTATCCCCTGTGTGCCTTGGCAGTC	518–543	62	Li et al. (2005)
Spiro-21F	Cluster 2 *Spirochaetes*	CTGCTGCCTCCCGTAGGAGT	21–40	55	This study

[a] Position indicates the numbering of *Escherichia coli* 16S rRNA.