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SHORT COMMUNICATION

Unusual bacterial populations observed in a full-scale municipal sludge digester affected by intermittent seawater inputs

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Abstract This study investigated the bacterial community of a full-scale anaerobic digester, which suffers from intermittent seawater contaminations, using 16S rRNA gene clone analysis over different seasons. *Bacteroidetes*, *Proteobacteria*, and unclassifiable bacteria were the three major bacterial groups within the clone library (a total of 290 clones). A significant portion of the total clones (29.3%) was not affiliated to any previously reported phylum, and 55.3% of the unclassifiable clones (16.9% of the total clones) showed potential relations to the species of *Thermotogae*, rarely present under normal mesophilic anaerobic conditions. These results suggested that the novel populations may have the potential to play an important role in anaerobic processes, particularly under abnormal environmental conditions. Additionally, statistical analysis supported that seasonal variations in influent characteristics, and potential competitions among different populations, may be related to the unusual bacterial diversity and community dynamics observed over the study period.

Keywords 16S rRNA gene · Anaerobic digestion · Clone library · Seawater, sewage sludge

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Introduction

Anaerobic digestion (AD) is important for carbon recycling in natural ecosystems [1, 2] and has been widely used for treating organic waste (water) due to the production of methane [3]. AD is a sequential reaction carried out by various symbiotic microorganisms broadly grouped into acidogens and methanogens [3]. The former group belonging to the *Bacteria* is responsible for the hydrolysis and fermentation of organic materials to form organic acids and alcohols, and the latter belonging to the *Archaea* converts the acidogenic product to methane. Completion of AD, therefore, depends on the concerted activity of diverse populations, which suggests that comprehensive microbial information is desired for improving AD technology.

Although significant efforts have been devoted to identifying microorganisms in AD, the great majority remain uncharacterized [1, 2]. Knowledge of anaerobic microbial communities has been greatly extended recently by culture-independent approaches based on molecular techniques, particularly targeting 16S rRNA genes. Application of molecular techniques has enabled us to reveal a number of unknown microbial strains that have never been isolated by conventional culture-dependent methods [4]. Recently, it has been reported that anaerobic digesters harbor several strains of novel high-order bacterial lineages of candidate divisions, such as OD1, WS3, OP3, NBL-UPA2, and BRC1 [5]. Such richness, regarding the potential for revealing novel populations, has made AD an attractive object of ecophysiological study. This microbial information would be useful for understanding the key factors underpinning the AD, which may lead to improved performance.

Many previous studies have focused on methanogens, as they are directly responsible for producing methane. However, the overall process should also be viewed in light of

Table 1 Operating conditions of the anaerobic digester during distinct seasonal periods

	Fall	Winter	Summer
Temperature (°C)	31.8 ± 1.8	28.8 ± 0.9	32.3 ± 1.6
pH	6.5 ± 0.1	6.6 ± 0.2	6.5 ± 0.1
Influent COD (g/l)	22.6 ± 3.9	20.2 ± 3.3	24.6 ± 3.5
COD removal (%)[a]	37.3 ± 9.8	49.9 ± 8.8	32.0 ± 8.5
VS removal (%)	27.6 ± 8.4	44.3 ± 7.6	31.4 ± 7.2
Methane content (%)[a]	48.2 ± 4.1	43.3 ± 8.2	45.9 ± 1.8
Alkalinity (mg/l)	1,901 ± 71	2,068 ± 179	2,017 ± 189
Na^+ (mg/l)	2,155 ± 312	2,614 ± 192	2,111 ± 469
Mg^{2+} (mg/l)	263 ± 53	300 ± 45	249 ± 57
Cl^- (mg/l)[a]	4,652 ± 895	6,875 ± 957	4,578 ± 852
SO_4^{2-} (mg/l)	109 ± 126	48 ± 58	78 ± 72
Influent carbohydrate (mg/l)[a]	3,507 ± 674	5,042 ± 861	4,760 ± 800
Influent protein (mg/l)	10,516 ± 1,878	11,896 ± 1,440	9,376 ± 1,110
Influent lipid (mg/l)[a]	1,976 ± 312	2,505 ± 484	2,015 ± 170

The observations are averaged values of weekly to monthly data (average ± standard deviation)

COD chemical oxygen demand, *VS* volatile solids

[a] Parameters with significant seasonal variations or correlations with other parameters

acidogens, since they initiate the breakdown of organic compounds and produce the major substrates for methanogens [6]. This study thus assessed the bacterial diversity in a full-scale AD system exposed to intermittent seawater contaminations, which has rarely been reported in the literature, during different seasons. We focused on this shortfall of information on the AD microbiology.

Materials and methods

Anaerobic digester and sampling

This study was carried out in a full-scale anaerobic digester treating sewage sludge from a municipal activated sludge plant (410,000 population equivalents) in Pohang, Korea. This completely stirred tank reactor (CSTR) was operated at 31.0 ± 2.5°C with a sludge retention time (SRT) of 33 ± 1 days. Anaerobic sludge was collected via a valve at the base of the digester. The operating conditions within the sampling period are summarized in Table 1. The seawater contamination was derived from an ill-sealed sewer pumping station located close to the sea.

Bacterial 16S rRNA gene clone library analysis

Total DNA was extracted using an automated nucleic acid extractor (Magtration, System 6GC, PSS Co.) as previously described [7]. From the extracted DNA, 16S rRNA genes were PCR-amplified with primers BAC338F (5′-ACTCCT ACGGGAGGCAG-3′) and BAC805R (5′-GACTACCA GGGTATCTAATCC-3′) [7]. Reaction was performed using the AccuPower PCR PreMix (Bioneer, Daejon, Korea) following the manufacturer's protocol. PCR products were gel-purified and cloned into a pGEM-T Easy Vector (Promega, Madison, WI). A total of 300 clones (i.e., 100 clones for each seasonal sample) were randomly selected and sequenced using T7 primer. The results were then compared against the GenBank and RDP II databases. Among the cloned sequences, ten chimeric sequences were identified and screened out using the Chimera Check program of RDP II; thereby, the total bacterial clone library of 290 clones was generated. Phylogenetic affiliation of each clone was determined by the RDP II Classifier with the default confidence threshold of 80% [8]. Sequence alignment was performed using MEGA ver. 4.0 [9]. The nucleotide sequences reported in this study were deposited in the GenBank under accession numbers EU743833–EU743919.

Analytical methods

Biogas composition was assessed by gas chromatography as previously described [10]. Anion and cation concentrations were measured using two ion chromatographs as previously described [11]. Chemical oxygen demand (COD) and volatile solids (VS) were analyzed according to Standard Methods [12]. Alkalinity, carbohydrate, and protein were measured by the titration method, the phenol-sulfuric acid method, and the Kjeldahl method, respectively [12]. Crude lipid was analyzed by the ether-extraction method using an automated soxhlet extraction system (Soxtec Avanti 2050, Foss Tecator) following the manufacturer's protocol. All analyses were done in duplicate. Statistical comparisons of the seasonal physicochemical data (Table 1) were performed using MANOVA in Minitab-package ver. 13 (Minitab Inc.)

Results and discussion

Statistical comparisons of the operating data showed a significantly higher COD removal during the winter (Table 1). The influent carbohydrate and lipid contents during the fall and winter, respectively, were significantly different from the values during the other seasons. A negative correlation between methane content and chloride ion content was

Table 2 Seasonal variations in the frequencies of bacterial clones and OTUs

Phylum	No. of clones/no. of OTUs[a]			
	Total	Fall	Winter	Summer
Acidobacteria	1/1	1/1	0/0	0/0
Actinobacteria	3/3	2/2	1/1	0/0
Bacteroidetes	99/30 (20%)[b]	21/17 (18%)	36/14 (20%)	42/3 (15%)
Firmicutes	11/8 (20%)	3/3 (19%)	3/3 (16%)	5/5 (20%)
Nitrospira	1/1	1/1	0/0	0/0
Proteobacteria	86/28 (21%)	39/17 (21%)	32/13 (18%)	15/9 (16%)
Spirochaetes	4/2	1/1	0/0	3/1
Unclassifiable bacteria	85/14 (30%)	27/6 (27%)	27/10 (30%)	31/5 (29%)
Sum	290/87	95/48	99/41	96/23

[a] *OTU* operational taxonomic unit (based on the cutoff point of 97% sequence similarity)

[b] Dissimilarities between the OTUs in the corresponding groups are shown in parentheses

observed during the sampling seasons ($\alpha \leq 0.01$). Although no other considerable correlation was found, it is also noteworthy that the sodium and chloride ion levels were significantly higher than in freshwater environments, but they were expectedly still lower than in seawater (Na^+: 20–24%, Cl^-: 24–35% of the seawater levels) [13]. This suggests that the seawater inputs significantly affected the chemical characteristics of the digester system.

Bacterial clones were grouped into 87 distinct operational taxonomic units (OTUs), based on the cutoff point of 97% sequence similarity [14], which were designated PA01 to 87. Most OTUs (83.9%) were distributed within seven known phyla, but the remains (16.1%) were yet unclassifiable at the phylum level (Table 2). The variations in OTU numbers of the two most abundant phyla, *Bacteroidetes* and *Proteobacteria*, indicated that the numerical diversities of their populations decreased from the fall to the summer. Both phyla had the lowest sequence dissimilarities (i.e., 100 − similarity) between the OTUs during the summer, implying that the potential genetic diversities of their populations decreased (Table 2). Unclassifiable clones made up the third largest group with regard to the number of OTU (16.1% of the total OTUs). The remaining 17.2% of the total OTUs were distributed in five distinct phyla (Table 2).

Among the 87 OTUs assessed, 14 OTUs (e.g., PA01, 24, and 25 in Table 3) showed high similarities (≥97%) to cultured bacterial species and 36 OTUs (e.g., PA52, 77, 32, 67, 19, 54, and 62 in Table 3) to classifiable but uncultured strains. On the other hand, 23 OTUs were not closely related (<97% similarity) to any reference sequences in the databases, but classifiable. The remaining 14 OTUs (e.g., PA46 and 50 in Table 3), representing 29.3% of the total clones, were uncultured and unclassifiable. These results, based on the phylogenetic analysis made in the RDP II classifier, indicated that those unclassifiable OTU sequences may correspond to novel bacterial lineages. Accordingly, an unclassifiable PA46 with the highest clone frequency of 12.8% was suggested to be a novel population potentially related to *Thermotogae* (Table 3), which is a thermophilic phylum including species frequently found in deep-sea sediments and hydrothermal vents [15]. PA46 was dominant during the fall and the summer, but not detected during the winter, and correlated negatively with PA 77, 86, and 87, which were detected during the winter only (Table 3). This negative correlation coincided with the changes in chloride ion and lipid contents. This raises the possibility that they may be in competitive relationships or play similar roles in the digester ecosystem. PA51, accounting for 4.1% of the total clones, also showed 82 to 87% similarities to several species of *Thermotogae*. Although evidence of the presence of *Thermotogae*-related organisms in AD processes, particularly under thermophilic conditions, has recently emerged, they have never been found in abundance (<2% frequency) in mesophilic systems [1, 16, 17]. Therefore, the high frequency of the potential novel OTUs potentially related to *Thermotogae*, PA46 and 51, observed in this study, is unusual and seems likely due to the effect of seawater inputs.

Thermotogaceae members are strict anaerobes that ferment a wide range of substrates to produce organic acids and hydrogen, and are able to grow over a wide salt concentration range of 0–10% NaCl [15]. This, together with their prevalent abundance, suggests that the PA46- and 51-related organisms may potentially play a vital role in high saline AD, although all bacterial clones recovered with high frequency may be active or viable under the high-salt conditions tested. Two unclassifiable OTUs, PA50 and 87 (with six and five clones, respectively), showed considerable similarities (>90%) to environmental clones from sea and hypersaline lakes (Table 3). The second largest OTU, PA52, occupying 12.8% of the total clones, showed a 100% similarity to an uncultured clone from marine sediment (Table 3).

The third largest OTU with a 4.8% clone frequency, PA01, showed a 99.5% similarity to a propionate-oxidizing bacterium *Smithella propionica* (Table 3) [18]. Under methanogenic conditions, propionate is first oxidized to acetate and hydrogen, which are then converted into methane. Due to the thermodynamic limitation, however, this

Table 3 Frequencies and phylogenetic affiliations of the OTUs with five or more clones within the library (i.e., >1.5% clone frequency)

OTUs[a]	Phylum[b]	No. of clones (% frequency)				Most closely related taxa (accession number)[b]	% Similarity
		Total	Fall	Winter	Summer		
PA46	U	37 (12.8)	13 (13.7)	0 (0.0)	24 (25.0)	*Thermosipho atlanticus* DV1140 (AJ577471)	84.8
PA52	B	35 (12.1)	4 (4.2)	2 (2.0)	29 (30.2)	Uncultured bacterium A4 (AY540495)	100.0
PA01	P	14 (4.8)	3 (3.2)	6 (6.1)	5 (5.2)	Uncultured bacterium MAD-74 (AB252685)	100.0
						Smithella propionica LYP (AF126282)	99.5
PA77	B	14 (4.8)	0 (0.0)	14 (14.1)	0 (0.0)	Uncultured bacterium DSBR-B004 (AY302113)	100.0
PA32	P	12 (4.1)	6 (6.3)	6 (6.1)	0 (0.0)	Uncultured bacterium UTFS-O04-12-02 (AB166775)	99.6
PA51	U	12 (4.1)	3 (3.2)	5 (5.1)	4 (4.2)	Uncultured bacterium ET10-38 (DQ443994)	99.3
PA67	B	12 (4.1)	0 (0.0)	0 (0.0)	12 (12.5)	Uncultured bacterium C-16 (DQ018790)	98.4
PA19	P	9 (3.1)	5 (5.3)	9 (9.1)	0 (0.0)	*Sphingomonadaceae* bacterium ASRB8-1 (AB299719)	100.0
PA24	P	7 (2.4)	5 (5.3)	2 (2.0)	0 (0.0)	*Thiobacillus sayanicus* 1HG (DQ390446)	99.6
PA25	P	7 (2.4)	4 (4.2)	3 (3.0)	0 (0.0)	*Ferribacterium limneticum* cda-1 (Y17060)	100.0
						Dechloromonas aromatica RCB (AY032610)	100.0
PA86	U	7 (2.4)	0 (0.0)	7 (7.1)	0 (0.0)	Uncultured bacterium HsB17 (AB291486)	98.5
PA50	U	6 (2.1)	5 (5.3)	0 (0.0)	1 (1.0)	Uncultured bacterium A543 (EU283591)	96.9
PA48	U	5 (1.7)	4 (4.2)	0 (0.0)	1 (1.0)	Uncultured bacterium 49 (DQ413108)	99.3
PA54	B	5 (1.7)	1 (1.1)	4 (4.0)	0 (0.0)	Uncultured bacterium LCA1-6D (EU522665)	98.1
PA62	B	5 (1.7)	2 (2.1)	3 (3.0)	0 (0.0)	Uncultured bacterium PHOS-HE28 (AF314421)	98.9
PA87	U	5 (1.7)	0 (0.0)	6 (6.1)	0 (0.0)	Uncultured bacterium SHA-9 (AJ306786)	100.0

[a] *OTU* operational taxonomic unit (based on the cutoff point of 97% sequence similarity)

[b] B *Bacteroidetes*, P *Proteobacteria*, U unclassifiable bacteria

series reaction can be achieved only by the syntrophic interactions of specialized bacterial and archaeal populations [19]. The abundant presence of PA01 clones thus suggests that propionate-oxidizing bacteria composed a considerable part of the bacterial community. This is an important observation because such organisms are often associated with the long-term stability of anaerobic digesters [20].

Conclusively, the most interesting finding in this study is that a significant amount of the total bacterial clones (29.3%) was not affiliated with any known phylum. This highlights that, as yet, very little is known about the microbial populations involved in AD, particularly under abnormal environmental conditions. The majority of the novel clones (55.3%) were, however, potentially related to *Thermotogae*, whose members are rarely present in normal mesophilic AD environments. This may be linked to seawater inputs, probably responsible for the high-salt conditions in the system studied. Despite the potential importance of novel populations in the overall process, it is difficult to link their roles and functions to the process characteristics due to the limitation of available information. It was, in this study, statistically supported that the changes in OTU frequency over sampling seasons may be potentially correlated with the seasonal variation in influent characteristics.

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