Comparative 16S rRNA gene-based microbial community analysis of different anaerobic bioreactors

16S rRNA 遺伝子情報に基づく嫌気性廃水処理システムの
微生物群集構造比較解析

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Chapter 1

General Information

1.1 Introduction

Anaerobic wastewater treatment system is essential technologies to treat several wastewater such as industrial, agricultural, and municipal wastewater. To effectively control this wastewater treatment system, the understanding of microbial ecology and functions is important. However, the detailed mechanisms in anaerobic wastewater treatment system remain unclear in respect of microbiology owing to presence of uncultured phyla and complicated microbial interaction for organic matter degradation.

So far, several molecular approaches have been performed to elucidate "*black box*" in wastewater treatment systems (Sekiguchi et al., 1999; Narihiro et al., 2009; Kuroda et al., 2015). 16S rRNA gene sequence analysis has been commonly used to understand microbial ecology (Sekiguchi et al., 2006). Owing to recent advance of DNA sequencing technology (next-generation DNA sequencer was developed), we became able to analyze massive DNA sequences (Mbp–Gbp) per run, and observe enough sequence data for analyses of microbial ecology, genomes, and transcripts (Mardis, 2011; Narihiro et al., 2014; Nobu et al., 2015). On the other hand, computational analysis became rate-limiting step due to massive DNA sequence data (Mardis, 2011). In recent advances, several bioinformatics tools have been developed to effectively analyze next-generation DNA sequencing data (e.g. fewer requirements of computer ability and novel computational logics for analysis (Koboldt et al., 2013). Massive DNA sequence data and bioinformatics tools can be able to deeply analyze microbial community compositions and microbial functions because these massive data can allow enough information for ecology and genomic analysis using statistics.

My laboratory has the wide range of network in the world (http://ecolab.nagaokaut.ac.jp/e/project). By using our network, I can collect several kinds of sludges from several anaerobic wastewater treatment systems. Therefore, in this dissertation, I attempted to understand comprehensive microbial ecology in anaerobic

wastewater treatment systems using high-throughput DNA sequencer. Comparison of different anaerobic bioreactors can indicate the presence of cultured or uncultured microorganisms and ecological heterogeneity of wastewater treatment sludges in the systems. In addition, I attempted to estimate uncultured bacterial and archaeal functions based on these detection patterns in their 16S rRNA gene sequences.

1.2 Outline

The present work in this dissertation performed comparative 16S rRNA gene-based microbial community analysis of several bioreactors using high-throughput DNA sequencer. Such analysis can observe comprehensive microbial ecology and diversity, uncultured microorganisms habitats, microbial community changes with wastewater treatment systems development, and ecological heterogeneity in industrial bioreactor.

Chapter 1 and **2** provide the background of this dissertation using important scientific literatures to understand this research field. In **Chapter 3**, I describe the results of microbial community analysis of core members and uncultured bacterial phyla in 54 biological wastewater treatment sludges using massive 16S rRNA gene sequencing data. **Chapter 4** mentions the results of archaeal community analysis of anaerobic or anoxic wastewater treatment sludges using archaeal specific primer set. In **Chapter 5**, I applied massively parallel 16S rRNA gene sequencing to molasses wastewater treatment systems. I could suggest the microbial mechanisms in this system. **Chapter 6** describes the results of granule microbial heterogeneity analysis. I attempted to elucidate granule microbial heterogeneity in three UASB reactors using single-granule 16S rRNA gene sequencing approach. **Chapter 7** concludes my findings of this study.

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Chapter 2

Literature Review

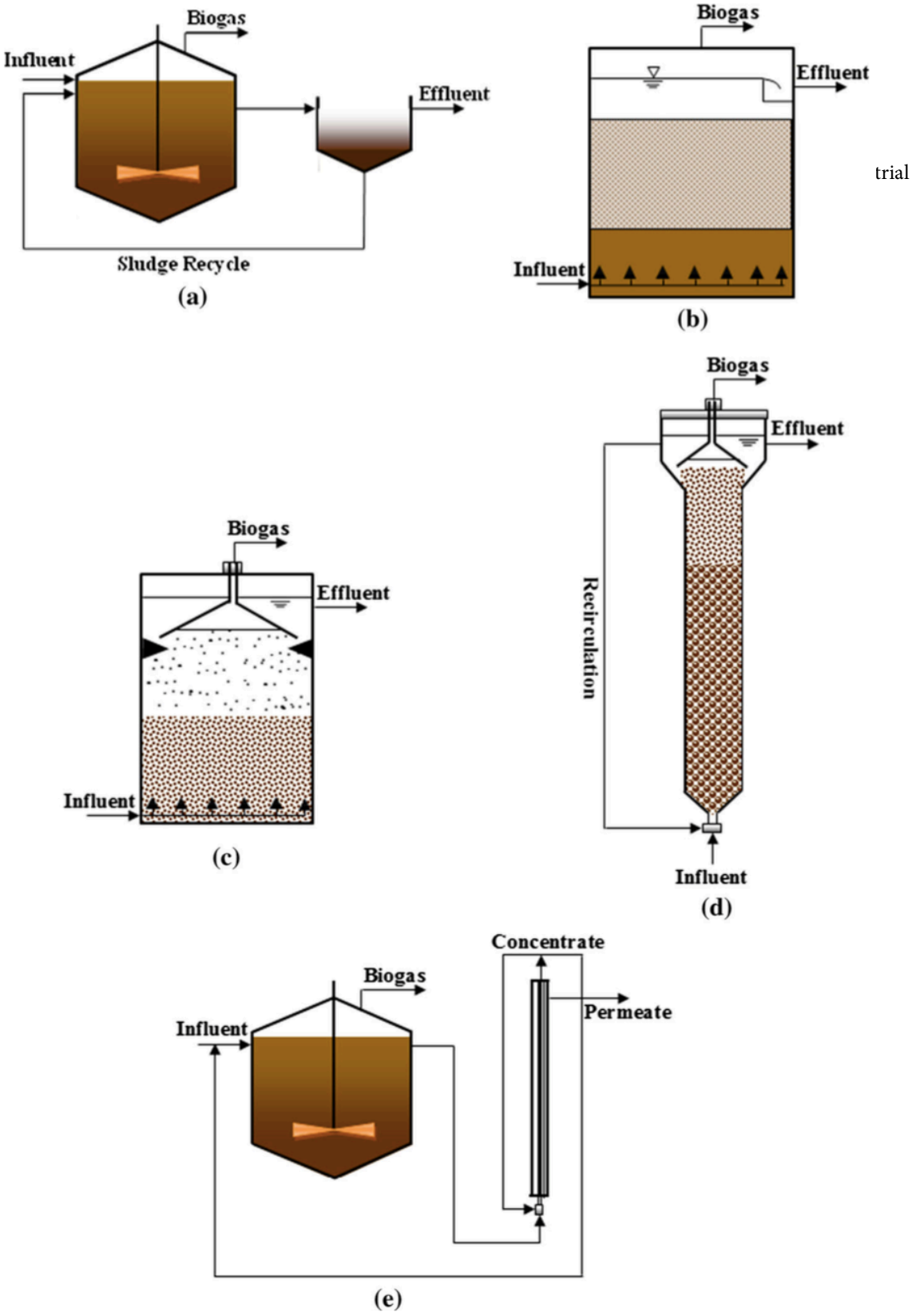
2.1 Anaerobic wastewater treatment systems

2.1.1 Anaerobic wastewater treatment technologies

Biological wastewater treatment systems are essential technologies for several different industries. Anaerobic wastewater treatment systems convert organic matter into methane gas; such systems have low energy requirements because their operation does not require an air supply, and they produce energy from methane gas in the treatment process. There are various types of wastewater treatment systems. Common systems include the up-flow anaerobic sludge blanket (UASB) reactor, the anaerobic baffled reactor, the fluidized bed reactor, the anaerobic membrane bioreactor, the expanded granular sludge bed reactor, the internal circulation reactor, the anaerobic contact process, and the continuous stirred-tank reactor (van Lier et al., 2015). Fig 2–1 shows a diagram of different anaerobic reactors. These systems are used for the treatment of different kinds of wastewater (Table 2–1) (Kleerebezem and Macarie, 2003). In section 2.1, I focus on the UASB system, which has been used in a wide range of applications globally and has been studied extensively. I review the UASB system applications and operational problems, the composition of microbial communities in UASB granular sludge, and recent microbial discoveries relevant to methanogenic wastewater treatment.

2.1.2 Application of UASB reactor

The UASB system, developed in the 1970s, has been widely used around the world. Proper wastewater treatment using UASB requires well-developed microbial aggregations (granular sludge) with good settling properties. Because of the important advantages of UASB, including low energy requirements and low amounts of excess sludge, UASB systems have been applied to treat many different kinds of wastewater.



trial

Fig.2–1 Examples of high-rate anaerobic reactors: a ACP, b AF, c UASB reactor, d EGSB reactor, e membrane coupled CSTR reactor (AnMBR) (van Lier *et al.*, 2015).

Table 2–1 Number of commercially operated reactors for treating different types of industrial wastewater and sewage (reported till 2003) (Kleerebezem and Macarie, 2003).

Type of wastewater	Low-rate[1)]	AC[2)]	Fixed-bed[3)]	Moving-bed[4)]	UASB[5)]	EGSB[6)]	Total number
Food & related Industries							
Brewery & malt	2	-	6	4	185	88	285
Distillery & ethanol	25	31	40	-	76	9	181
Other beverage	-	3	11	2	88	15	119
Sugar production	-	49	7	1	32	3	92
Potato processing	14	4	2	-	46	10	76
Dairy, ice-cream & cheese	12	10	10	2	27	6	67
Starch production	2	9	10	2	34	7	64
Yeast production	7	8	6	-	25	8	54
Candy & confectionery	4	-	3	-	15	2	24
Citric acid production	2	3	1	1	3	5	15
Coffee processing	-	-	7	-	4	1	12
Wine processing	-	-	6	1	3	1	11
Fish & seafood processing	1	4	-	-	2	1	8
Miscellaneous	10	22	40	5	112	25	214
Non-food industries							
Pulp- & -paper	1	16	5	3	75	37	137
(Petro) chemical	3	4	43	1	20	20	91
Leachates	-	-	6	-	18	-	24
Pharmaceutical	4	1	2	-	6	3	16
Pig, cow manure & poultry	5	3	6	-	1	-	15
Textile	-	-	1	-	4	2	7
Natural rubber	-	-	3	-	3	-	6
Sludge & sludge liquor	1	-	2	1	1	-	5
Tobacco manufacture	-	-	-	-	4	-	4
Tannery	-	-	-	-	3	-	3
Fluegas desulfurization	-	-	-	-	-	1	1
Electronic components	-	-	-	-	1	-	1
Sewage	-	-	2	1	64	-	67
Number of reactor per type	93	167	219	24	852	244	1,599

1): Low-rate reactors include CSTR, lagoons and BVD reactors (80 % of the plant reported correspond to BVD)., 2): AC indicates an anaerobic contact process., 3): The fixed-bed systems reported corresponded for 44 % to upflow anaerobic filter (UAF); 26 % to downflow filters (DAF), and 30 % to hybrid reactors., 4): Moving-bed reactors contain both an upflow fluidized-bed reactors and an anaerobics mobilized film technology., 5): UASB indicates an upflow anaerobic sludge blanket process., 6): EGSB indicates an expanded granular sludge bed process.

2.1.2.1 High-organic wastewater treatment under thermophilic conditions

Granular sludge can retain high concentrations of microorganisms, and different kinds of organisms can degrade several different types of organic matter such as proteins, lipids, carbohydrates, and fatty acids to produce methane as the final product. Because industrial wastewater contains high concentrations of organic matter, high-organic-loading treatments are required. Compared with mesophilic conditions, under thermophilic conditions, both the activity of methanogenic bacteria (Fig. 2–2) and the temperature of wastewater produced are higher (Lettinga et al., 2001). Several thermophilic UASB reactors have been studied with respect to their capacity to treat molasses-based wastewater and alcohol distillery wastewater (Kongjan et al., 2013; Yamada et al., 2006; Yamada et al., 2013). Additionally, there is some evidence that thermophilic wastewater treatment reduces pathogen concentrations (Blais et al., 2004; Narayanan and Sreekrishnan, 2009). While there are several benefits of high-organic wastewater treatment methods, the following problems frequently occur: 1) high accumulation of volatile fatty acids (VFAs) with high partial pressure of H_2, 2) sludge washout due to high methane flux, and 3) sensitivity of external shocks, such as temperature changes and high concentrations of inhibitors.

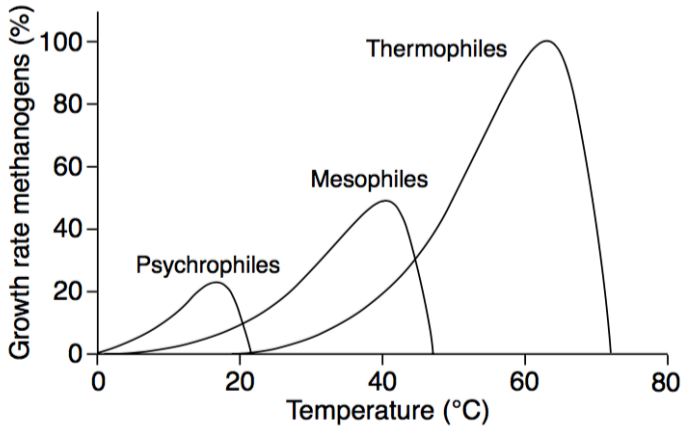


Fig.2–2 Relative growth rates of psychrophilic, mesophilic and thermophilic methanogens (Lettinga *et al.*, 2001).

To avoid the accumulation of VFAs and high partial pressure of H_2 in a thermophilic UASB reactor, acidification tanks have been installed upstream of UASB reactors as a pre-treatment step for high-organic wastewater (Kongjan et al., 2011; Kuroda et al., 2015a). As a result, studies were able to achieve higher efficiency wastewater treatment (Lettinga, 1995; van Lier et al., 2015). However, a high degree of pre-acidification step can have negative effects on the treatment process because of the potential for carry-over of the acidifying microorganisms into the UASB reactor. Additionally, there is an extra cost associated with the construction and operation of an additional unit (van Lier et al., 2015). Therefore, we must consider the optimal pre-acidification conditions for each substrate and system by considering the trade-off relationship between operational costs and wastewater treatment efficiency improvement.

High methane gas flux during high-organic wastewater treatment can cause sludge washout from the UASB reactor. To reduce the influence of high gas flux, multiple gas-solid separators (GSS) have been installed into UASB systems (Kucivilize et al., 2001; Kuroda et al., 2015a; van Lier et al., 1996; van Lier et al., 2015; Yamada et al., 2006;

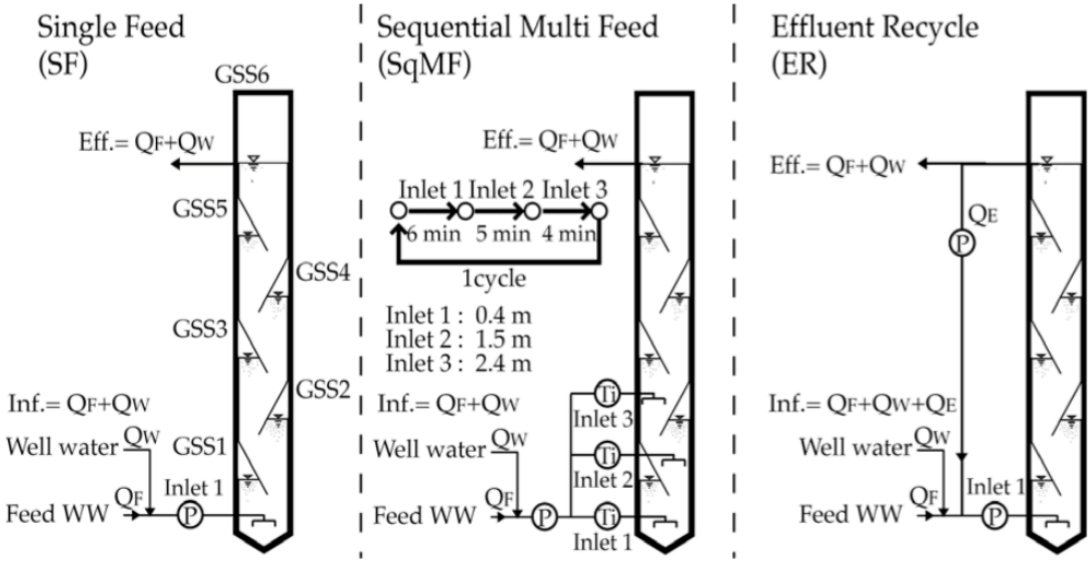


Fig.2–3 Illustration of MS-UASB reactors and feeding paterns (Yamada *et al.*, 2013).

Yamada et al., 2013) (Fig. 2–3). These studies have reported that the use of multiple GSSs decreases the risk of sludge washout from the reactor because they reduce the vertical biogas flux. Therefore, multiple GSSs could be useful in high-organic loading wastewater treatment, in particular under thermophilic conditions.

It is well known that the operation of thermophilic reactors is more sensitive to external factors than is the operation of mesophilic reactors. Lettinga (1995) reported that acetate- and butyrate-degrading sludge under thermophilic conditions were sensitive to temperature to only a minor extent. With respect to temperature, the highest methanogenic activity of cane-molasses vinasse-degrading granular sludge were reported to occur at 65°C, 60°C, and 55°C for H_2/CO_2, acetate, and vinasse, respectively (Harada et al., 1996). Though methanogenic activity for vinasse is higher at higher temperatures, optimal methanogenic activity for acetate occurs at 55°C, which might be the result of the influence of other intermediate degradation conditions, such as the presence of propionate. Indeed, the optimal temperature for propionate degradation was reported to be 55°C (Van Lier et al., 1993). Additionally, several studies have reported that high ammonia concentrations inhibit methanogenic reactions under thermophilic conditions because of the high ratio of free ammonia to total ammonium at higher temperatures (Chen et al., 2008; Chen et al., 2014).

By taking into account the problems associated with the operation of thermophilic UASB reactors, several researchers have successfully treated high-organic wastewater. For the treatment of alcohol distillery wastewater, an organic loading rate (OLR) of over 100 $kgCOD \cdot m^{-3} \cdot d^{-1}$ was achieved at 55°C in laboratory-scale and pilot-scale multi-staged (MS)-UASB reactors (Kucivilize et al., 2001; Yamada et al., 2006). When there is a high concentration of carbohydrates in wastewater, a decrease in pH will create a requirement for higher alkalinity. To overcome this problem, previous studies have developed a sequential multi-feed mode and an effluent recycle mode for MS-UASB reactors treating alcohol distillery wastewater (Fig. 2–3), which reduced the alkalinity

requirements to 67.2% (OLR: 45 kgCOD·m^{-3}·d^{-1}) and 0% (OLR: 34.8 kgCOD·m^{-3}·d^{-1}), respectively (Yamada et al., 2013).

2.1.2.2 Wastewater treatment under mesophilic conditions

The UASB system under mesophilic conditions is used globally and has been studied extensively. UASB systems have been applied to various types of wastewater, including municipal sewage, industrial wastewater, and agricultural wastewater under low-, middle-, and high-strength OLRs (Kleerebezem and Macarie, 2003).

Mesophilic UASB reactors have also been applied to low-strength organic wastewater, such as municipal sewage. Even though methane gas production from low-strength organic wastewater is lower than from middle- or high-strength organic wastewater, the installation of a UASB reactor still has advantages, such as low-cost operation, the removal of organics and suspended solids with a short retention time, and the smaller size of the reactor (therefore a smaller required construction area) compared with an activated sludge system. Indeed, full-scale UASB reactors are used around the world to treat municipal sewage (Sato et al., 2006; Khan et al., 2013). However, the treatment of wastewater with UASB systems always requires post-treatment to remove remaining organics, nitrogen, and pathogens. In India, polishing units, polishing ponds, activated sludge processes, and aeration-polishing ponds are widely used, and down-flow hanging sponge (DHS) reactor for post-treatment of the anaerobic wastewater treatment has been demonstrated (Tandukar et al., 2006).

Despite several full-scale UASB reactors are commercially operated, sludge-bulking and sudden sludge washout from the reactor have been frequently reported (Li et al., 2008; Sekiguchi et al., 2001; Yamada et al., 2007). Although the bulking mechanisms are still unknown, bacteria causing bulking have been identified based on rRNA approaches, cultivation, and genomic analysis (in a lab-scale reactor treating synthetic wastewater containing sucrose, acetate, propionate, and yeast extract at 55°C, researchers detected

Anaerolinea thermophila UNI-1; in a full-scale UASB reactor treating sugar-manufacturing wastewater at 35–40°C, previous studies detected "*Ca.* Moduliflexus flocculan YM-1") (Sekiguchi et al., 2001; Sekiguchi et al., 2003; Sekiguchi et al., 2015; Yamada et al., 2007).

2.1.2.3 Wastewater treatment under psychrophilic conditions

Significant quantities of low-temperature wastewater are discharged from various industries (*i.e.,* brewery and soft drinks manufacturing plants). Under psychrophilic conditions, energy requirements for wastewater treatment are lower compared with mesophilic conditions, while the growth rate of methanogens is much slower than under mesophilic and thermophilic conditions (Fig. 2–2). Additionally, energy requirements for organic matter degradation are higher under psychrophilic conditions than under mesophilic conditions (Table 2–2) (Lettinga et al. 2001). However, hydrogenotrophic methane production, hydrogenotrophic sulfate reduction, and acetate formation from H_2/CO_2 require less energy than mesophilic conditions (Table 2–2). In methane activity and sulfate-reducing activity (SRA) tests of sewage treatment using UASB granules at

Table 2–2 Stoichiometry and Gibbs free-energy changes[a] of acetate, propionate, butyrate and hydrogen anaerobic conversion in the presence and absence of sulfate (Lettinga *et al.*, 2001).

	Reactions	ΔG′ kJ reaction^{-1} (37°C)	(10°C)
1	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+71.8	+82.4
2	$CH_3CH_2COO^- + 0.75SO_4^{2-} \rightarrow CH_3COO^- + HCO_3^- + 0.75HS^- + 0.25H^+$	–39.4	–35.4
3	$CH_3CH_2COO^- + 1.75SO_4^{2-} \rightarrow 3HCO_3^- + 1.75HS^- + 0.25H^+$	–88.9	–80.7
4	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H^2$	+44.8	+52.7
5	$CH_3CH_2CH_2COO^- + 0.5SO_4^{2-} \rightarrow 2CH_3COO^- + 0.5HS^- + 0.5H^+$	–29.3	–25.9
6	$CH_3CH_2CH_2COO^- + 2.5SO_4^{2-} \rightarrow 4HCO_3^- + 2.5HS^- + 0.5H^+$	–128.3	–116.4
7	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	–49.5	–45.3
8	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	–32.5	–29.2
9	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	–148.2	–157.1
10	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	–131.3	–140.9
11	$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$	–98.7	–111.8

[a]Energy changes were calculated by using the Van 't Hoff equation, standard enthalpy values of compounds[6] and Gibbs free-energy changes[7] at 25°C.

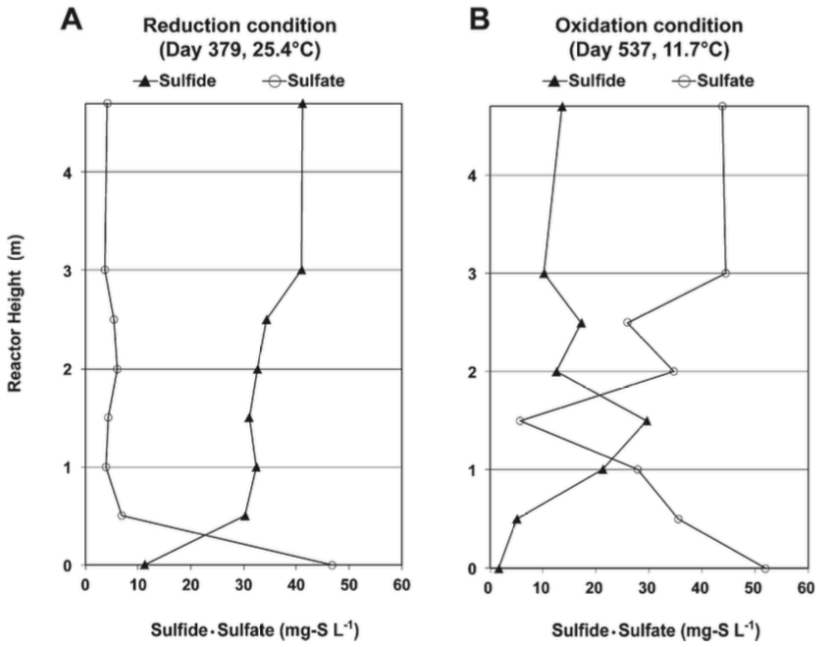


Fig.2–4 UASB profiles of sulfide and sulfate.A-non-occurrenceof anaerobicsulfur oxidation; N-occurrence of anaerobic sulfur oxidation. (Aida *et al*., 2015).

10°C, SRA (0.008 gCOD·gVSS^{-1}·d^{-1}) was higher than methane activity (0 gCOD·gVSS-1^{-1}·d^{-1}) when H_2/CO_2 was the substrate. When acetate was the substrate, SRA and methane activity were similar (SRA: 0.003 gCOD·gVSS^{-1}·d^{-1}; methane activity: 0.005 gCOD·gVSS^{-1}·d^{-1}) (Sumino et al., 2007). These results indicate that the utilization of sulfate-reducing bacteria under psychrophilic conditions is effective in removing organic matter from wastewater. Indeed, sulfate-reducing bacteria contributed to the degradation of organic matter in pilot-scale UASB reactors that were treating sewage that contained > 40 mgS L^{-1} of sulfate (Takahashi et al., 2011; Hatamoto et al., 2016). Anaerobic sulfur oxidation reactions have also been used in UASB reactors for treating sewage in recent years (Aida et al., 2014; Aida et al., 2015; Hatamoto et al., 2016). This novel reaction occurs in the following steps: 1) sulfide is generated by sulfate-reducing bacteria at the bottom of the UASB reactor; and 2) the generated sulfide oxidizes to sulfate in the middle of the reactor (Fig. 2–4). However, the anaerobic sulfur oxidizing mechanism is still unclear.

2.1.3 Anaerobic organic matter degradation under methanogenic conditions

Methanogenic organic matter degradation occurs through the activity of different functional microorganisms (Fig. 2–5) (Abbasi et al., 2012). First, the hydrolysis of complex organic matter, such as proteins and polysaccharides, results in their conversion to fatty acids, monophyletic sugars, alcohol, and amino acids. In the second step, fermentation bacteria utilize the products. The fermentation products contain several VFAs, methanol, and H_2/CO_2. The third step is the conversion of those products to H_2/CO_2 or acetate by VFA-degrading bacteria, sulfate-reducing bacteria, and acetogens. VFA-degrading bacteria (syntrophs) associate with H_2-utilizing organisms because a low partial pressure is required for the degradation reaction to proceed (Schink and Stams, 2006). The degradation of aromatic compounds, long-chain fatty acids, and some amino acids also occur through syntrophic associations (Nobu et al., 2015). The final step is methanogenesis, during which hydrogenotrophic, acetoclastic, or methylotrophic methanogens produce methane using H_2/CO_2, acetate, or methanol (with or without H_2), respectively. In this section, I describe recent new discoveries relevant to methanogenesis.

2.1.3.1 Aromatic compound-degrading bacteria

Aromatic compounds are present in wastewater produced from plastic and coke industries (Macarie et al., 2000; Veeresh et al., 2005). The degradation of aromatics requires well syntrophic association development such as closer spatial distribution among the organisms because of the difficulty to push the reaction (Nobu et al., 2014; Nobu et al., 2015). *Syntrophorhabdus*, *Pelotomaculum*, and *Syntrophus* are known to be aromatic compound degraders (Nobu et al., 2015). A recent study revealed the metabolic pathways of *Syntrophorhabdus*, including aromatic metabolism (phenol, TA, benzoate, and 4-hydroxybenzoate) and Benzoyl-CoA metabolism (from Benzoyl-CoA to acetate or

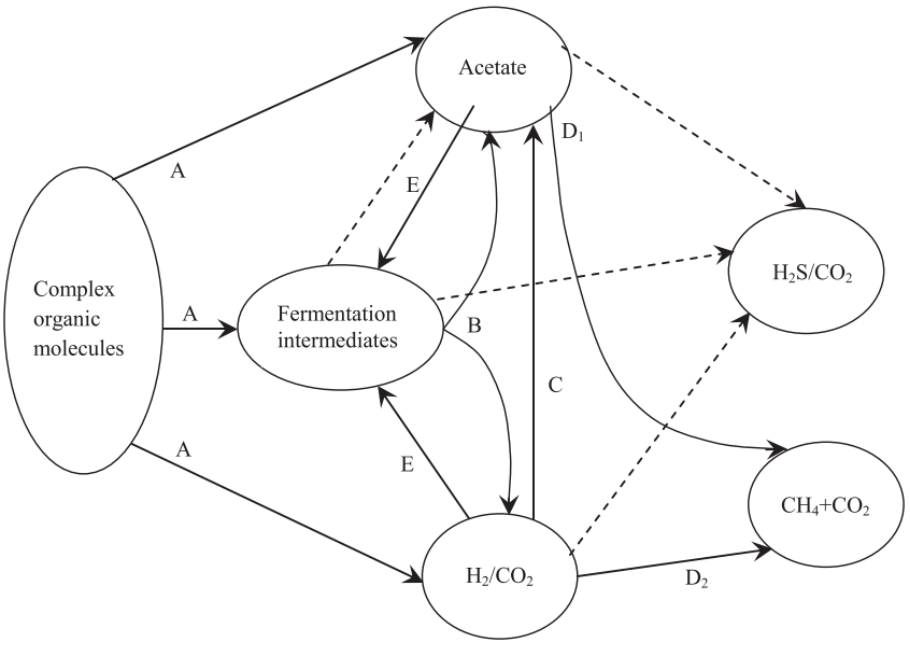


Fig.2–5 Web of interactions leading to progressive degradation of complex organic molecules to CH4, CO2, and traces of H2S in a UASB reactor. A: hydrolytic/fermentative bacteria; B: obligate hydrogen producing bacteria; C: homoacetogenic bacteria; D1: acetoclastic methanogens (Abassi, T. and Abassi, S., 2012).

butyrate) (Nobu et al., 2014). This study revealed not only the pathways of aromatic compound metabolism, but also newly proposed energy conservation mechanisms (*i.e.,* utilization of a novel thiol-disulfide redox pair by electron-confurcating hydrogenase and benzoyl-CoA reductase). In another study, a TA-degrading bioreactor under hypermesophilic conditions (46–50°C) was analyzed using a genomic approach (Nobu et al., 2015). In this environment, *Pelotomaculam* mainly degraded TA, as indicated by the higher expression level (accounting for 31.4% in total bacteria) and expression of the TA-degradation pathway were observed (Nobu et al., 2014). Under these temperature conditions, *Syntrophorhabdus* abundance was low, and *Syntrophus* expressed butyrate and long-chain fatty acids degradation pathways. It is suggested that *Pelotomaculam* is the main TA-degrader under hypermesophilic conditions. While *Pelotomaculam* predominates under such conditions, high abundances of *Syntrophorhabdus* and *Syntrophus* have been obtained from TA- or purified TA wastewater-treating

methanogenic bioreactors under mesophilic conditions (Perkins et al., 2011; Wu et al., 2001). Further investigation is required to understand mesophilic aromatic compound-degrading conditions.

2.1.3.2 Acetate-utilizing bacteria

Acetate, which is produced from the fermentation step, is an important final intermediate for methanogenesis. In methanogenesis, information relevant to acetate-utilizing bacteria is more scarce than information on aceticlastic methanogen because of the presence of several uncultured bacteria in the anaerobic digestion process. Group 4 of the PD-UASB-13 group belonging to the phylum *Synergistes* is frequently observed in anaerobic digesters (Chouari et al., 2005a). Through microautoradiography-fluorescence in situ hybridization (MAR-FISH) and stable-isotope probing of 16S rRNA (RNA-SIP) with acetate as a substrate, researchers have detected this organism (Ito et al., 2011). Additionally, researchers observed a higher K_m for acetate for *Synergistes* group 4 (2.5–10 mM) compared with *Methanosaeta* (0.5–1.0 mM). Therefore, the co-existence of acetate with both acetate-degrading bacteria and aceticlastic methanogens in anaerobic digesters can be explained by acetate concentration levels in their habitats. In a recent study, Nobu et al. (2015) speculated that *Mesotoga* ("*Ca*. Mesotoga acetoxidans") belonging to the phylum *Thermotogae* is also an acetate-oxidizing bacteria (Nobu et al., 2015). Genomic and transcriptomic analyses have demonstrated that "*Ca*. M. acetoxidans" possesses a novel syntrophic acetate-oxidizing pathway (Nobu et al., 2015). Researchers have also demonstrated that "*Ca*. Mesotoga" shows high transcriptomic activity (11.3% in total bacteria) in a terephthalate-degrading methanogenic bioreactor, suggesting that this organism may play an important role in anaerobic bioreactors under methanogenic conditions (Nobu et al., 2015).

2.1.3.3 Specific syntrophic partnership

The development of a strong syntrophic association is essential for methanogenic degradation of organic matter. Interactions between syntrophs and methanogens requires a low partial pressure of H_2 (Schink and Stams, 2006). Though it is known that a syntrophic association is developed by H_2-utilizing organisms and syntrophs, detailed information on this partnership remains unclear. Recent 16S rRNA gene sequence analysis of a methanogenic culture enriched with several substrates (propionate, butyrate, benzoate, acetate, formate, and H_2/CO_2) demonstrated the presence of different syntrophic partnerships (different taxonomies of methanogens and syntrophs), suggesting that syntrophic partners may be dependent on substrate type (Narihiro et al., 2014). However, specific syntrophic partnerships are still unknown because few studies have examined them (Narihiro et al., 2014).

2.2 16S rRNA gene analysis

Ribosomes play important roles in the synthesis of proteins, which contain ribosomal RNA (rRNA). As a result of the work of C.R. Woese *et al.* (Woese and Fox, 1977; Woese et al., 1990), small subunit (SSU) rRNA gene sequences have been widely used as phylogenetic marker genes; SSU rRNA genes have conserved regions and variable regions, are ubiquitous in all organisms, and their sequence length is adequate for phylogenetic calculation. Using SSU rRNA gene sequences, we are able to systematically observe phylogenetic information. For microbial community analysis of granules in the UASB reactor, researchers generally target 16S rRNA genes of *Bacteria* and *Archaea*. In this section, I review fundamental knowledge about 16S rRNA gene sequence analysis using NGS technologies.

2.2.1 Traditional 16S rRNA gene sequence analysis

Molecular techniques are powerful tools to analyze microbial community composition in anaerobic wastewater treatment sludge. Several applications are used to understand sludge microbial ecology (Sanz and Köchling, 2007). In this section, I review traditional and recent applications of molecular techniques for 16S rRNA gene sequence analysis.

The PCR cloning method is widely used by microbial ecologists because it can separately observe target genes in complex environments. The PCR cloning step for 16S rRNA gene sequence consists of DNA extraction, amplification of the 16S rRNA gene, purification, transformation into *Escherichia* coli plasmids, performing PCR on transformed *E. coli* colonies, DNA sequencing, and phylogenetic analysis (Fig. 2–6) (Sanz and Köchling, 2007). Narihiro et al. (2009) reported that granule core community composition containing groups such as *Proteobacteria*, *Firmicutes*, *Spirochaetes*, *Bacteroidetes*, *Methanomicrobia*, and *Methanobacteria* were detected in several UASB reactors treating high-strength wastewater from different food-processing sources. Additionally, PCR cloning methods are frequently used along with other molecular techniques, such as the FISH method, which can visualize the spatial distribution of target microorganisms in the environment. For example, Sekiguchi et al. (2001) identified the bacteria causing bulking through the following steps: 1) identification of predominant organisms in bulking sludges based on cloning, 2) design of new DNA probes to target the predominant organisms, 3) visualization of the spatial distribution of the organism in the bulking sludge, and 4) cultivation of the organism by a combination of FISH and cultivation methods. While the PCR cloning method is a very useful tool, cloning methods are time-consuming and less suitable for the analysis of large datasets (Sanz and Köchling, 2007).

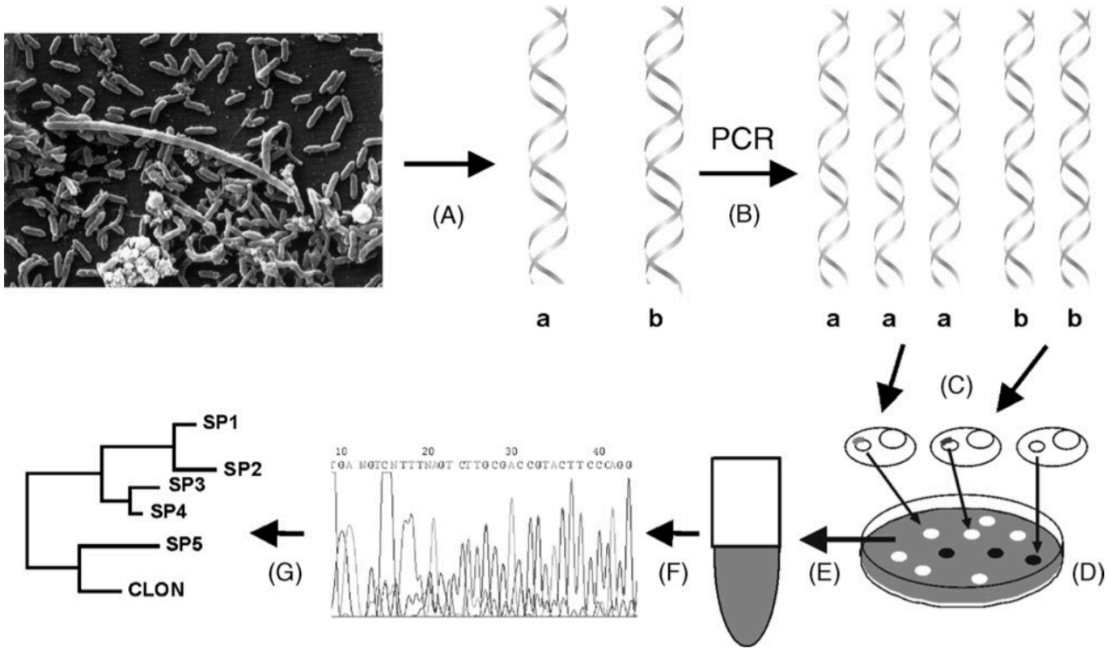


Fig.2–6 Outline of the cloning procedure for studying a microbial community. The work cycle is as follows: (A) direct nucleic acid extraction, without the need for previous isolation of microorganisms; (B) amplification of the genes that code for 16S rRNA by polymerase chain reaction (PCR), commonly using universal primers for bacteria or archaea, resulting in a mixture of rDNA copies corresponding to the microorganisms present in the sample; (C) cloning of the PCR products obtained into a suitable high copy number plasmid and transformation of competent E. coli cells with this vector; (D) selection of transformed clones with an indicator contained in the plasmid (the white colonies in the figure); (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP) (Sanz and Köchling, 2007).

Other molecular approaches, such as denaturant gradient gel electrophoresis and restriction fragment length polymorphism, can illustrate the differences in microbial diversity between samples. While these methods can simply monitor the microbial diversity of an entire sample, observations of short-length 16S rRNA gene sequences and non-quantitative band/peak intensities hinder understanding of phylogenetic positions and organism abundances (Sanz and Köchling, 2007).

2.2.2 Next-generation DNA sequencers-based 16S rRNA gene sequencing

Recent advances in DNA sequencing technology have been remarkable. In

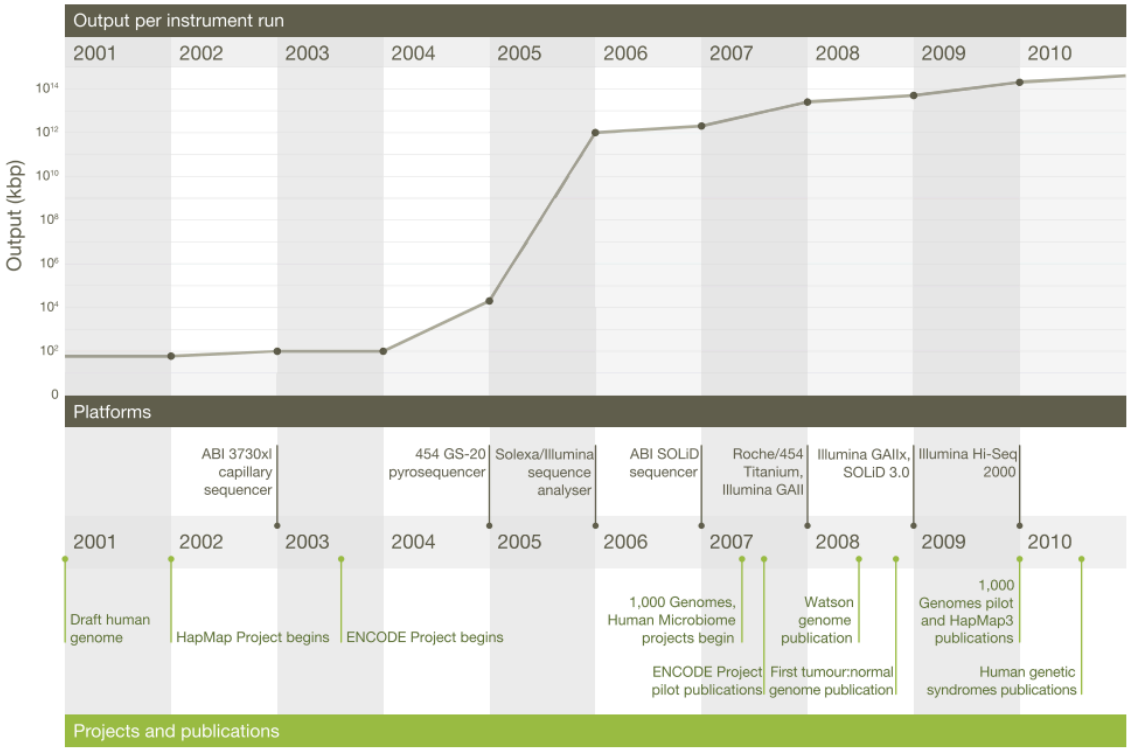


Fig.2–7 Changes in instrument capacity over the past decade, and the timing of major sequencing projects. Top, increasing scale of data output per run plotted on a logarithmic scale. Middle, timeline representing major milestones in massively parallel sequencing platform introduction and instrument revisions. Bottom, the timing of several projects and milestones described in the text (Mardis, 2011).

particular, DNA sequencing depth has been markedly improved by such advances; previous Sanger sequencing was only capable of reading kbp sequences per run, while new DNA sequencers (called "next-generation DNA sequencers" (NGSs)) can read Mbp–Gbp sequences per run (Fig. 2–7) (Mardis, 2011). Although the main disadvantage of NGSs was short read length, NGSs from Illumina and Roche are now able to read lengths (>500 bp per run) more similar to those of Sanger DNA sequencers. Recently, NGSs have been more frequently used for 16S rRNA gene sequence analysis. Because of its convenience and high sequencing depth, the Illumina DNA sequencer is used frequently all over the world. New DNA sequencing technologies, such as third- (single-molecule sequencing) and fourth-generation (Nanopore-based sequencing) DNA sequencers have also been developed in recent years (Feng et al., 2015; Schadt et al., 2010).

After using NGS to perform 16S rRNA gene sequencing, we used computer programming to analyze the massive 16S rRNA gene database. Therefore, we required advanced computational knowledge. In 2010, an excellent new software, "quantitative insights into microbial ecology" (QIIME), was developed for 16S rRNA gene data produced from NGS (Caporaso et al., 2010b). Using this software package, we are able to easily produce massive 16S rRNA gene analysis data in a short period of time. Additionally, several universal and archaeal 16S rRNA gene target primer sets for NGS have been designed (Table 2–3).

2.2.3 16S rRNA gene sequence data processing for NGS technology

When raw sequences are observed from NGS, we have to treat the sequences to maintain their qualities. In this section, I review data processing techniques for MiSeq-based 16S rRNA gene sequences. Illumina DNA sequences are produced as fastq files, which encode the sequence IDs (header), observed sequences, and sequence quality as ASCII code + 33 (Phred quality score). Additionally, general Illumina-based 16S rRNA gene sequences were produced from two different directions (forward and reverse sequences) (Fig. 2–8) (Caporaso et al., 2010a). Therefore, if we want to observe connected 16S rRNA gene sequences, we must assemble sequences from two different directions. For quality trimming and assembly, several types of software are available. Mothur is one of the most applicable for data processing because it can perform quality trimming and assembly using a "make.contigs" command (Schloss et al., 2009). Fastx-toolkit is also a powerful tool for NGS data processing; it has several scripts, such as fastq_quality_filter, fastx_barcode_splitter.pl, and fastx_trimmer (http://hannonlab.cshl.edu/fastx_toolkit/). After trimming the raw sequences, we can assemble forward and reverse sequences using an assembler (*i.e.,* PAired-eND

Table 2–3 Summary of 16S rRNA-target primer pair for illumina next-generation DNA sequencer.

Primer pair	Target	Variable region	Primer sequences (5'–3')	References
Univ8F BSR357	Prokaryotes	V1–V2	AGAGTTTGATCCTGGCTCAG CTGCTGCCTYCCGTA	Claesson et al., 2010
Univ101F Univ534R	Prokaryotes	V2–V3	AGYGGCGNACGGGTGAGTAA ATTACCGCGGCTGCTGG	Claesson et al., 2010
Univ338F Univ802R	Prokaryotes	V3–V4	ACTCCTACGGRAGGCAGCAG TACNVGGGTATCTAATCC	Claesson et al., 2010
Univ341F Univ518R	Prokaryotes	V3	CCTACGGGAGGCAGCAG CCTACGGGAGGCAGCAG	Bartram et al., 2011
Univ319F Univ806R	Prokaryotes	V3–V4	CCTACGGGAGGCAGCAG GTGCCAGCMGCCGCGGTAA	Kozich et al., 2013
Univ319F Univ806R	Prokaryotes	V3–V4	ACTCCTACGGGAGGCAGCAG GGACTACHVGGGTWTCTAAT	Fadrosh et al., 2014
Pro341F Pro805R	Prokaryotes	V3–V4	CCTACGGGNBGCASCAG GACTACNVGGGTATCTAATCC	Takahashi et al., 2014
Arch344F Arch806R	Archaea	V3–V4	ACGGGGYGCAGCAGGCGCGA GGACTACVSGGGTATCTAAT	Takahashi et al., 2014
Univ515F Univ806R	Prokaryotes	V4	GTGCCAGCMGCCGCGGTAA GGACTACHVGGGTWTCTAAT	Caporaso et al., 2012
Arch516F Univ806R	Archaea	V4	TGYCAGCCGCCGCGGT AAHACCVGC GGACTACHVGGGTWTCTAAT	Kuroda et al., 2015
Univ515F Univ909R	Prokaryotes	V4–V5	GGACTACHVGGGTWTCTAAT CCCGTCAATTCMTTTRAGT	Kozich et al., 2013
Univ563F BSR926	Prokaryotes	V4–V5	AYTGGGYDTAAAGNG CCGTCAATTYYTTTRAGTTT	Claesson et al., 2010
BSF784 Univ1064R	Prokaryotes	V5–V6	RGGATTAGATACCC CGACRRCCATGCANCACCT	Claesson et al., 2010
Univ926F Univ1392R	Prokaryotes	V6–V8	AAACTYAAAKGAATTGRCGG (CTG)ACGGGCGGTGTGTRC	Tremblay et al., 2015
BSF1099 BSR1407	Prokaryotes	V7–V8	GYAACGAGCGCAACCC GACGGGCGGTGWGTRC	Claesson et al., 2010
Univ1114F Univ1392R	Prokaryotes	V7–V8	GCAACGAGCGCAACCC (CTG)ACGGGCGGTGTGTRC	Tremblay et al., 2015

Assembler for DNA sequences (pandaseq), FLASH, or mothur) (Magoc and Salzberg 2011; Masella et al., 2012; Schloss et al., 2009). After quality trimming and assembly, we can perform 16S rRNA gene sequence analysis using free software such as QIIME or mothur.

Fig.2–8 Protocol for barcoded Illumina pyrosequencing. First, conserved regions within the target gene (in this case, 16S rRNA) are identified (blue), together with an amplicon that clipping studies along the lines of ref. 15 indicate are especially good for community sequence analysis (green). Second, PCR amplifications are performed, using primers that include a linker sequence not homologous to any 16S rRNA sequence at the corresponding positions, the barcode, and the Illumina adaptor. Thus, the match between the primer and the template sequence ends at the end of the black region of the primer, and the linker and adaptors (shown in color) do not match the template. This procedure yields a library of amplification products that contain the barcode and Illumina adaptors. Finally, three separate primers are used to yield the 5′ read, the 3′ read, and the index read (that yields the barcode sequence) (Caporaso *et al.*, 2010).

We generally analyze microbial community data using the operational taxonomic unit (OTU) based on sequence similarity because a large amount of NGS data may lead to an overestimation of microbial diversity and community composition. However, this threshold of sequence similarity may produce false microbial communities as a result of artificial DNA sequences. Kunin *et al.* (2010) performed 16S rRNA gene sequencing of V1/V2 and V8 using a 454-pyroseqencer on a pure culture of *E. coli* MG1655. The results showed that error rates ≤ 0.2% and OTU thresholds ≤ 97% are closest to pure culture, and a lot of artificial DNA sequences were observed to occur outside of these

ranges. Additionally, it was reported that higher PCR cycle numbers cause substitution error, which was tested using 454-pyrosequencing technology (Patin et al., 2012). It was also reported that with the Illumina platform, DNA sequencing errors occur rarely, with Phred quality scores <21 (Kozich et al., 2013). However, a recent study confirmed that the majority of DNA sequencing errors occurred in the PCR step (Schirmer et al., 2015). We therefore must consider PCR cycle numbers, error rates of sequence results, and OTU-clustering thresholds in the analysis of 16S rRNA gene sequences using NGS.

For taxonomic classification, we can choose different taxonomic references, such as Greengenes, the Ribosome Database project (RDP), or the SILVA project. Greengenes taxonomy, which uses FastTree, is based on non-chimeric sequences of phylogenetic positions in *de novo* phylogenetic trees, which are aligned based on Near Alignment Space Termination (DeSantis et al., 2006). Taxonomic names are based on Grouping, Ungrouping, Naming Tool (GRUNT), cyanoDB (only Cyanobacteria), and NCBI taxonomy (DeSantis et al., 2006; McDonald et al., 2011). The RDP uses the naïve Bayesian classification method, which can achieve rapid and accurate prokaryote classification in assignment with Bergey's taxonomic references without an alignment step (Wang et al., 2007). SILVA taxonomy for SSU rRNA gene sequences is first based on the official ssu_jan04 release of the ARB Project. It parsimoniously adds the aligned sequences with strict thresholds using SILVA Incremental Aligner and manual curation (Pruesse et al., 2007). SILVA taxonomic names have used Bargey's taxonomic outlines and List of Prokaryotic names with Standing in Nomenclature (LPSN) resources for candidate taxonomic groups (Yilmaz et al., 2014). While the Greengenes database can assign several candidate groups (Table 2–4) (McDonald et al., 2011), SILVA has three domains references, and its database is more frequently updated (http://www.arb-silva.de).

The formation of artifact sequences, such as chimera, frequently occurs during the PCR step (Schloss et al., 2011). Minor species that are produced by 454-pyrosequencing

Table 2–4 Greengenes classifications of NCBI-defined candidate phyla (divisions) based on tree_16S_candiv_gg_2011_1. SILVA_106 and RDP classifications are included for reference (McDonald *et al.*, 2011).

Candidate bacterial divisions (phyla) in the NCBI taxonomy[a]	*Number of NCBI representative sequences; full (partial)*[b]	*Consensus phylum-level classification*[c]		
		Greengenes	*SILVA*	*RDP*
AC1	6 (7)	p__AC1[d]	TA06	
OS-K	3 (7)	p__Acidobacteria[d]	Acidobacteria	Acidobacteria
OP10	69 (279)	p__Armatimonadetes	OP10	OP10
KSA1	0 (2)	p__Bacteroidetes[d]		Bacteroidetes
KSB1	13 (23)	p__Caldithrix	Deferribacteres	
MSBL5	0 (1)	p__Chloroflexi		Chloroflexi
NT-B4	0 (1)	p__Chloroflexi		
CAB-I	7 (59)	p__Cyanobacteria	Cyanobacteria	Cyanobacteria
OP2	1 (25)	p__Elusimicrobia[e]	Thermotogae	
GN01	10 (12)	p__GN01	Spirochaetes	
GN02	4 (10)	p__GN02	BD1-5	
GN10	3 (4)	p__GN02	BD1-5	
GN11	3 (0)	p__GN02	BD1-5	
GN07	0 (4)	p__GN02		
GN08	0 (1)	p__GN02		
GN04	7 (7)	p__GN04	TA06	
GN12	0 (2)	p__GN04		
GN15	0 (2)	p__GN04		
GN13	0 (2)	p__GN13		
GN14	0 (2)	p__GN14		
GN06	1 (2)	p__KSB3	Proteobacteria	
NC10	6 (27)	p__NC10	Nitrospirae	Firmicutes
NKB19	4 (11)	p__NKB19	BRC1	
KB1 group	7 (20)	p__OP1	EM19	
OP1	10 (38)	p__OP1	EM19	
MSBL6	0 (5)	p__OP1		
Sediment-3	0 (1)	p__OP1		
MSBL4	0 (3)	p__OP3		
kpj58rc	0 (1)	p__OP3		
OP8	36 (390)	p__OP8	Nitrospirae	
JS1	26 (89)	p__OP9	OP9	Firmicutes
VC2	0 (2)	p__Proteobacteria[d]		
Marine group	0 (2)	p__SAR406		
SBR1093	9 (1)	p__SBR1093	Proteobacteria	
SPAM	8 (1)	p__SPAM	Nitrospirae	
GN05	4 (9)	p__Spirochaetes[d]	Spirochaetes	
WWE1	3 (2)	p__Spirochaetes[d]	Spirochaetes	
OP4	1 (1)	p__Spirochaetes[d]	Spirochaetes	
MSBL2	0 (6)	p__Spirochaetes[d]		
KSA2	0 (1)	p__Spirochaetes[d]		
Sediment-4	0 (3)	p__Spirochaetes[d,f]		
Sediment-2	0 (2)	p__Spirochaetes[d];p__SAR406[g]		
GN09	6 (4)	p__TG3	Fibrobacteres	
TG3	41 (40)	p__TG3	Fibrobacteres	
MSBL3	0 (1)	p__Verrucomicrobia[d]		
Sediment-1	0 (3)	p__WS3		
GN03	0 (27)	p__WS3		
KSB4	0 (1)	p__WS3		WS3
WS5	1 (2)	p__WS5	WCHB1-60	
WWE3	116 (0)	p__WWE3	OD1	
ZB3	11 (0)	p__ZB3	Cyanobacteria	
TG2	4 (0)	p__ZB3	Cyanobacteria	
SAM	1 (0)	Chimera[h]	Chloroflexi	

Abbreviation: NCBI, National Center for Biotechnology Information.
[a]The following candidate phyla are not shown because they were consistent between NCBI, Greengenes, SILVA and RDP (where classifications were available): BRC1, KSB2, KSB3, OD1, OP11, OP3, OP6, OP7, OP9, SR1, TM6, TM7, WS1, WS2, WS3, WS4, WS6 and WYO.
[b]Full-length representatives ⩾1200 nt, partial length <1200 nt, not all sequences are 16S rRNA. Phylogenetic placements based only on partial sequences should be considered probationary until full-length or genomic sequence data become available.
[c]Name of phylum that encompasses the majority of the NCBI representative sequences, except where specifically noted. Gaps indicate no classification.
[d]Not robustly supported as a monophyletic group in tree_408135 (jackknife <70%).
[e]On the basis of the position of the single full-length representative after which the group was originally named, the 25 partial length representatives are not affiliated with the full-length sequence and belong to the Chlorobi.
[f]On the basis of the longest representative of this proposed group (AF142890), the two shorter sequences are members of the Firmicutes.
[g]One representative belongs to each phylum; AF142866—Spirochaetes, AF142828—SAR406.
[h]Between Planctomycetes and Chloroflexi.

were detected, with chimeric sequence rates exceeding 70% (Haas et al., 2011). Therefore, the confirmation of chimeric sequences requires complete microbial community data. There are web-based (*i.e.,* Bellerephon, EZ-Taxon, greengenes, RDP, and SILVA) and local-based resources (*i.e.* ,ChimeraSlayer, Perseus, and UCHIME) available to check chimeric sequences (Chun et al., 2007; DeSantis et al., 2006; Haas et al., 2011; Huber et al., 2004; Pruesse et al., 2007; Schloss et al., 2009; Wang et al., 2007). For NGS data processing, ChimeraSlayer and UCHIME are frequently used because they are the most sensitive software (Edgar et al., 2011). While ChimeraSlayer searches multiple alignments of chimera-free reference sequences, UCHIME can search chimera sequences similar to ChimeraSlayer (reference-based approach) and Perseus (*de novo* approach), which achieve the highest sensitivity and have the shortest time requirement for chimera detection (Edgar et al., 2011).

2.3 Unknown and uncultivated microorganisms in wastewater treatment systems

In biological wastewater treatment systems (in particular anaerobic environments) there are many unknown and uncultivated microorganisms. The presence of these unknown organisms often inhibits a comprehensive understanding of the wastewater treatment mechanisms. Based on 16S rRNA gene sequencing, several kinds of uncultured bacteria and archaea at class or phylum levels have been detected in aerobic, anoxic, and anaerobic wastewater treatment systems (Table 2–5). Owing to the development of NGS, we now have the ability to uncover environmental genomes. As a result, several single-cell genomics, metagenome, and metatranscriptome studies have been reported (Table 2–6), resulting from our ability to easily observe approx. 100 microbial genomes using NGS technology (Gasc et al., 2015). In this chapter, I discuss

Table 2–5 Main candidate phylum genomes obtained with metagenomics and/or single-cell genomics approaches. (These table and legend were modified from Gasc *et al*., 2015).

Kingdom	Candidate phylum	First description in	Sequenced genomes	
			Metagenomics	SCG
Bacteria	AD3	Sandy surface soils [Zhou et al., 2003]	–	–
	BD1-5 group/GN02 [Gracilibacteria]	Guerrero Negro hypersaline microbial mat [Ley et al., 2006]	5 [Wrighton et al., 2012]	2 [Rinke et al., 2013]
	BH1	Near-boiling silica-depositing thermal springs [Blank et al., 2002]	–	–
	BRC1/NKB19 [Hydrogenedentes]	Bulk soil and rice roots (BRC1 means Bacterial Rice Cluster) [Derakshani et al., 2001]	1 [Nobu et al., 2015]	4 [Nobu et al., 2015] and [Rinke et al., 2013]
	CD12/BHI80-139 [Aerophobetes]	– [Rinke et al., 2013]	–	1 [Rinke et al., 2013]
	EM3 (former OP2)	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	–	1 [Rinke et al., 2013]
	GN01	Guerrero Negro hypersaline microbial mat [Ley et al., 2006]	–	–
	GN04	Guerrero Negro hypersaline microbial mat [Ley et al., 2006]	–	–
	GOUTA4	Monochlorobenzene-contaminated groundwater [Alfreider et al., 2002]	–	–
	KSB1	Sulfide-rich black mud from marine coastal environments [Tanner et al., 2000]	–	–
	LD1	Anoxic marine sediments [Freitag and Prosser, 2003]	–	–
	Marine Group A/SAR406 [Marinimicrobia]	Subsurface of Atlantic and Pacific oceans [Fuhrman et al., 1993]	1 [Nobu et al., 2015]	22 [Nobu et al., 2015] and [Rinke et al., 2013]
	MVP-15	Suboxic freshwater pond [Briee et al., 2007]	–	–
	NC10	Aquatic microbial formations in flooded caves [Holmes et al., 2001]	1 [Ettwig et al., 2010]	–
	OD1/WWE3 [Parcubacteria]	Obsidian Pool, Yellowstone National Park (OD1 means OP11-derived 1) [Harris et al., 2004]	28 [Kantor et al., 2013], [Wrighton et al., 2014] and [Wrighton et al., 2012]	9 [Rinke et al., 2013]
	OP1/KB1 group [Acetothermia]	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	1 [Takami et al., 2012]	–
	OP11 [Microgenomates]	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	17 [Wrighton et al., 2014] and [Wrighton et al., 2012]	–
	OP3 [Omnitrophica]	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	–	4 [Rinke et al., 2013]
	OP8 [Aminicenantes]	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	–	36 [Rinke et al., 2013]
	OP9/JS1 [Atribacteria]	Obsidian Pool, Yellowstone National Park [Hugenholtz et al., 1998]	2 [Dodsworth et al., 2013] and [Nobu et al., 2015]	18 [Dodsworth et al., 2013], [Nobu et al., 2015] and [Rinke et al., 2013]
	Poribacteria	Marine sponge-associated [Fieseler et al., 2004]	–	1 [Siegl et al., 2011]
	SBR1093	Activated sludge from an industrial wastewater treatment system [Layton et al., 2000]	–	–
	SC4	Arid soil from Arizona [Dunbar et al., 2002]	–	–
	SPAM	Alpine soil in the Colorado Rocky Mountains (SPAM means SPring Alpine Meadow) [Lipson and Schmidt, 2004]	–	–
	SR1	Hydrocarbon-contaminated aquifer (SR means "Sulfur River") [Dojka et al., 1998]	2 [Kantor et al., 2013] and [Wrighton et al., 2012]	1 [Campbell et al., 2013]
	TM6	Peat bog (TM means Torf, Mittlere schicht) [Rheims et al., 1996]	–	1 [McLean et al., 2013]
	TM7	Peat bog (TM means Torf, Mittlere schicht) [Rheims et al., 1996]	5 [Albertsen et al., 2013] and [Kantor et al., 2013]	2 [Marcy et al., 2007] and [Podar et al., 2007]
	WPS-2	Wittenberg polluted soil [Nogales et al., 2001]	–	–
	WS1	Wurtsmith Air Force Base, Michigan [Dojka et al., 1998]	–	2 [Nobu et al., 2015] and [Rinke et al., 2013]
	WS2	Wurtsmith Air Force Base, Michigan [Dojka et al., 1998]	–	–
	WS3 [Latescibacteria]	Wurtsmith Air Force Base, Michigan [Dojka et al., 1998]	–	7 [Rinke et al., 2013]
	WS6	Wurtsmith Air Force Base, Michigan [Dojka et al., 1998]	–	–
	WWE1 [Cloacimonetes]	Municipal Anaerobic Sludge Digester [Chouari et al., 2005a]	2 [Nobu et al., 2015] and [Pelletier et al., 2008]	3 [Nobu et al., 2015]
Archaea	ZB3	Mesophilic sulfide-rich spring [Elshahed et al., 2003]	–	–
	Korarchaeota	Obsidian Pool, Yellowstone National Park [Elkins et al., 2008]	1 [Elkins et al., 2008]	–
	Nanoarchaeota	Submarine hot vent [Huber et al., 2002]	–	–

Table 2–6 Putative functions of uncultured taxa frequently existing in wastewater treatment systems.

Kingdom	Phylum or class	Proposed name		Habitats	Putative functions
		Phylum level	Genus and specie		
Bacteria	TM7	"*Ca*. Saccharibacteria"	"*Ca*. Saccharimonas aalborgensis"	activated sludge systems	oligosaccharides and arginine utilization
	WWE1	"*Ca*. Cloacimonetes"	"*Ca*. Cloacimonas acidaminovorans"	mesophilic anaerobic digester	propionate or/and amino acids utilization cellulolytic?
	GN02	"*Ca*. Gracilibacteria"	"*Ca*. Altimarinus pacificus"	hypersaline microbial mat drinking water distribution system	unknown (fermentation)
	GN04	–	–	hypersaline microbial mat mesophilic methanogenic reactors	unknwon (anaerobe)
	OD1	"*Ca*. Parcubacteria"	"*Ca*. Paceibacter normanii"	hot spring freshwater environments	CO_2-fixation (anaerobe) methane oxidation?
	OP8	"*Ca*. Aminacenantes"	"*Ca*. Aminicenans sakinawicola"	aerobic and anaerobic conditions low, medium, and high temperature from non-salinity to hypersaline conditions	amino acids fermentation
	OP9	"*Ca*. Atribacteria"	"*Ca*. Caldatribacterium californiense"	anaerobic terephthalate-degrading bioreactor thermophilic sulfur-rich environment organic-rich microbiomes at middle or low temperature	cellulolytic? (anaerobe)
	WS3	"*Ca*. Latescibacteria"	"*Ca*. Latescibacter anaerobius"	activated sludge systems hydrothermal vent lagoon	aerobe and anaerobe are present? CO_2-fixation
	FCPU426	–	–	subsurface peat layers	unknwon (anaerobe)
	Hyd24-12	–	–	hypersaline lake marine sponge	unknown halophilic?
	KSB3	"*Ca*. Modulibacteria"	"*Ca*. Moduliflexus flocculan" "*Ca*. Vecturathrix granuli"	up-flow anaerobic sludge blanket (UASB) reactor	glucose/maltose fermentation (anaerobe)
Archaea	WSA2	–	–	mesophilic anaerobic digester	unknown (methanogen?)
	"Parvarchaeota"		"*Ca*. Parvarchaeum acidophilus"	anaerobic/anoxic sequencing batch reactor treating sewage	unknown aerobe and anaerobe are present?

the genomic and ecological information of uncultured bacterial and archaeal classes or phyla relevant to biological wastewater treatment.

2.3.1 Uncultured organisms in aerobic or/and anoxic environments

TM7 ("*Candidatus* Saccharibacteria")

Uncultured phylum TM7 has been detected at a rate of approximately 2% in several activated sludge samples, based on results of 16S rRNA gene sequencing analysis (Nielsen et al., 2009; Zhang et al., 2012; Hugenholtz et al., 2001). Using TM7-specific DNA probes for a FISH method, the organisms belonging to TM7 have filamentous, rod-like (10–30 μm), or coccoid (~0.7 μm) morphologies (Hugenholtz et al., 2001; Marcy et al., 2007; Albertsen et al., 2013). Recently, near-complete genomes (approx. 1 Mbp genome size) of TM7 were uncovered by metagenomics, and the names of this phylum and representative species were proposed (phylum: "*Candidatus* Saccharibacteria"; representative species: "*Ca.* Saccharimonas aalborgensis") (Albertsen et al., 2013). It was speculated that "*Ca.* Saccharimonas aalborgensis" has coccoid morphology, is gram-positive, and has an obligate fermentative lifestyle (Albertsen et al., 2013; Marcy et al., 2007). This organism also has oxygen tolerance genes, such as superoxide dismutase and glutathione peroxidase. In the other TM7 group (TM7a), different genome sizes (approx. 3 Mbp) were observed. This group possesses a tricarboxylic acid cycle, glycolysis, nucleotide biosynthesis, and some amino acids biosynthesis, suggesting that this organism may be able to utilize oligosaccharides and arginine as growth substrates (Marcy et al., 2007). However, although a significant amount of metabolic information have been obtained, this phylum remains uncultivated.

2.3.2 Uncultured organisms in anaerobic environments

2.3.2.1 *Bacteria*

WWE1 ("*Ca*. Cloacimonetes")

The uncultured phylum WWE1 is frequently detected in mesophilic anaerobic digesters. Using the FISH method with WWE1-specific probes, rod- or filamentous-type bacteria belonging to WWE1 were detected at a rate of approx. 12% in a mesophilic digester (Chouari et al., 2005a). Additionally, Pelletier et al. (2008) and Nobu et al. (2015) speculated on the metabolic function of WWE1 based on genomic studies and proposed phylum and representative names (Phylum: "*Ca*. Cloacimonetes"). Results of these reports indicate that WWE1 may be a propionate-oxidizing syntroph or an amino acids fermenter. However, to date, no isolation successions have been reported. Based on results of isotope-probing methods and secondary ion mass spectrometry–in situ hybridization, it has been speculated that some WWE1 members could perform other functions, such as cellulose hydrolysis (Limam et al., 2014).

GN02 ("*Ca*. Gracilibacteria")

In the Guerrero Negro hypersaline microbial mat, several unknown clone clusters at the class and phylum levels were detected by 16S rRNA gene analysis (Ley et al., 2006; Harris et al., 2013). The GN02 clones were observed in low-H_2S (non-oxic) and high-H_2S zones. In another report, GN02 clones were detected in high abundance (3.1–15.6%) in a drinking water distribution system (Lautenschlager et al., 2013). Recently, researchers have performed genome analysis of GN02 taxa, which demonstrated that these bacteria may obtain energy from fermentation (Wrighton et al., 2012; Rinke et al., 2013). Although these studies have provided metabolic and genetic information on GN02, no isolation reports have been published to date.

GN04

GN04 phylotypes were detected in the Guerrero Negro hypersaline microbial mat (Ley et al., 2006; Harris et al., 2013). Several GN04 phylotypes were also detected in methanogenic wastewater treatment environments, including a methanogenic reactor that was treating soft-drink wastewater, a UASB reactor that was treating sugar-containing wastewater, and a mesophilic anaerobic digester (Narihiro et al., 2009; Narihiro et al., 2014; Narihiro et al., 2015). Evidence from previous 16S rRNA-based analyses indicates that GN04 may be an anaerobe, however, the functions of this bacterium remain unclear.

OD1 ("*Ca*. Parcubacteria")

16S rRNA gene sequences belonging to OD1 clone clusters have been uncovered from many terrestrial and marine environments (*e.g.,* anaerobic sulfide- and sulfur-rich springs, the East Pacific Rise, Homestake Mine, Sakinaw Lake, and freshwater ponds and lakes) (Berdjeb et al., 2011; Elshahed et al., 2005; Rinke at al., 2013; Wrighton et al., 2012). Some taxa belonging to OD1 were analyzed using genome analysis, such as metagenome and single-cell genomics. The results showed that OD1 (proposed phylum name: "*Ca*. Parcubacteria") has an anaerobic lifestyle, 17 hydrogenases, and RuBisCO type III (Rinke at al., 2013; Wrighton et al., 2012). Additionally, based on correlation analysis with 16S rRNA gene sequences, environmental conditions, and stable isotope experiments, OD1 was positively correlated with concentrations of methane and ammonium (Peura et al., 2012), suggesting that this bacterium might play a role in methane oxidation in the environment, however, its function remains unknown.

OP8 ("*Ca*. Aminacenantes")

Candidate phylum OP8 was first discovered in Obsidian Pool in Yellowstone National Park, USA, which is rich in sulfide, CO_2, hydrogen, and reduced ions at

thermophilic temperatures (75–95°C) (Hugenholtz et al., 1998). Metagenomic or 16S rRNA gene-based approaches have produced evidence suggesting that "*Ca*. Aminicenans sakinawicola" belonging to "*Ca*. Aminacenantes" (proposed phylum name based on genomic study) can degrade amino acids. The members of this phylum exist in diverse environments: under aerobic and anaerobic conditions, at low, medium, and high temperatures, and in environments ranging from non-saline to hypersaline (Rinke et al., 2013; Farag et al., 2014). Results of 16S rRNA gene analysis have demonstrated that "*Ca*. Aminicenantes" have eight clades at order- or class- level (Farag et al., 2014). Despite the availability of some information, most of the "*Ca*. Aminicenantes" functions remain unclear.

OP9 ("*Ca*. Atribacteria")

OP9 clones were also first found in Obsidian Pool in Yellowstone National Park, USA (Hugenholtz et al., 1998). Based on genomic analysis, OP9 phylotype (proposed name "*Ca*. Atribacteria") has an anaerobic lifestyle, depending on glycolysis for energy production, and might utilize cellulose by catabolism in thermophilic environments (Dodsworth et al., 2013; Rinke et al., 2013). Phylotypes belonging to "*Ca*. Atribacteria" have been observed in anaerobic terephthalate-degrading bioreactors and organic-rich microbiomes at intermediate or low temperatures (Gittel et al. 2009; Rivére et al. 2009; Rinke et al., 2013). Therefore, further genomic analysis of several phylotypes from different environmental conditions are required to understand "*Ca*. Atribacteria" physiologies.

WS3 ("*Ca*. Latescibacteria")

Candidate phylum WS3 16S rRNA gene sequences were obtained from diverse environments, such as activated sludge systems, hydrothermal vents, subsurface sediments, lagoons, and lakes (Dhillon et al., 2003; Hiras et al., 2015; Rinke et al., 2013;

Zhang et al., 2012). Observed distribution patterns of this phylum suggest that WS3 organisms might be aerobic, anoxic, or anaerobic bacteria. Genomic studies conducted to predict the function of WS3 revealed that the phylum contains RuBisCO type III. WS3 is in the same clade as methanogens in *Euryarchaeota* (Rinke et al., 2013). However, further information from transcriptome or proteomics analyses is required to fully understand the role of WS3 in its natural environment.

FCPU426 and Hyd24-12

The taxonomy of the phylum-level clone cluster FCPU426 is unknown because no genomic- or culture-based studies have been performed on it. Previously, FCPU426 was detected in subsurface peat layers (Serkebaeva et al., 2013). However, there is no available information on FCPU426 metabolism.

Hyd24-12 is the candidate phylum, and its genome has not been uncovered to date. Schneider et al. (2013) and Simister et al. (2012) detected relatively high abundances of Hyd24-12 in high-salinity environments, including a hypersaline lake and a marine sponge. This indicates that Hyd24-12 may be able to grow under high-salinity conditions. However, detailed information on the metabolic functions of phylum Hyd24-12 members is unavailable.

KSB3 ("*Ca*. Modulibacteria")

Clone cluster KSB3 was present predominantly in mesophilic granular sludge used to treat high-strength organic wastewater discharged from sugar-producing facilities (Yamada et al., 2007; Yamada et al., 2011). This organism is known as sludge-bulking causative bacteria in UASB reactors (Yamada et al., 2007). 16S rRNA- and microautoradiography-based methods have suggested that this organism has filamentous morphology, predominantly outside of the UASB granules, and glucose/maltose fermentation metabolism. Two types of KSB3 genomes ("*Ca*.

Moduliflexus flocculan" and "*Ca*. Vecturathrix granuli") were uncovered from methanogenic sludge samples (Sekiguchi et al., 2015). The genomic study predicted that KSB3 members are sensitive to glucose and maltose, which cause a gliding motility response (Sekiguchi et al., 2015). Previous bulking reports from that study as well as from UASB reactors have suggested that controlling sugar concentrations in wastewater is important for maintaining reactor operation stability.

2.3.2.2 *Archaea*

WSA2

Clone cluster WSA2 is a putative methanogen clade, and was discovered in a mesophilic anaerobic digester in 2005 (Chouari et al., 2005b). Evidence from a previous study in which researchers applied a cultured-based approach with the FISH method suggests that WSA2 might be a hydrogenotrophic methanogen (Chouari et al., 2005b). WSA2 is commonly detected in a wide variety of natural and engineered environments, including lakes, marine sediments, contaminated groundwater, and bioreactors. Though this organism is thought to be a hydrogenotrophic methanogen, there have been very few successful reports of enrichment cultivation and FISH results (Chouari et al., 2005b; Narihiro et al., 2007; Saito et al., 2015). Though results of 16S rRNA gene and *mcrA* gene analyses suggest that WSA2 is a methanogen, detailed information on the functions of WSA2 remains unknown (Saito et al., 2015).

"Parvarchaeota"

"*Parvarchaeota*" is a newly-discovered phylum that is present in chemo-autotrophic biofilms in acidic (pH <1.5), metal-rich solutions from Richmond Mine (Iron Mountain, CA, USA) (Baker et al., 2006; Baker et al., 2010). Based on the environments in which it lives, and FISH and genome analysis results, it is thought that "*Ca*. Parvarchaeum" has an aerobic lifestyle (it has a near complete TCA cycle) and has a

small cell size (< 1-μm diameter). Recent genomic studies have proposed a new DPANN superphylum, which includes "*Ca*. Diapherotrites", "*Ca*. Parvarchaeum acidophilus", and "*Ca*. Micrarchaeum acidiphilum" in "*Parvarchaeota*", "*Ca*. Aenigmarchaeota", "*Ca*. Nanoarchaeota", and "*Ca*. Nanohaloarchaeota" (Castelle et al., 2015; Rinke et al., 2013). Additionally, our research group recently published a study in which we detected "*Ca*. Parvarchaeum" at an archaeal abundance rate of approx. 10% in an anaerobic/anoxic sequencing batch reactor used to treat sewage (Kuroda et al., 2015b). This indicates that this archaea occurs in diverse environments (aerobic, anoxic, and anaerobic). However, the role of this *Archaea* in the anaerobic bioreactor is still unknown.

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Chapter 3

Patterns of uncultured Bacteria phyla in different wastewater treatment sludges

Comprehensive understanding of biological wastewater treatment mechanisms was prevented because a wide range of uncultured and unknown lineages existed in the wastewater treatment sludges. In this chapter, to understand the patterns of uncultured phyla in wastewater treatment sludges, I analyzed a total of 54 aerobic, anoxic and anaerobic sludge samples collected from 17 different wastewater treatment reactors by massively parallel 16S rRNA gene sequencing. I analyzed a total of 1,041,539 sequence reads of 16S rRNA gene. The results of microbial community analysis at the phylum level and phylogenetic diversity analyses indicated that the patterns of microbial communities depended heavily on types of wastewater and types of treatment technologies. I could estimate the putative habitats and environmental conditions of these uncultured lineages by the distribution pattern of the microbial communities in different wastewater treatment sludges.

Kyohei Kuroda, Masashi Hatamoto, Akinobu Nakamura, Kenichi Abe, Masayoshi Yamada, Masahito Yamauchi (2014). Patterns of uncultured *Bacteria* phyla in different wastewater treatment sludges, *Journal of JSCE, Division G (Environmental Research)* in Japanese with English Abstract, Vol. 70, No. 3, pp. 42–52.

3.1 Introduction

Biological wastewater treatment systems are essential for the treatment of various types of wastewater. Activated sludge reactors, membrane bioreactors, and down-flow hanging sponge (DHS) reactors, all of which are types of aerobic wastewater treatment technology, are widely used and studied globally. Up-flow anaerobic sludge blanket (UASB) reactors are also widely used; these can treat higher quantities of organic loading, have lower energy requirements compared with aerobic systems, and provide energy recovery in the form of methane gas production (Sato et al., 2007; Kleerebezem et al., 2003). Recently, the UASB reactor has been applied to the treatment of low-organic wastewater, psychrophilic wastewater, and wastewater that is toxic to microorganisms (Uemura et al., 2000; Lettinga et al., 2001; Veeresh et al., 2005). One disadvantage of UASBs is that a sludge-bulking phenomenon often occurs in the activated sludge and the UASB systems. The causative agent of this phenomenon might be filamentous bacterium. In particular, bacteria belonging to KSB3 and *Anaerolineae* are speculated to cause bulking, however, this mechanism remains unclear because these microbes are uncultivated (Yamada et al., 2007; Sekiguchi et al., 2001).

To elucidate this phenomenon, microbial community analysis based on 16S rRNA gene sequences, such as PCR-cloning, denaturing gradient gel electrophoresis, and terminal restriction fragment length polymorphism have been performed (Sekiguchi et al., 1998; Liu et al., 1997; Muyzer et al., 1993). In recent advances, researchers have developed a next-generation DNA sequencer that can perform high-throughput DNA sequences (>1 million reads per run). Researchers have used this latest technology to perform various types of microbial community analyses (Sundberg et al., 2013; Zhang et al., 2012). Additionally, these analyses have demonstrated that many kinds of uncultivated taxa are present in wastewater treatment sludge (Ye et al., 2011; Chouari et al., 2010).

As a result of recent developments in molecular biology, many microbial genomes have been analyzed, and their physiological functions have been estimated. Rinke et al. (2013) analyzed 201 uncultivated microorganism genomes, including those of 20 uncultured phyla, and estimated the relationships of each phylum, such as DPANN superphylum (Rinke et al., 2013). Previous studies have completely constructed the genome of WWE1, the group that is detected at an abundance rate >12% in mesophilic anaerobic digesters (Chouari et al., 2005; Pelletier et al., 2008). These studies have suggested that this organism may perform amino acid fermentation or propionate degradation along with hydrogen-utilizing microbes. However, a complete understanding of biological wastewater treatment mechanisms, such as the removal of organics and nutrients, is not possible because of the presence of several uncultivated and unknown microorganisms (Rinke et al., 2013; Dinis et al., 2011).

For this chapter, I analyzed a total of 54 aerobic, anoxic, and anaerobic sludge samples from 17 different reactors by 16S rRNA gene sequencing with the goal of understanding the distribution of uncultivated bacterial phyla in wastewater treatment systems. I then estimated environmental conditions of putative habitats based on distribution patterns. This chapter provides ecological information relevant to core microbial members and uncultured bacteria at the phylum level in biological wastewater treatment systems.

3.2 Materials and Methods

3.2.1 Sludge sample collection and DNA extraction

I collected wastewater treatment sludge samples from 17 wastewater treatment systems (Table 3–1). I collected samples No. 1–16 (Table 3–1) from a pilot-scale UASB reactor that was treating municipal sewage at an ambient temperature (10–28°C) on

different operational days (Days 91, 111, 167, 214, 255, 284, 335, 363, 379, 406, 421, 453, 537, 634, 699, and 747). The influent of the UASB reactor contained sulfate at a concentration of 40–150 mgS·L^{-1} of sulfate from day 98. The chemical oxygen demand (COD) concentration was approximately 300 mg L^{-1}. I collected samples No. 17–20 from a mesophilic digester that was treating rice husk (1000–2000 mgCOD·L^{-1}) at days 1, 34, 48, and 66. The seed sludge of this reactor was mesophilic UASB granular sludge that was treating food-industry wastewater. I obtained samples No. 21–23 from different heights of a mesophilic UASB reactor that was treating rubber-industry wastewater (approx. 13,000 mgCOD·L^{-1}). Sample No. 24 was thermophilic multi-staged-(MS-)UASB granule that was treating alcohol distillery wastewater. I collected sample No. 25 from a MS-UASB reactor that was treating molasses wastewater under thermophilic conditions (influent concentration: 17,000 mgCOD·L^{-1}), and I collected sample No. 26 from a mesophilic UASB reactor installed before No. 25 for post-treatment processing. I obtained samples No. 27–30 from a full-scale mesophilic UASB reactor that was treating alcohol-producing wastewater (300–2500 mgCOD·L^{-1}). This UASB reactor was recirculating DHS effluent for nitrogen removal before samples No. 29 and 30 were collected. I obtained samples No. 31–36 from an anaerobic/anoxic sequencing batch reactor (A_2SBR) at days 2, 33, 89, 152, 207, and 244. The influent wastewater of this reactor was from a DHS reactor that was treating sewage, in which the carbon:phosphorus ratio was maintained at 25:1 with acetate (approx. 100 mg COD·L^{-1}). I collected sample No. 37 from a sand filter that was treating the DHS effluent (sewage treatment) at an ambient temperature (10–28°C). I collected samples No. 38–45 from different up-flow sludge blanket reactors that were treating marine aquarium water for nitrogen removal. I obtained samples No. 46 and 47 from methane-oxidizing upflow fixed bed (UFB) sludge that contained nitrate and nitrite, respectively. I collected samples No. 48 and 49 from methane-oxidizing DHS sludge that contained nitrate and nitrite, respectively. Sample 50 was methane-oxidizing UFB sludge with ferric ion. I

Table 3–1 Summary of sludge samples used in this chapter.

Sample No.	*Reactor Type*	*Teatment Type*	*Temp. (°C)*	*Wastewater Type*	*Diversity Index*[¶]						
					No. of Sequence	*No. of OTU*	*Chao1*[¶¶]	*Shannon*[§]	*PD*[§§]	*Coverage**	*Simpson**
1–16	UASB	Methanogenic	10–28	Sewage	17282±2472	6534±1030	30917±5670	9.24±0.47	257±23	0.69±0.03	0.97±0.01
17–20	CSTR	Methanogenic	35	Rice husk	20876±4713	1784±934	9409±7341	5.97±0.40	86±20	0.94±0.03	0.95±0.01
21–23	UASB	Methanogenic	35	Industrial rubber wastewater	15719±495	753±78	2595±439	4.79±0.12	65±4	0.97	0.86±0.01
24	MS-UASB	Methanogenic	55	High-strength alcohol distillation wastewater	22118	1030	4599	5.47	69	0.97	0.94
25	MS-UASB	Methanogenic	55	High-strength molasses	23007	771	3182	5.15	45	0.98	0.92
26	UASB	Methanogenic	30–35	Molasses	25466	1507	3744	6.96	96	0.97	0.97
27–28	UASB	Methanogenic	35	Industrial beer wastewater	16050±2131	974±177	2737±282	6.16±0.24	81±10	0.96	0.96
29–30	UASB	Methanogenic and Denitrification	35	Industrial beer wastewater	16453±472	1004±68	2709±283	6.58±0.11	88±1	0.96	0.97
31–36	A_2SBR	Nitrogen and phosphorus removal	10–28	Sewage (DHS eff.)	31764±7060	4410±1213	17630±4161	7.76±0.64	155±18	0.90±0.01	0.97±0.03
37	Sand filter	Nitrogen and coliform removal	10–28	Sewage (DHS eff.)	20512	2507	5423	7.84	157	0.93	0.98
38–45	USB	Nitrogen removal	26	Marine aquarium water	12558±2917	1322±486	4370±2044	6.46±1.01	111±24	0.93±0.02	0.95±0.03
46	UFB	Denitrifying methane oxidation	30	Nitrite and methane	39305	2971	21723	6.02	75	0.94	0.95
47	UFB	Denitrifying methane oxidation	30	Nitrate and methane	29222	2753	24131	6.07	88	0.92	0.95
48–49	DHS	Denitrifying methane oxidation	30	Nitrite, nitrate and methane	16698±39	1563±170	8524±998	6.63±0.15	89±8	0.93±0.01	0.96±0.02
50	UFB	Fe-reducing methane oxidation	30	Fe and methane	9548	799	2168	5.25	90	0.95	0.86
51–52	DHS	Nitrification and organic removal	35	Industrial rubber wastewater (UASB eff.)	13290±1326	1478±349	5928±72	6.78±0.31	108±19	0.92±0.01	0.96
53–54	DHS	Nitrification and organic removal	35	Molasses (UASB eff.)	24758±9837	1744±407	6022±4744	7.20±0.71	115±20	0.96±0.01	0.98±0.01

[¶]Calculations based on the operational taxonomic units determined at an evolutionary distance of 0.03.

collected samples No. 51 and 52 from the top and bottom, respectively, of a DHS reactor installed after the UASB reactor (No. 21–23). I collected samples 53 and 54 from a DHS reactor that was treating mesophilic UASB effluent (No. 26). I performed DNA extraction using FastDNA Spin Kit for Soil (MP Biomedicals, Carlsbad, CA, USA), according to the manufacturer's protocol.

3.2.2 16S rRNA gene sequencing

I performed a PCR reaction with a universal forward primer of Univ515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and a universal reverse primer of Univ806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). The PCR reaction was performed under the following conditions: initial denaturation, 94°C for 3 min; denaturation, 94°C for 45 s; annealing, 50°C for 60 s; elongation, 72°C for 90 s; final elongation, 72°C for 10 min (Caporaso et al., 2012). I purified the PCR products using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). I performed DNA sequencing using the MiSeq reagent Kit v2 (500 cycles) and MiSeq (Illumina Inc., San Diego, CA, USA).

3.2.3 Data analysis

I analyzed all raw data using QIIME software package ver. 1.7.0 (Caporaso et al., 2010). To maintain the quality of observed 16S rRNA gene sequences, I trimmed the low quality DNA sequences (Phred quality score>30) using the fastx trimmer tool (http://hannonlab.cshl.edu/fastx_toolkit/). I used the paired-end assembler for Illumina sequences software package (PANDAseq) for assembly (Masella et al., 2012). I observed operational taxonomic units (OTUs) using UCLUST at 97% sequence similarity (Edgar, 2010). I identified the taxonomy of observed OTUs with the Greengenes database ver. 13_5 using blast (DeSantis et al., 2006; Altschul et al., 1990). I performed BLAST searches to confirm that observed OTUs were related species (http://blast.ncbi.nlm.nih.gov/Blast.cgi). I removed chimeric sequences using ChimeraSlayer (Haas et al., 2011). I calculated alpha diversities based on the observed species, and the Chao1, Shannon, Simpson, phylogenetic diversity (PD), and Good's coverage indexes. I calculated Shannon, Simpson, and PD at a sampling depth of 8000 reads. I calculated beta diversity with weighted UniFrac at resampled 8000 reads, and displayed them using principal coordinate analysis (Lozupone and Knight, 2005). I constructed the phylogenetic tree based on 16S rRNA gene sequences using neighbor-joining and parsimony methods in ARB, using the Greengenes 16S rRNA gene database (Ludwig et al., 2004). The topology of the constructed tree was confirmed by 1000 bootstrap replicates (Felsenstein, 1985). The definitions of phylum and genus level analyses were performed according to QIIME scripts.

3.3 Results and Discussion

3.3.1 Overview of microbial community analysis in wastewater treatment reactors

I performed microbial community analysis based on 16S rRNA gene sequences of 54

sludge samples from 17 wastewater treatment reactors using the next generation DNA sequencer. In this study, I observed a total of 1,041,539 sequence reads and approximately 9500–39,000 reads per sample (Table 3–1). The number of OTUs were 753–6534, and Chao1 was 2.2–8.8-fold higher than the OTU numbers. Good's coverage values for all samples, except for UASB sludge used to treat sewage (No .1–17), were >0.90. This study covered a greater diversity and number of microbial communities than did a previous study (coverage 0.67–0.81), in which microbial communities of 12 methanogenic wastewater treatment sludge samples were analyzed using PCR cloning (Narihiro et al., 2009). Samples No. 1–17 had higher biodiversity than other sludge samples (Shannon, 9.24; PD, 257; Simpson, 0.97). High diversity of sewage-treating wastewater treatment sludge has also been reported (Sundberg et al., 2013), suggesting that this high diversity might be the result of processes occurring inside the biological sewage treatment reactor. The diversities of samples No. 24 and 25, which were thermophilic sludge samples, were the lowest of all the sludge samples (Table 3–1). Lower diversities in these samples might be the result of the limited archaeal community present because >90% OTUs in *Archaea* were genus *Methanothermobacter*; this finding is consistent with previous results (Sekiguchi et al., 1998; Cheng et al., 2011; Levén et al., 2007).

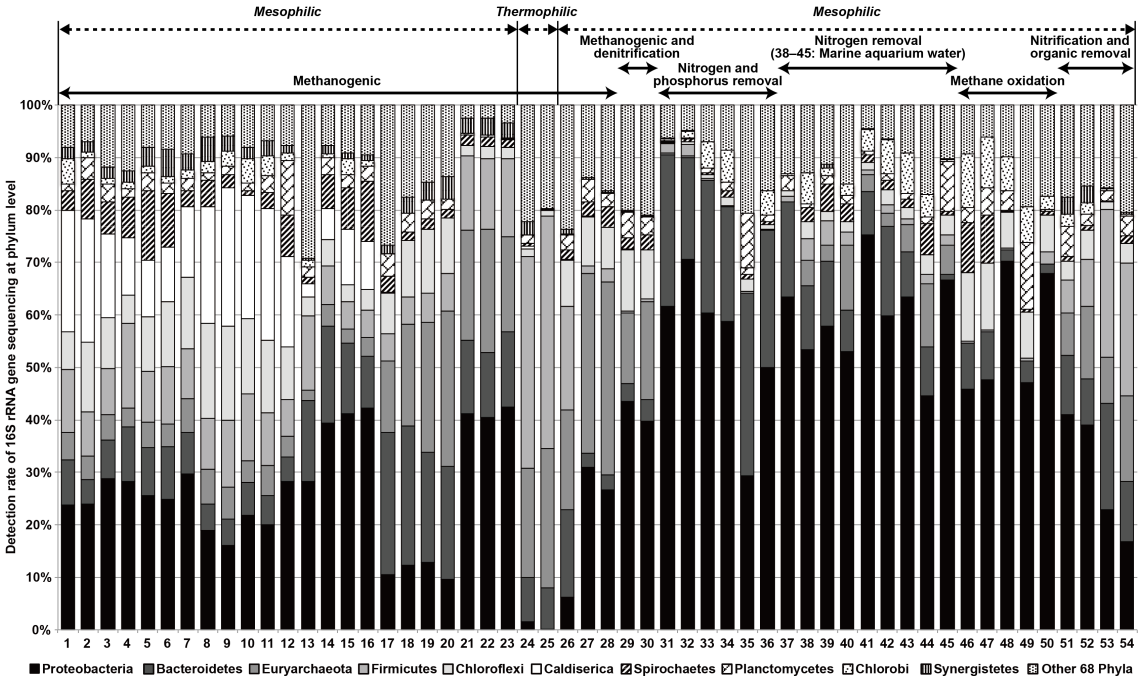

Fig.3–2 Microbial community compositions at phylum level in 54 wastewater treatment sludge samples.

The microbial community analysis conducted at the phylum level demonstrates that the distribution patterns of methanogenic and nitrogen-removal sludge are different (Fig. 3–1). In mesophilic methanogenic sludge (samples No. 1–23 and 26–28), phyla *Proteobacteria* (abundance rate of 6.2–42.4%), *Euryarchaeota* (2.0–36.7%), *Firmicutes* (1.4–19.7%), *Bacteroidetes* (2.8–27.0%), *Caldiserica* (0–26.3%), and *Chloroflexi* (1.9–18.1%) are the predominant taxa, and the predominance of those phyla, except for *Caldiserica*, are consistent with previous reports of mesophilic methanogenic communities (Sekiguchi et al., 2006; Riviére et al., 2009). The *Caldiserica* detected in this study belong to the WCHB1-03 group (DeSantis et al., 2006), and is distantly related to genus *Caldisericum*, which is the cultured bacterium of this phylum (Mori et al., 2009). These uncultured *Caldiserica* are uniquely present in samples No. 1–16. This indicates that this microbe might play a specific role in the sewage-treating UASB reactor. In thermophilic methanogenic samples No. 24 and 25, I observed microbial community compositions that were unique compared with other mesophilic methanogenic samples,

which mainly consist of phyla *Firmicutes* (42.4±2.7%), *Euryarchaeota* (23.7±4.0%), and *Bacteroidetes* (8.1±0.3%). The community composition of denitrifying sludge was different than that of mesophilic methanogenic sludge, which was composed of phyla *Proteobacteria* (No. 31–36, 55.1±14.3%; No. 38–45, 59.3±9.4%) and *Bacteroidetes* (No. 31–16, 26.1±5.5%; No. 38–45, 9.6±4.6%). In particular, the phylum *Proteobacteria* had a higher abundance rate in these samples because of the presence of denitrifying bacterium (Heylen et al., 2006). Similarly, I observed that the abundance rate of *Proteobacteria* was >50% in methane-oxidizing microbial communities (No. 46–50). The results of the aerobic DHS samples (No. 51–54) were similar to those of mesophilic methanogenic sludge, including the predominance of phyla *Proteobacteria* (29.9±11.9%), *Firmicutes* (17.2±11.1%), *Bacteroidetes* (13.0±4.9%), and *Euryarchaeota* (11.7±4.0%). This may be because the anaerobes in UASB effluent might be trapped on the sponges in these reactors. Indeed, the *Methanothermobacter* population made up >50% of the archaeal community in the DHS reactor that was treating molasses wastewater (No. 53 and 54).

The results of PCoA analysis with unweighted UniFrac demonstrated that the microbial community composition of the sewage-treating UASB granules were quite different from those of other samples (Fig. 3–2). One reason for this could be the presence of uncultured *Caldiserica*, which uniquely exists in samples No. 1–16, as discussed above. Additionally, the influent contains low COD (approx. 300 $mgCOD \cdot L^{-1}$) and high sulfate (40–150 $mgS \cdot L^{-1}$) concentrations; these findings demonstrate that the methanogenic microbial communities were altered as a result of the presence of sulfate-reducing bacteria.

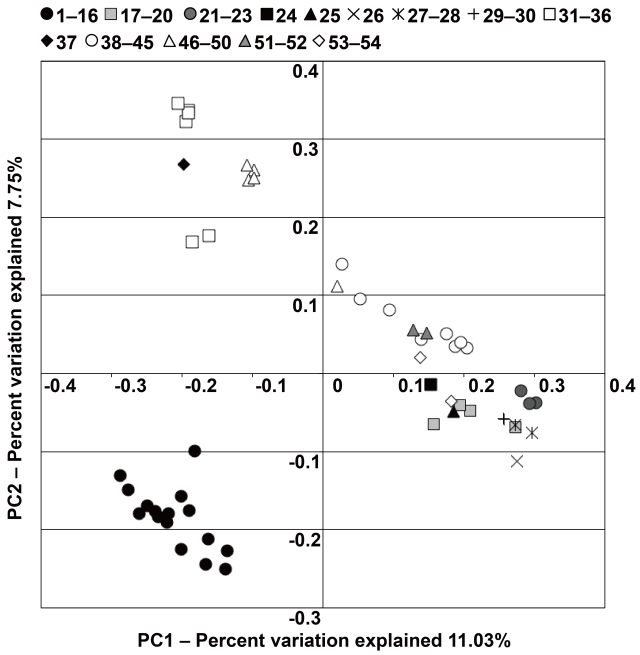

Fig.3–2 Principal coordinate analysis (PCoA) plots from unweighted UniFrac distance for 54 wastewater treatment sludge samples..

3.3.2 Distribution patterns of uncultured bacterial phyla

Based on the results of the 16S rRNA gene sequences from 54 sludge samples, I evaluated the distribution patterns of uncultured bacterial phyla. Using a heatmap of bacterial phyla detection rates, I speculated on their habitats and functions (Fig. 3–3 and Table 3–2). Based on the detected predominant OTUs (>1% maximum abundance rate in each sample), I constructed phylogenetic positions using 16S rRNA gene phylogenetic tree based on the Greengenes database (Fig. 3–4).

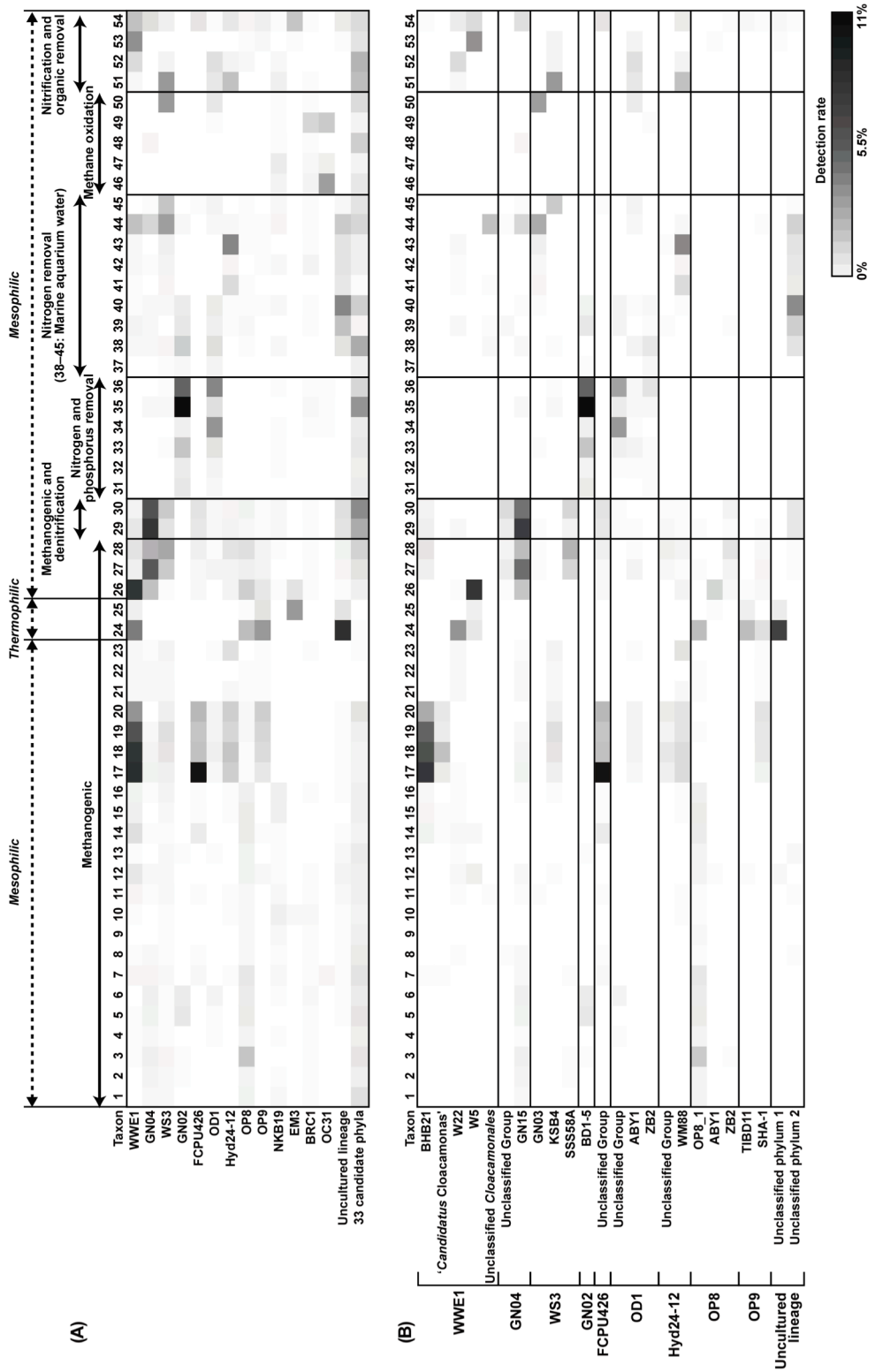

Fig. 3–3 Microbial community compositions of uncultured bacterial phyla in 54. (A) phylum level and (B) genus level.

Table 3–2 Summary of putative habitats and environmental conditions of uncultured phyla.

Taxon			Environmental conditions of uncultured lineages		References of genome information
phyla	uncultured group	temp. (C°)	conditions	characteristics of wastewater (waste)	
WWE1	BHB21	35	anaerobic	Unknown (food-processing wastewater)	Pelletier *et al*., 2008[16] Rinke *et al*., 2013[14]
	'*Candidatus* Cloacamonas'	35	anaerobic	Cellulosic biomass	
	W22	55	anaerobic	High contents of sugar and protein	
	W5	30–35	anaerobic	High contents of sugar, cations and sulfate	
	Unclassified Cloacamonales	26	anoxic	Contents of nitrate, nitrite and salt (3.0%)	
GN04	Unclassified	30	anaerobic or anoxic	Unknown (alcohol producing wastewater after circulation)	–
	GN15	30	anaerobic or anoxic	Unknown (alcohol producing wastewater after circulation)	
WS3	GN03	26–35	anoxic	–	Rinke *et al*., 2013[14]
	KSB4	26–35	anoxic or aerobic	–	
	SSS58A	30	anaerobic	Unknown (alcohol producing wastewater)	
GN02	BD1-5	10–28	anaerobic or anoxic	Contents of nitrate, phosphate, acetate and sulfate	Wrighton *et al*., 2012[38] Rinke *et al*., 2013[14]
FCPU426	Unclassified group	35	anaerobic	Unknown (food-processing wastewater)	–
OD1	Unclassified group	10–28	anaerobic or anoxic	Contents of nitrate, phosphate, acetate and sulfate	Wrighton *et al*., 2012[38] Rinke *et al*., 2013[14]
Hyd24-12	WM88	26	anoxic	Contents of nitrate, nitrite and salt (3.0%)	–
OP8	OP8_1	55	anaerobic	High contents of sugar and protein	Rinke *et al*., 2013[14]
	ABY1	30–35	anaerobic	High contents of sugar, cations and sulfate	
OP9	TIBD11	55	anaerobic	High contents of sugar and protein	Rinke *et al*., 2013[14]
	SHA-1	55	anaerobic	High contents of sugar and protein	Dodsworth *et al*., 2013[43]
Unclassified phylum	Unclassified phylum 1	55	anaerobic	High contents of sugar and protein	–
	Unclassified phylum 2	26	anoxic	Contents of nitrate, nitrite and salt (3.0%)	

WWE1

The uncultured bacteria belonging to WWE1 ("*Ca*. Cloacimonetes") were detected at rates of approx. 6.5% and 8.2% in mesophilic rice husk digester sludge (No. 17–20) and mesophilic UASB sludge (No. 26), respectively (Fig. 3–3A, Fig. 3–4, and Table 3–2). This phylum has been detected in anaerobic sludge, for example in anaerobic digesters (Chouari et al., 2005). According to the genus level analysis, "*Ca*. Cloacimonas" are only present in sample No. 18 at a rate of approximately 2.0% (Fig. 3–3B). Recent genomic analysis suggested that "*Ca*. Cloacimonas" may perform propionate degradation with hydrogenotrophic methanogens or amino acid fermentation (Pelletier et al., 2008). Clone cluster BHB21 was predominant in samples No. 17–20 at a rate of approximately 5.5% (Fig. 3–3B). This genus had an abundance rate of approximately 7.8% in sample No. 17 (seed sludge), while in sample No. 20, its abundance rate was approx. 2.8%. This indicates that BHB21 might favor the substrates derived from food-processing wastewater, because this seed sludge was collected from the UASB reactor that was treating this wastewater. Besides, several uncultured groups of WWE1 are present in thermophilic MS-UASB granule No. 24 (W22 group, 3.5%); mesophilic USB sludge that was treating marine aquarium water No. 44 ("*Ca*. Cloacimonales", 2.0%); mesophilic

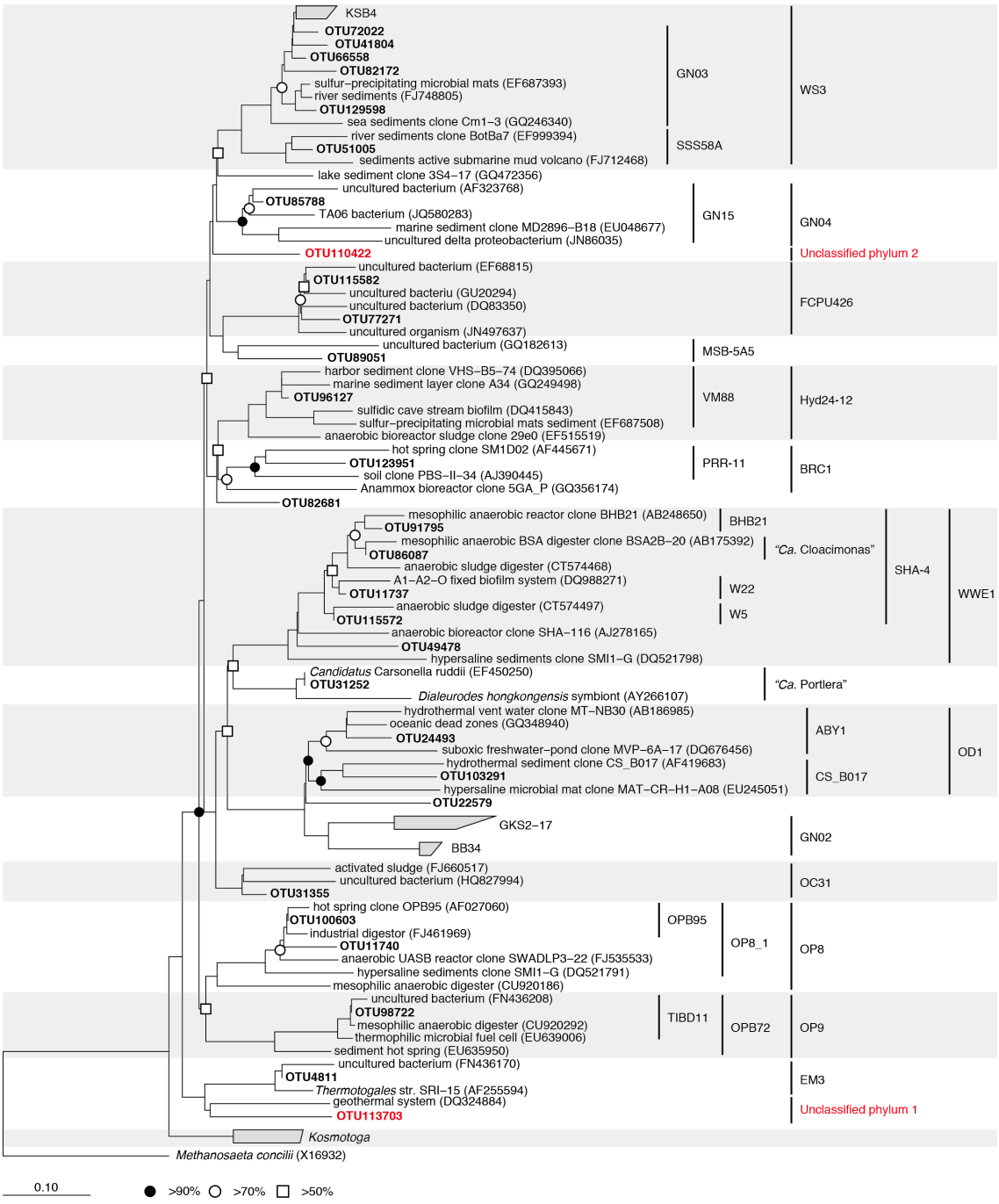

Fig.3–4 Phylogenetic tree representing predominant (>1% of maximum abundance rates in each environment) OTUs in 54 sludge samples using the neighbor-joining and parsimony methods based on 16S rRNA gene sequences. The solid circle, open circle, and open square indicate the bootstrap-supported probabilities at >90%, >70%, and >50%, respectively. The red highlights indicate the uncultured phylum clade in Fig. 3–3 and Table 3–2.

("*Ca*. Cloacimonales", 2.0%); mesophilic DHS sludges No.52–54 and mesophilic UASB granules treating molasses wastewater No.26 (W5 group, 2.6% and 8.0%). Consequently, it is estimated with previous reports that most of uncultured group in WWE1 are present in anaerobic condition (Table 3–2) (Chouari et al., 2005; Zhang et al., 2009).

GN04

Phylum GN04 was detected in UASB sludge samples No. 27–28 (4.0%), which were treating alcohol-producing wastewater and in sludge samples No. 29–30 (6.9%), which were from the same reactor and were treating recirculated of DHS effluent. These findings indicate that the nitrogen components might be influencing the GN04 abundance rate because GN04 abundance increased following recirculation. Although GN04 was detected in high-sodium concentration environments, deep-sea methane seeps, and sediments from water storage tanks (Ley et al., 2006; Nunoura et al., 2012; Röske et al., 2012; Harris et al., 2013), no genomic analyses of GN04 have been reported to date. In high-salinity microbial mats, GN04 had a relatively high abundance (approximately 2.0%), and the maximum abundance rate was observed at a maximum depth of 49 mm in this mat (collected from a depth of 1–49 mm) (Harris et al., 2013). This environment has a high sulfur concentration because sulfate reduction occurs from the surface to a depth of 49 mm in the mat. These findings, along with those of previous reports, indicate that GN04 might be present under anaerobic or anoxic environments (Table 3–2).

WS3

WS3 was widely detected in mesophilic UASB granules that were treating alcohol-producing wastewater (No. 27 and 28), denitrifying and methanogenic sludge (No. 29 and 30), mesophilic USB sludge that was treating marine aquarium water (No. 44 and 45), methane-oxidizing sludge with ferric ion (No. 50), and aerobic DHS sludge

that was treating industry wastewater (No. 51) (Fig. 3–3A and 3–4). As indicated by genomic analysis, some taxa belonging to WS3 may utilize a wide range of sugar and amino acids; however, detailed information is not available (Rinke et al., 2013). The distribution patterns suggest that WS3 are widely present under anaerobic, anoxic, and aerobic conditions (Fig. 3–3A and Table 3–2). Genus-level analysis of WS3 demonstrates that clone cluster group KSB4 was present in mesophilic USB sludge that was treating marine aquarium water (No. 44, 2.7%) and methane-oxidizing DHS sludge with ferric ion (No. 50, 3.2%). Additionally, the clone cluster group KSB4 was detected in mesophilic USB sludge (No. 45) and aerobic DHS sludge (No. 51) at rates of 1.7% and 3.4%, respectively (Fig. 3–3B). GN03 and KSB4 are likely aerobic or anoxic microorganisms because most of these taxa were not detected under anaerobic conditions. I also detected SSS58A group in the UASB reactor that was treating alcohol-producing wastewater (No. 27 and 28) at a rate of approximately 1.9%. Although this group was also detected in the same reactor in sludge that was treating recirculated DHS effluent, the abundance rate was lower (approximately 0.8%). These findings indicate that the SSS58A group could be anaerobes.

GN02 and OD1

GN02 and OD1 were detected in high abundances in A_2SBR sludge that was treating sewage (nitrogen and phosphorus removal) (Fig. 3–3A, 3–3B, and 3–4). In particular, I measured high abundance (approximately 8.6%) in samples No. 35 (operational day 207) and 36 (day 244), suggesting that these organisms are suitable for growth in a A_2SBR environment. In previous studies, researchers analyzed GN02 and OD1 genomes by single-cell genomics (Rinke et al., 2013; Wrighton et al., 2012). BD1-5 belonging to GN02 and OD1 might be strict anaerobes because they lack an electron transport chain and a tricarboxylic acid cycle (Wrighton et al., 2012). Additionally, OD1 may play an important role in the sulfur cycle under anaerobic conditions, as indicated

by the distribution patterns of OD1 (Wrighton et al., 2012; Peura et al., 2012; Elshahed et al., 2005). Results of this study, along with those of previous studies, indicate that GN02 and OD1 may play a role in the removal of organics or in the sulfur cycle (Table 3–2).

FCPU426 and Hyd24-12

FCPU426 was present in mesophilic anaerobic digester sludge that was treating rice husk (No. 17–20) at a rate of approximately 4.2% (Fig. 3–3A and 3–4). However, the detection rate of FCPU426 decreased from >10% to 2.3% during operation. This indicates that FCPU426 favors substrates present in food-industry wastewater (Table 3–2). Although FCPU426 has been highly detected in peat layers of northern wetlands (Serkebaeva et al., 2013), its metabolic functions remain unclear.

Hyd24-12 was present in high abundance in nitrogen removal sludge and aerobic DHS sludge (Fig. 3–3A, B, and 3–4). In particular, USB sludge that was treating marine aquarium water (No. 43) had a high abundance rate (approximately 4.0%, Fig. 3–3A). To date, Hyd24-12 has been observed in high-sodium environments, such as microbial mats and marine sponges (Harris et al., 2013; Simister et al., 2012), indicating that Hyd24-12 can be optimally grown at high sodium concentrations.

OP8 and OP9

OP8 and OP9 were detected in high abundances in methanogenic sludge samples (Fig. 3–3A and 3–4). In particular, abundance rates of these organisms were high (OP8, 2.2%; OP9, 3.4%) in thermophilic methanogenic sludge that was treating alcohol-distillery wastewater (No. 24). The distribution patterns of OP8 are consistent with those of a previous study, in which OP8 was detected in high-organic-loading wastewater treatment methanogenic sludge (Sekiguchi et al., 2006). The results of genome analyses of OP8 (OP8_1 group) and OP9 indicate that these microbes may be able to utilize wide ranges of amino acids and sugar (Rinke et al., 2013; Dodsworth et al.,

2013). However, detailed information, such as the roles they play in the environment remains unknown.

Unknown lineage

Results of phylogenetic analysis using Greengenes ver. 13_5 indicated that there were two types of unknown lineages: unclassified phylum1 and unclassified phylum2, identified in thermophilic MS-UASB sludge (No. 24, 7.1%) and mesophilic USB sludge that was treating marine aquarium water (No. 40, 3.9%), respectively (Fig. 3–3B and 3–4). Results of a blast search of the nr database demonstrated that the closest taxonomy of unclassified phylum1 was *Dictyoglomus turgidum* DSM6724 (NR_043385) in phylum *Dictyoglomus* (84%, 215/257 bp). *Dictyoglomus turgidum* is known to be a thermophilic, strictly anaerobic, and chemo-organotrophic organism (Saiki et al., 1985). Results of this study suggested that this unclassified phylum1 might play a role similar to that of the genus *Dictyoglomus*, given that this unknown taxonomy was observed in a thermophilic methanogenic sludge sample (Fig. 3–3A and B).

Unclassified phylum2 was observed in mesophilic USB sludge samples that were treating marine aquarium water (No. 39, 1.6%; No. 40, 3.9%; No. 44, 1.5%,) (Fig. 3–3B and 3–4). This unknown taxonomy was most closely related to *Caldicoprobacter guelmensis* D2C22 (NR_109614) (87%, 220/254 bp), which is a hyperthermophilic, anaerobic, and xylanolytic organism (Yokoyama et al., 2010; Bouanane-Darenfed et al., 2011). This uncultured phylum2 is completely unknown because environments in this study would not support organisms with a certain type of physiology.

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Chapter 4

Community composition of known and uncultured archaeal lineages in anaerobic or anoxic wastewater treatment sludge

Microbial systems are widely used to treat different types of wastewater from domestic, agricultural, and industrial sources. Community composition is an important factor in determining the successful performance of microbial treatment systems; however, a variety of uncultured and unknown lineages exist in sludge that requires identification and characterization. The present study examined the archaeal community composition in methanogenic, denitrifying, and nitrogen-/phosphate-removing wastewater treatment sludge by Archaea-specific 16S rRNA gene sequencing analysis using Illumina sequencing technology. Phylotypes belonging to Euryarchaeota, including methanogens, were most abundant in all samples except for nitrogen-/phosphate-removing wastewater treatment sludge. High levels of Deep Sea Hydrothermal Vent Group 6 (DHVEG-6), WSA2, Terrestrial Miscellaneous Euryarchaeotal Group, and Miscellaneous Crenarchaeotic Group were also detected. Interestingly, DHVEG-6 was dominant in nitrogen-/phosphate-removing wastewater treatment sludge, indicating that unclear lineages of Archaea still exist in the anaerobic wastewater treatment sludges. These results reveal a previously unknown diversity of Archaea in sludge that can potentially be exploited for the development of more efficient wastewater treatment strategies.

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4.1 Introduction

Archaea play a significant role in the Earth's geochemical cycles and are widely distributed in various environments including soil, freshwater, the ocean and deep sea, and hot springs (Liu and Whitman, 2008; Auguet et al., 2010; Offre et al., 2013). *Archaea* are also key players in industry, and methanogenic archaea are especially important for successful anaerobic wastewater treatment processes. Methanogenic archaea produce methane as the end product of anaerobic metabolism in the carbon cycle (Offre et al., 2013), which is not only a major greenhouse gas, but also an important alternative energy source carrier that can be harnessed to meet current global energy demands.

Culture-independent approaches such as 16S rRNA gene sequence analysis have shown that methanogens belonging to classes "*Methanomicrobia*" and *Methanobacteria* are the primary hydrogenotrophic and acetoclastic methanogens found in methanogenic sludges (Sekiguchi et al., 1998; Liu and Whitman, 2008; Narihiro et al., 2009; Tabatabaei et al., 2010; Sundberg et al., 2013). Genera *Methanosarcina* and *Methanosaeta* in the *Methanosarcinales* can use the important substrate acetate, and *Methanosarcina* and other members of this order can use various methylated compounds (Liu and Whitman, 2008). In addition, members of the uncultured group WSA2 (also known as "ArcI" or "WCHA1-57" (Chouari et al., 2005; Pruesse et al., 2007) were detected at relatively high abundance in anaerobic mesophilic sludge digesters (Chouari et al., 2005), indicating that they are also likely to be methanogens (Chouari et al., 2005; Narihiro et al., 2009). Culture-dependent approaches have also enabled isolation of various anaerobic archaea (Liu and Whitman, 2008; Offre et al., 2013), and recent studies described members of the family *Methanoregulaceae* and order *Methanomassiliicoccales*, which are novel methanogenic taxonomies (Sakai et al., 2012; Iino et al., 2013).

In addition to methanogens, several types of halophilic and denitrifying methane oxidizing archaea were detected from anaerobic wastewater treatment processes

(Bandara et al., 2012; Ghanimeh et al., 2013; Shi et al., 2013), suggesting that they played crucial roles in these environments. However, investigations of archaeal communities in anaerobic wastewater treatment processes have been limited to methanogens and denitrifying methane oxidizing archaea to date (Tabatabaei et al., 2010; Shi et al., 2013). 16S rRNA gene sequencing studies have suggested that a variety of unidentified taxa at the phylum or class levels are present in wastewater treatment sludge (Chouari et al., 2010; Ye et al., 2011). To date, most 16S rRNA gene sequencing approaches using next-generation sequencing technology have employed universal primer sets (Caporaso et al., 2012; Sundberg et al., 2013), which has likely led to underestimation of the diversity of *Archaea* owing to their smaller numbers relative to bacterial populations. In addition, studies focusing on *Archaea*-specific populations have been limited in 454-pyrosequencing technology (Huber et al., 2007; Roh et al., 2010).

In this chapter, I selected the *Archaea*-specific primer set consisting of Arch516F and Univ806R for Illumina DNA sequencing analysis to investigate known and uncultured archaeal lineages in anaerobic and anoxic wastewater treatment systems under different conditions (Takai et al., 2001; Caporaso et al., 2012). Additionally, the archaeal 16S rRNA gene sequence was analyzed from 12 sludge samples under methanogenic, denitrifying, or nitrogen-/phosphate-removing conditions, and the phylogenetic diversity of each sample was examined relative to wastewater treatment conditions. *Archaea*-specific microbial community analysis using high-throughput DNA sequencer can provide unknown archaeal ecology in anaerobic wastewater treatment systems.

4.2 Materials and Methods

4.2.1 Sample collection and analysis

A total of 12 sludge samples were collected from 10 wastewater treatment reactors (Table 4–1). The SEU sample was collected from an upflow anaerobic sludge blanket (UASB) reactor treating sewage (324 ± 101 mgCOD/L) at ambient temperature throughout the year (10–29 °C, average 19 °C). Samples RHS (fed with 1,000 mgCOD/L) and RHC (2,000 mgCOD/L) were collected on days 1 and 66, respectively, from a methanogenic, continuous stirred-tank reactor treating rice husk that was maintained at 35 °C and seeded with UASB granular sludge from the treatment of industrial food wastewater. ADU (day 1) and AMU (day 357) samples were taken from a thermophilic multi-stage (MS)-UASB reactor treating molasses wastewater, which was seeded with granular sludge from a thermophilic MS-UASB reactor treating high-strength alcohol distillery wastewater. The influent COD of ADU and AMU were 1,494 ± 317 mgCOD/L and 17,114 ± 1,329 mgCOD/L, respectively. ASU and IRU samples were taken from mesophilic UASB reactors treating molasses (AMU effluent, 8,493 ± 491 mgCOD/L) and high-strength industrial rubber wastewater (13,100 ± 730 mgCOD/L), respectively. Sample MLU was taken from a psychrophilic UASB reactor treating molasses (281 ± 71 mgCOD/L). MAU, MAD, and MAP samples were collected from different upflow sludge blanket (USB) reactors treating marine aquarium water with a NaCl concentration of about 3.0%. Sample SAS was collected from an anaerobic/anoxic sequencing batch reactor (A_2SBR) treating nitrogen and phosphorus from sewage (153 ± 45 mgCOD/L). Sludge samples were gently washed with 1 × phosphate-buffered saline and stored at −20 °C until DNA extraction.

4.2.2 DNA extraction, PCR amplification, and 16S rRNA gene sequencing

DNA extraction was performed using a FastDNA SPIN Kit for Soil (MP

Biomedicals, Eschwege, Germany) according to the manufacturer's protocols. PCR amplification of archaeal 16S rRNA gene was performed with the *Archaea*-specific forward primer Arch516F (5′-TGYCAGCCGCCGCGGTAAHACCVGC-3′) and universal reverse primer Univ806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Takai and Horikoshi, 2000; Caporaso et al., 2012). This primer set covers most of the anaerobic archaea present in anaerobic wastewater treatment sludges (Table 4–2). The Arch516F was chosen based on better coverage of *Archaea* (81.5% as 0 mismatch in probeBase) compared with Univ515F (54.5% archaeal coverage as 0mismatch in probeBase) and similar E.coli position (516–514)/GC contents (64%) with Univ515F (E.coli position, 515–533; GC contents, 68.4%) (Caporaso et al., 2012). The PCR reaction mixture (20 μL) contained 2 μL template DNA (10 ng/μL), 0.5 μM forward and reverse primers, and 10 μL Premix Ex Taq Hot Start Version (Takara Bio Inc., Shiga, Japan). Amplification was performed using a thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: 94 °C for 3 min; 30 cycles of 94 °C (35 cycles for SAS) for 45 s, 50 °C for 60 s, and 72 °C for 90 s; and then final extension at 72 °C for 10 min. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. 16S rRNA gene sequencing was performed as previously described (Caporaso et al., 2012). DNA was sequenced using a MiSeq reagent Kit v2 and the MiSeq system (Illumina Inc., San Diego, CA, USA).

Table 4–1 Summary of sludge samples used in this study.

Sample Name	Reactor Type	Wastewater (Waste) Type	Treatment Type	Volume (L)	Temp. (°C)	ORP§§§ eff.§§§ (mV)	Total COD# inf.## (mgCOD • L^{-1})	COD removal rate (%)	Total Sulfur inf. (mgS • L^{-1})	NO_3^- inf. (mgN • L^{-1})	NH_4^+ inf. (mgP • L^{-1})
SEU	UASB*	Sewage	Methanogenic	1178	10–29	-287±42	324±101	43±22	56±38	0.3±1.3	23±9.0
RHS	CSTR**	Rice husk (Start-up)	Methanogenic	10	35	N.D.###	1000	N.D.	N.D.	N.D.	N.D.
RHC	CSTR	Rice husk (After 2 months)	Methanogenic	10	35	N.D.	2000	13±6	N.D.	N.D.	N.D.
ADU	MS¶-UASB	Molasses (Start-up)	Methanogenic	10	55	-336±25	1,494±317	0.2±34	27±9	0.2±0.2	1.4±1.0
AMU	MS-UASB	Molasses	Methanogenic	11	55	-478±10	17,114±1329	49±5	244±92	0.4±0.9	140±30
ASU	UASB	Molasses (AMU eff.)	Methanogenic	10	35–40	-456±13	8,493±491	52±5	45±20	0.0±0.0	162±31
IRU	UASB	Industrial rubber wastewater	Methanogenic	10	35	-243±50	13,100±730	88±7	N.D.	N.D.	N.D.
MLU	UASB	Molasses	Methanogenic	13.7	15	-247±55	281±71	38±8	50±5	N.D.	N.D.
MAU	USB¶¶	Marine aquarium water	Nitrogen removal	800	26	N.D.	N.D.	N.D.	N.D.	28±13	N.D.
MAD	USB	Marine aquarium water	Nitrogen removal	800	26	N.D.	N.D.	N.D.	N.D.	11±3.0	N.D.
MAP	USB	Marine aquarium water	Nitrogen removal	800	26	N.D.	N.D.	N.D.	N.D.	33±16	N.D.
SAS	A_2SBR¶¶¶	Sewage (DHS§ reactor effluent)	Nitrogen and Phosphorus removal	100	10–29	26±76	153±45	N.D.	46±16	20±7.0	8.4±8.7

*Upflow anaerobic slude blanket
**Continuous stirred tank reactor
¶Multi-staged
¶¶Upflow sludge blanket
¶¶¶Anaerobic/anoxic sequencing batch reactor
§Down-flow hanging sponge
§§Oxidation-reduction potential
§§§effluent
#Chemical oxygen demand
##influent
###Not determined

4.2.3 Data analysis

All data were analyzed using the QIIME software (version 1.8.0) (Caporaso et al., 2010). To maintain the Phred quality score of the reads, sequences of low quality were trimmed using the fastx_trimmer tool (http://hannonlab.cshl.edu/fastx_toolkit/) before being assembled with the paired-end assembler (Masella et al., 2012). Operational taxonomic units (OTUs) were selected at 97% identity according to a closed-reference protocol using UCLUST (Edgar, 2010). Taxonomies were assigned using BLAST based on the SILVA database ver. 111 (Altschul et al., 1990; Pruesse et al., 2007), and predominant OTUs were confirmed to be related species by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011). Observed bacterial OTUs were removed using the QIIME software package. Alpha diversity was determined using observed species, Chao1, and Good's coverage at the sampling depth of each sequencing read. The Shannon and Simpson indices and phylogenetic diversity (PD) were calculated at a sampling depth of

5,000 reads. Principal coordinate analysis (PCoA) was conducted using weighted UniFrac distances at a sampling depth of 5,000 reads (Lozupone and Knight, 2005). A phylogenetic tree of uncultured archaea was constructed using the neighbor-joining and parsimony methods based on 16S rRNA gene sequences (Hugenholtz et al., 2001). Archaeal uncultured OTUs and related reference sequences were aligned using the ARB program (Pruesse et al., 2007). Archaeal uncultured OTUs were extracted based on the results of uncultured groups assigned by SILVA ver. 111 and >1% maximum abundance in each sludge sample, respectively.

4.2.4 Analytical methods

The pH was measured using a portable pH meter (AS-212; Horiba, Kyoto, Japan). COD was determined using a water quality analyzer (DR-2800; Hach, Loveland, CO, USA). Volatile fatty acids concentrations and biogas compositions were determined using a gas chromatograph equipped with a flame ionization detector (GC-1700; Shimadzu, Kyoto, Japan) and a thermal conductivity detector (GC-8A; Shimadzu), respectively. The nitrogen compounds and sulfate were determined by HPLC (LC-10Avp; Shim-pack IC-C1, IC-A1, Shimadzu, Japan).

4.2.5 Accession numbers of nucleotide sequences

The representative 16S rRNA gene sequences were deposited into DDBJ/EMBL/Genebank databases under accession numbers AB968192–AB968211. The raw sequence data were submitted to the DDBJ Sequence Read Archive database (DRA002433).

Table 4–2 Alignment of the Arch516F and Univ806R primer sequences, target sites, and target sequences with reference strains or clones.

Target organisms (Accesion number)	Arch516F 3'- G C B G G T D T T A C C G C G G C G G C T G R C A -5' 5'- T G Y C A G C C G C C G C G G T A A H A C C V G C -3'	Univ806R 3'- A T T A G A W A C C C B D G T A G T C C -5' 5'- G G A C T A C H V G G G T W T C T A A T -3'
Methanosaeta concilii NBRC103675 (AB679168)	. . . . .	. . . . .
Methanosaeta thermophila PT (AB071701)	. . . . .	. . . . .
Methanosarcina acetivorans DSM2834 (M59137)	. . . . .	. . . . .
Methanosarcina barkeri DSM 800T (AJ012094)	. . . . .	. . . . .
Methanosarcina vacuolata DSM1232T (FR733661)	. . . . .	. . . . .
Methanosarcina mazei Go1 (AE008384)	. . . . .	. . . . .
Methanosarcina siciliae DSM3028T (FR733698)	. . . . .	. . . . .
Methanosarcina lacustris (DQ058823)	. . . . .	. . . . .
Methanosarcina baltica AK-4 (AY663809)	. . . . .	. . . . .
Methanohalobium evestigatum Z-7303 (CP002069)	. . . . .	. . . . .
Methanococcoides burtonii DSM6242 (CP000300)	. . . . .	. . . . .
Methanohalophilus mahii DSM5219 (CP001994)	. . . . .	. . . . .
Methanomethylovolans thermophila (AY672821)	. . . . .	. . . . .
Methanomethylovolans hallandica (AF120163)	. . . . .	. . . . .
Methanolobus bombayensis DSM7082T (FR733684)	. . . . .	. . . . .
Methanolobus taylorii (U20154)	. . . . .	. . . . .
Methermicoccus shengliensis ZC-1 (DQ787474)	. . . . .	. . . . .
Methanolinea tarda NOBI-1 (NR_028163)	. . . . .	. . . . .
Methanolinea mesophila (AB447467)	. . . . .	. . . . .
Methanoregula formicicum SMSP (AB479390)	. . . . .	. . . . .
Methanoregula boonei 6A8 (DQ282124)	. . . . .	. . . . .
Methanospirillum hungatei NBRC100397 (NR_112982)	. . . . .	. . . . .
Methanospirillum lacunae (AB517986)	. . . . .	. . . . .
Methanocorpusculum labreanum DSM4855 (AY260436)	. . . . .	. . . . .
Methanocorpusculum sinense DSM4274T (FR749948)	. . . . .	. . . . .
Methanomicrobium mobile DSM1539 (M59142)	. . . . .	. . . . .
Methanoculleus bourgensis (AB065298)	. . . . .	. . . . .
Methanoculleus palmolei (Y16382)	. . . . .	. . . . .
Methanoculleus marisnigri DSM1498 (M59134)	. . . . .	. . . . .
Methanoculleus receptaculi ZC-1 (DQ787474)	. . . . .	. . . . .
Methanosalsum zhilinae DSM4017 (CP002101)	. . . . .	. . . . .
Methanimicrococcus blatticola (AJ238002)	. . . . . T	. . . . .
Methanococcus voltae NBRC100457 (KC139248)	. . . . . A .	. . . . .
Methanococcus vannielii SB (CP000742)	. . . . . A . . . . . A .	. . . . .
Methanococcus aeolicus Nankai-3 (CP000743)	. . . . . A .	. . . . .
Methanothermococcus thermolithotrophicus DSM2095 (M59128)	. . . . . A .	. . . . .
Methanotorris formicus Mc-S-70 (AB095167)	. . . . . A .	. . . . .
Methanocaldococcus vulcanius M7 (CP001787)	. . . . . A .	. . . . .
Archaeoglobus profundus DSM5631 (CP001857)	. . G .	. . . . .
Ferroglobus placidus DSM10642 (CP001899)	. . G .	. . . . .
Geoglobus ahangari 234 (AF220165)	. . G .	. . . . .
Methanobacterium beijingense 8-2 (AY350742)	. . . . .	. . . . .
Methanobacterium lacus 17A1 (HQ110085)	. . . . .	. . . . .
Methanobacterium bryantii MOH (AY196657)	. . . . .	. . . . .
Methanobacterium formicicum (M36508)	. . . . .	. . . . .
Methanobacterium subterraneum C2BIS (X99045)	. . . . .	. . . . .
Methanobacterium palustre (AF093061)	. . . . .	. . . . .
Methanobacterium alcaliphilum (AB496639)	. . . . .	. . . . .
Methanobrevibacter wolinii SH (NR_044790)	. . . . .	. . . . .
Methanobrevibacter millerae ZA-10 (AY196673)	. . . . .	. . . . .
Methanobrevibacter thaueri CW (U55236)	. . . . .	. . . . .
Methanobrevibacter smithii ATCC35061 (CP000678)	. . . . .	. . . . .
Methanobrevibacter woesei GS (U55237)	. . . . . A . . . . . T .	. . . . .
Methanobrevibacter acididurans ATM (AF242652)	. . . . . G	. . . . .
Methanobrevibacter olleyae KM1H5-1P (AY615201)	. . . . .	. . . . .
Methanobrevibacter curvatus (U62533)	. . . . .	. . . . . G . . . . .
Methanothermobacter crinale Tm2 (HQ283273)	. . . . .	. . . . .
Methanothermobacter thermautotrophicus Delta (AE000666)	. . . . .	. . . . . A . . . . .
Methanothermobacter wolfeii (HI592318)	. . . . .	. . . . .
Methanothermobacter marburgensis NR_102881 (NR102881)	. . . . .	. . . . .
Methanothermus fervidus DSM2088 (CP002278)	. . . . .	. . . . .
Methanosphaera stadtmanae DSM3091 (CP000102)	. . . . .	. . . . .
Methanosphaera cuniculi DSM4103T (HE582783)	. . . . .	. . . . .
Methanomassiliicoccus luminyensis B10 (HQ896499)	. . . . .	. . . . .
Candidatus Methanomassiliicoccus intestinalis Issoire-Mx1 (CP005934)	. . . . .	. . . . .
Thermoplasma volcanium GSS1 (NR_074223)	. . . . .	. . . . .
Thermoplasmata archaeon Kjm51a (AB749767)	. . . . . T . .	. . . . .
Ferroplasma acidiphilum (AJ224936)	. . . . .	. . . . .
Haloterrigena longa ABH32 (DQ367242)	. . . . .	. . . . .
Natrinema pallidum JCM8980 (JF421973)	. . . . .	. . . . .
Natrialba magadii ATCC43099 (CP001932)	. . . . .	. . . . .
Natronococcus amylolyticus (D43628)	. . . . .	. . . . .
Halococcus dombrowskii H4 (AJ420376)	. . . . .	. . . . .
Halobacterium sp. NCIMB763 (AB073365)	. . . . .	. . . . .
Haloquadratum walsbyi C23 (HM165235)	. . . . .	. . . . .
Haloamina pelagica TBN21 (GU208826)	. . . . .	. . . . .
Haloferax volcanii DS2 (CP001956)	. . . . .	. . . . .
Haloarcula marismortui ATCC43049 (AY596297)	. . . . .	. . . . .
Halorubrum lacusprofundi ATCC49239 (CP001366)	. . . . .	. . . . .
Thermococcus peptonophilus DSM10343 (AJ298871)	. . G .	. . . . .
Thermococcus barossii DSM9535 (AY099173)	. . G .	. . . . .
Pyrococcus furiosus DSM 3638 (NR_074375)	. . G .	. . . . .
Nanoarchaeum equitans Kin4-M (AJ318041)	. . G . . . . . G . . . . . C .	. . . A . . . . . C . . . . .
Methanopyrus kandleri AV19 (AE009439)	. . . . . G . . .	. . . . .
Candidatus Nitrosocaldus yellowstonii HL72 (EU239960)	. . . . .	. . . . .
Nitrosopumilus maritimus SCM1 (NR_102913)	. . . . .	. . . . .
Sulfolobales archaeon KOZ01 (DQ350777)	. . . . .	. . . . .
Pyrobaculum calidifontis JCM11548 (CP000561)	. . . . .	. . . . .
Thermodiscus maritimus (X99554)	. . . . .	. . . . . N . . . . .
Candidatus Korarchaeum cryptofilum OPF8 (NR_074112)	. . . . .	. . . . .

4.3 Results and Discussion

4.3.1 Overview of 16S rRNA gene sequencing with *Archaea*-specific primer set

A total of 12 anaerobic or anoxic sludge samples were collected from 10 different types of bioreactors. A total of 186,077 sequencing reads were determined, and the median sequence length of the 16S rRNA genes was 245 bp. Approximately 5,500–44,000 sequencing reads per sample were analyzed, and 172–822 OTUs per sample were found at 97% identity (Table 4–3). The number of gene sequences was adequate for analysis of microbial communities in the sludge samples because the coverage was within the range of 0.90–0.98, which is sufficient to estimate biodiversity in bioreactors (Narihiro et al., 2009). However, the Chao1 estimation suggested that the number of OTUs was 2.2- to 8.2-fold greater observed. Specifically, the rarefaction curve revealed undetected OTUs present in each sludge sample, indicating that saturation of observed OTUs was not achieved in this study (Fig. 4–1). Nonetheless, the coverage indicated high values, suggesting that a minor population containing several OTUs belonging to *Archaea* exist in wastewater treatment sludge.

Samples MAU, MAD, and MAP of denitrifying sludge from marine aquarium water treatment had a higher diversity than other sludge samples based on the rarefaction

Table 4–3 Diversity indices of sludge samples used in this study.

Sample Name	*Reactor Type*	*Wastewater (Waste) Type*	*Treatment Type*	*Diversity Indices*¶						
				No. of Sequence	*No. of OTU*	*Chao1*	*Shannon*¶¶	*PD*¶¶, §	*Coverage*¶¶	*Simpson*
SEU	UASB*	Sewage	Methanogenic	9225	285	1049	3.39	10.9	0.98	0.79
RHS	CSTR**	Rice husk (Start-up)	Methanogenic	14379	399	1758	2.93	11.7	0.98	0.68
RHC	CSTR	Rice husk (After 2 months)	Methanogenic	14023	327	1300	2.58	10.2	0.98	0.65
ADU	MS***-UASB	Molasses (Start-up)	Methanogenic	8323	172	1413	0.70	8.9	0.98	0.15
AMU	MS-UASB	Molasses	Methanogenic	24926	332	2565	1.03	7.3	0.99	0.26
ASU	UASB	Molasses (AMU eff.§§)	Methanogenic	43632	822	4166	3.35	12.6	0.99	0.75
IRU	UASB	Industrial rubber wastewater	Methanogenic	14177	260	707	3.01	8.8	0.99	0.80
MLU	UASB	Molassess	Methanogenic	7921	234	520	3.59	9.2	0.98	0.85
MAU	USB#	Marine aquarium water	Nitrogen removal	15112	545	1562	4.00	15.5	0.98	0.85
MAD	USB	Marine aquarium water	Nitrogen removal	18258	551	1660	3.79	14.5	0.98	0.83
MAP	USB	Marine aquarium water	Nitrogen removal	10620	450	1743	3.51	15.7	0.97	0.77
SAS	A_2SBR##	Sewage (DHS### reactor eff.)	Nitrogen and Phosphate removal	5481	173	784	3.94	11.4	0.98	0.88

¶Calculations based on the operational taxonomic units (OTUs) determined at an evolutionary distance of 0.03.
¶¶Caluculation at the sampling depth of 5,000 reads
§Phylogenetic diversity
§§ effluent
*Upflow anaerobic slude blanket
**Continuous stirred tank reactor
***Multi-staged
#Upflow sludge blanket
##Anaerobic/anoxic sequencing batch reactor
###Down-flow hanging sponge

curve and PD (Table 4–3 and Fig. 4–1). One of the possible reasons is that methanogenic archaea were not predominant in denitrifying sludges because of unfavorable condition for methanogens, whereas only a few methanogens were predominant in methanogenic condition. In the other reason, high archaeal diversity has been reported in deep sea water samples with high concentrations of nutrients such as nitrate, phosphate, and silicate, and relatively high salinity and temperature compared with other marine environments (Alonso-Saez et al., 2011). Therefore, the relatively higher diversity in MAU, MAD, and MAP could be due to exposure of these USB reactors to higher levels of salinity (NaCl concentration approximately 3.0%) relative to the other sampled reactors. The thermophilic, methanogenic sludge samples ADU and AMU had the lowest diversity, with Shannon indices of 0.70 and 1.03, respectively, which was less than one-third the value observed for other sludge samples.

The similarities in the phylogenetic diversity of sludge samples were investigated using PCoA plots (Fig. 4–2). The thermophilic sludge samples ADU and AMU were distant from mesophilic or psychrophilic samples because the presence of thermophilic methanogens was limited to thermophilic methanogenic sludges (Liu and Whitman, 2008). Indeed, 97.9% and 95.3% of archaeal phylotypes in ADU and AMU, respectively, belonged to the genus *Methanothermobacter* as found by others (Fig. 4–3) (Luo et al., 2013; Sundberg et al., 2013). The microbial community of marine aquarium denitrification was similar to that of mesophilic or psychrophilic samples owing to the presence of methanogens in MAU, MAD, and MAP (Fig. 4–3). Conversely, SAS of nitrogen/phosphate removal sludge was distantly related to other methanogenic or denitrifying sludge samples, which may have been due to the higher redox conditions in SAS than the other sludges (Table 4–1) and because phosphate removal sludge was limited to SAS. Thus, the microbial community composition in sludge samples from nitrogen- and phosphate-removal systems is likely to be different from that of other samples.

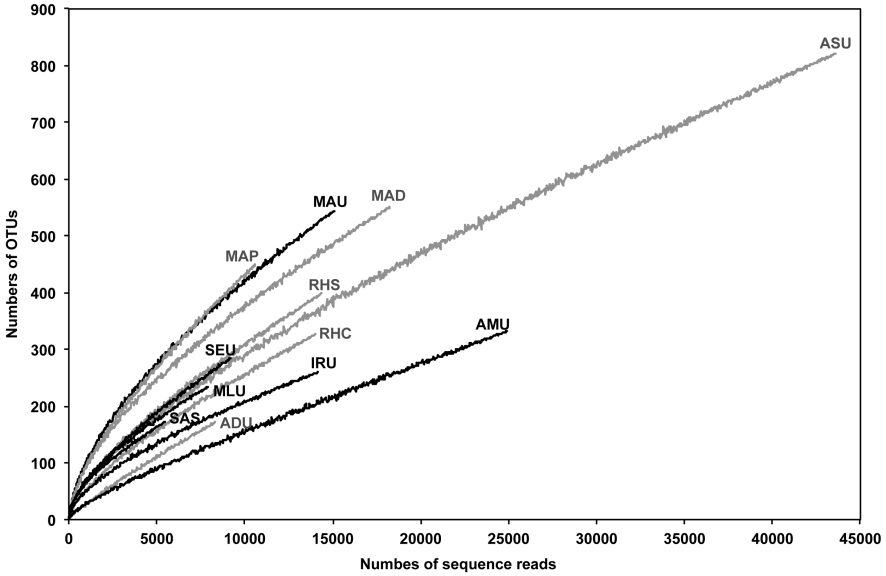

Fig. 4–1 Rarefaction curve of archaeal operational taxonomic units (OTUs) in 12 wastewater treatment sludge samples.

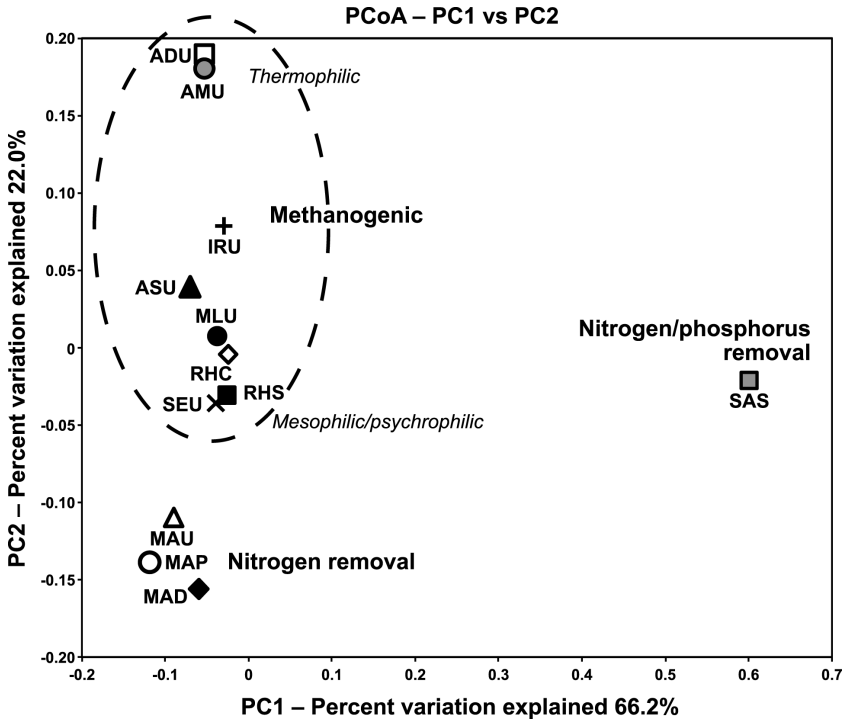

Fig. 4–2 Principal coordinate analysis (PCoA) plots from weighted UniFrac distance for 12 wastewater treatment sludge samples.

4.3.2 Known archaeal populations in anaerobic or anoxic wastewater treatment sludge

The archaeal community composition at the phylum level was similar across sludge samples. *Euryarchaeota*, which comprises all known methanogens, was the most abundant phylum in all samples, with an average detection rate of 98.8% ± 1.4%. The detection rate of the other phyla, *Thaumarchaeota* and *Crenarchaeota*, was 1.0% ± 1.2% and 0.2% ± 0.2%, respectively.

Methanobacteriales

The order *Methanobacteriales* was the predominant phylotype in all samples except for SAS, accounting for 9.4–97.9% of all sequencing reads. In this order, *Methanobacterium* or *Methanothermobacter*, which are known as hydrogenotrophic methanogens, were the predominant phylotypes in all sludge samples except for SAS (Fig. 4–3) (Demirel and Scherer, 2008). The genera *Methanobacterium* or *Methanothermobacter* were typically detected in mesophilic or thermophilic anaerobic digester sludges, respectively (Narihiro et al., 2009; Ritari et al., 2012; Sundberg et al., 2013). Thus, these methanogens play a crucial role in the final degradation of anaerobic digestion via H_2 or formate in methanogenic sludge samples. *Methanobacterium* was detected in salty environments of MAU, MAD, and MAP at 40.3–50.1% (Fig. 4–3). Recently, a methanogen belonging to the genus *Methanobacterium* was isolated from salty environments and shown to produce methane from H_2 (Mori and Harayama, 2011). Although the occurrence of methane production in MAU, MAD, and MAP is unknown, *Methanobacterium* could be grown in marine denitrifying sludges.

Methanosarcinales

The second most abundant order was *Methanosarcinales*, which was present at 0.4–43.6%. *Methanosarcinales* includes acetoclastic methanogens such as *Methanosarcina*

and *Methanosaeta* (Kendall and Boone, 2006), which are frequently present in methanogenic sludges such as those formed during treatment of industrial or sewage wastewater and anaerobic digestion sludge (Kendall and Boone, 2006; Demirel and Scherer, 2008). However, a low level of *Methanosarcinales* was observed in ADU (1.7%) and AMU (0.4%) in the present study (Fig. 4–3). Under thermophilic conditions, acetoclastic methanogens are more susceptible to inhibition by H_2S than hydrogenotrophic methanogens, and the 50% inhibitory concentration of unionized H_2S for the methane production from H_2/CO_2 or acetate for acetoclastic methanogens are one-tenth the values for hydrogenotrophic methanogens (Pender et al., 2004). Molasses wastewater contains a high concentration of sulfate (Table 4–1) (Onodera et al., 2013); therefore, the abundance of *Methanosarcinales* was likely to be small in samples of ADU and AMU. In this study, the *Archaea*-specific primer pair Arch516F-Univ806R was used for amplification of the 16S rRNA gene. Arch516F has been designed for an *Archaea*-specific qPCR method (Takai and Horikoshi, 2000), and has not been used for community analyses such as cloning; therefore, I cannot compare the results of the present study with those of previous studies using the same primer set. However, the Arch516F and Univ806R primers perfectly matched most of the general methanogens in methanogenic sludges, such as genera *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanomassiliicoccus*, *Methanolinea*, and *Methanothermobacter* (Table 4–2). Under inhibitory or specific conditions, low populations of acetoclastic methanogens could occur [37], which may explain my findings; accordingly, additional research should be conducted to investigate the populations in greater detail.

Methanomicrobiales

The third most abundant order was *Methanomicrobiales*, which was present at 0.1–46.8% in each sludge sample. This order consists of hydrogenotrophic methanogens (Liu and Whitman, 2008). MAU, MAD, and MAP samples of treated marine aquarium water

had high levels of *Methanolinea* (35.8 ± 9.8%), which are found in diverse environments such as anaerobic digesters, river sediment, and rice field soil (Imachi et al., 2008; Sakai et al., 2012; Chen and Yin, 2013). In addition, *Methanolinea* were detected in an oilfield under high salinity, pressure, and temperature conditions (Tang et al., 2012; Lenchi et al., 2013). Therefore, *Methanolinea* could exist under salty conditions. Other methanogenic genera, *Methanoregura* and *Methanospirillum*, also frequently exist in biological wastewater treatment systems and various anaerobic environments (Garcia et al., 2006), and these genera were detected at 0.01–4.4% and 0.004–3.6% in all sludge samples except for ADU (Fig. 4–3).

Methanomassiliicoccales

The genus *Methanomassiliicoccus* belonging to the order *Methanomassiliicoccales* was represented in samples MAD (7.4%), MAU (7.3%), RHC (4.3%), RHS (4.2%), and MAP (2.7%) (Fig. 4–3). *Methanomassiliicoccus*, which was recently isolated from human feces, produces methane from H_2 and methanol (Dridi et al., 2012a). In addition, the phylogenetic position of the order *Methanomassiliicoccales* was systematically described in recent reports (Iino et al., 2013). Although *Methanomassiliicoccus* has primarily been studied as part of the human microbiome (Dridi et al., 2012b; Borrel et al., 2013), this methanogen might be important to anaerobic wastewater treatment system owing to its frequencies in anaerobic wastewater treatment sludges (Fig. 4–3).

4.3.3 Uncultured archaeal community compositions in anaerobic or anoxic wastewater treatment sludge

Deep Sea Hydrothermal Vent Group 6 (DHVEG-6)

The predominant uncultured archaeal community in wastewater treatment sludge

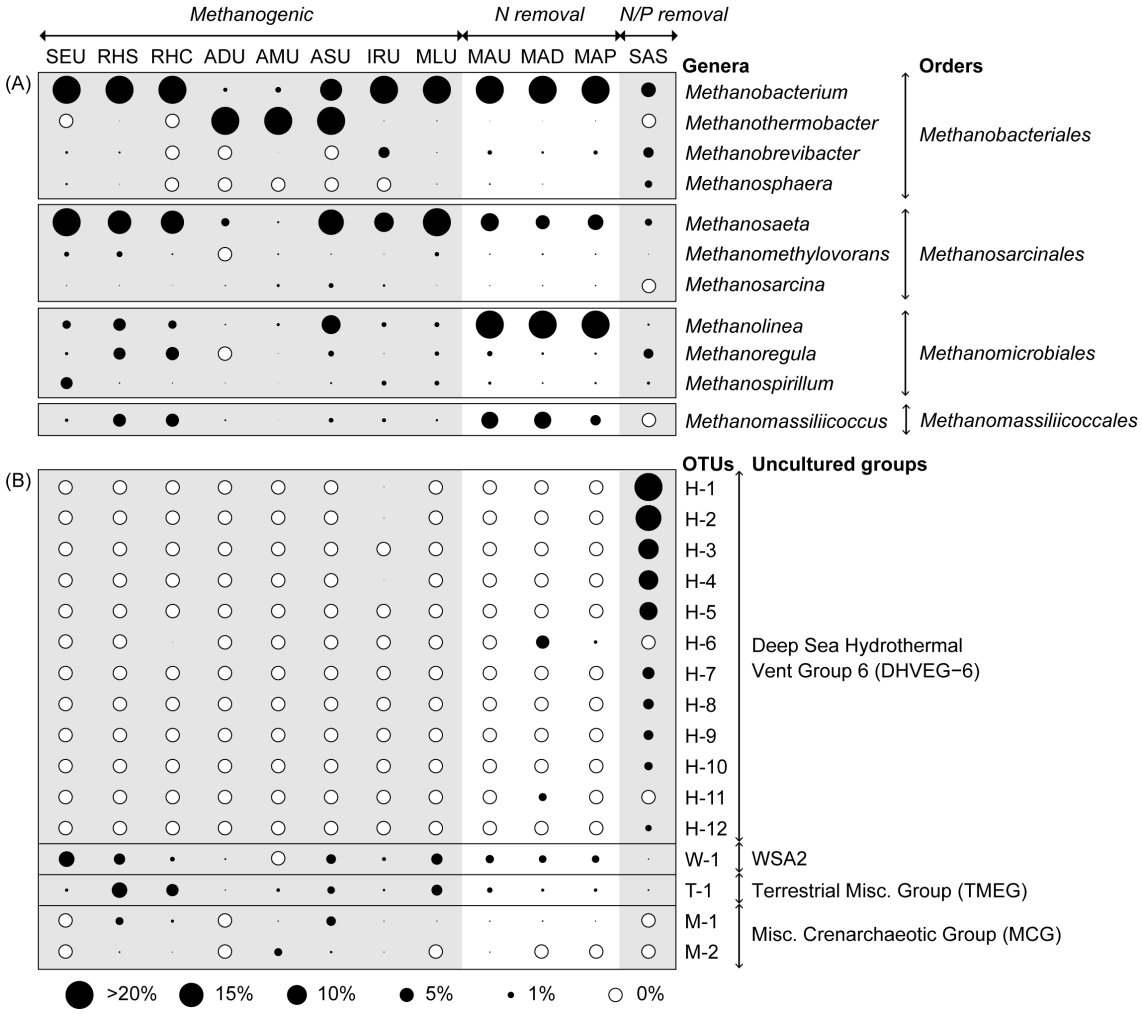

Fig. 4–3 Archaeal community composition of 12 sludges. (A) Known archaeal population at genus level. (B) Uncultured archaeal groups at the operational taxonomic unit level. Circle size shifts correspond to detection rate as shown in the bottom of this figure..

was DHVEG-6, being the most abundant in sample SAS at 85.1%, and also detected in MAD at 7.6% (Fig. 4–3). DHVEG-6 is known as haloarchaea, which is distantly related to *Halobacteriales* (Casamayor et al., 2013). DHVEG-6 has been detected in marine environments, terrestrial soils, and saline lakes such as coastal waters, hydrothermal sediments, deep sea methane seep sediments, rice paddy soil, and shallow saline and hypersaline lakes (Grosskopf et al., 1998; Nunoura et al., 2009; Nunoura et al., 2012; Balcazar et al., 2013; Casamayor et al., 2013; Hugoni et al., 2013). In addition, DHVEG-6 has been observed in municipal wastewater-treating methanogenic bioreactors

(Bandara et al., 2012). Overall, 12 OTUs belonging to DHVEG-6 were observed in SAS and MAD sludge samples (Figs. 4–3 and 4–4). The OTUs fell in different phylogenetic positions of the SAS sample (Fig. 4–4), suggesting that a wide diversity of DHVEG-6 existed in the A_2SBR ecosystem. Although the physiological and metabolic functions of DHVEG-6 are unknown, its distribution suggests that it is more likely to be heterotrophic than inhabitants of known heterotrophic archaea in deep-sea environments (Nunoura et al., 2009). The presence of DHVEG-6 in SAS and MAD sludge, as opposed to methanogenic sludges (Table 4–1), suggested that the relatively high ORP

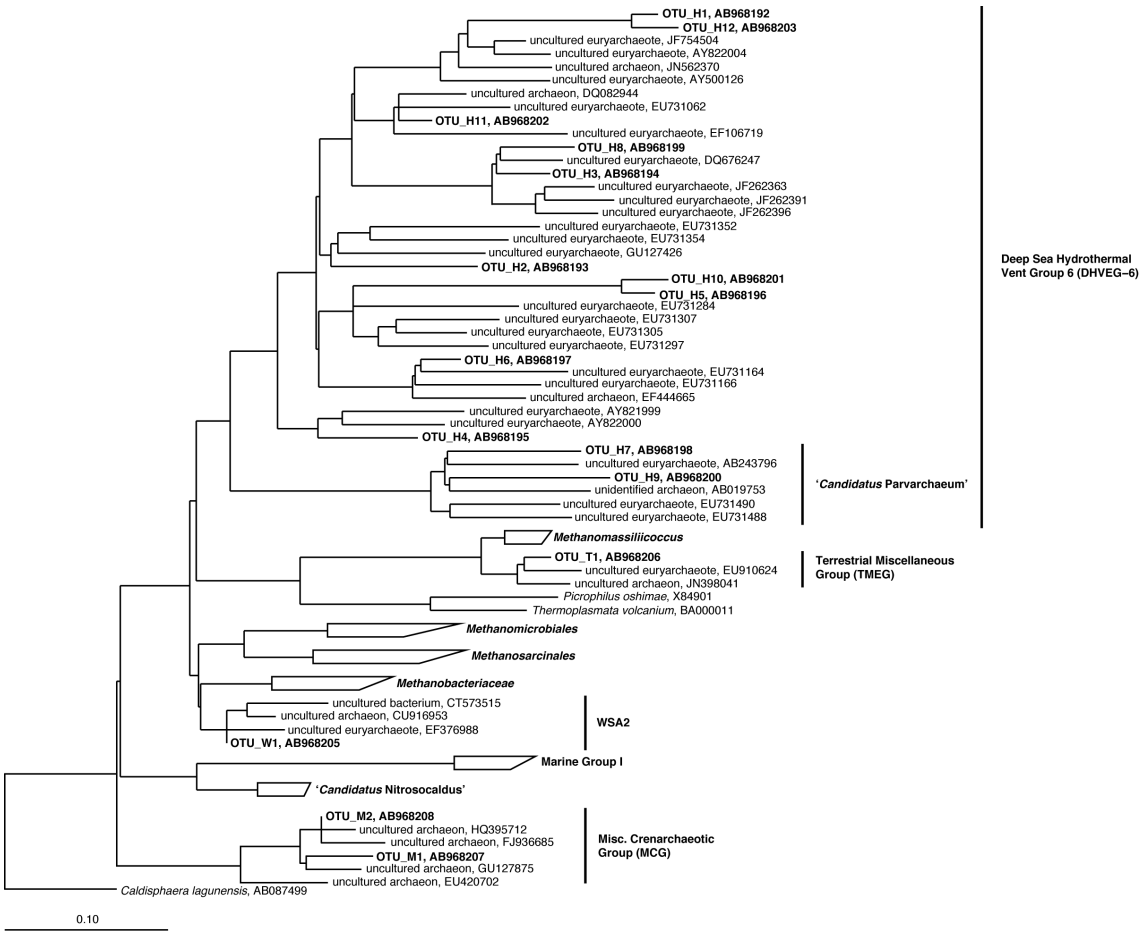

Fig. 4–4 Phylogenetic tree representing predominant, uncultured, archaeal operational taxonomic units (OTUs) in wastewater treatment sludge constructed using the neighbor-joining and parsimony methods based on 16S rRNA gene sequences. OTUs and related reference sequences were aligned with the ARB program. Accession numbers are shown after the name of each OTU, strain, or clone.

might influence the proliferation of DHVEG-6 in the SAS reactor (Nunoura et al., 2009; Nunoura et al., 2012). Interestingly, OTUs H-7 and -9 formed clusters that were distinct from other OTUs belonging to DHVEG-6 (Fig. 4–4). SILVA database assignment and phylogenetic analysis suggested that OTUs H-7 and -9 were related to members of the genus '*Candidatus* Parvarchaeum', which was originally discovered in chemoautotrophic biofilms in acidic (pH < 1.5) metal-rich solutions from Richmond Mine (Iron Mountain, CA), and belongs to the Archaeal Richmond Mine Acidophilic Nanoorganisms (ARMAN)-4 group (Baker et al., 2006). ARMANs have complete or near-complete tricarboxylic acid cycles (Baker et al., 2010). These results suggest that the genus '*Candidatus* Parvarchaeum' was highly abundant in the SAS sample owing to the oxidative conditions (Table 4–1). Conversely, the pH was 7.4 in the SAS environment, suggesting that members of '*Candidatus* Parvarchaeum' can grow in diverse environments. However, a comparison of bacterial and archaeal communities is required to enable a better understanding of the roles of DHVEG-6 in wastewater treatment systems.

WSA2

WSA2 was observed at frequencies of 0.4–6.8% in mesophilic methanogenic sludges and marine denitrifying sludges (Fig. 4–3). The representative OTU in WSA2 was OTU W-1 (Figs. 4–3 and 4–4), for which the most closely related *Archaea* was *Methanothermobacter marburgensis* strain Marburg (NR_102881), with a sequence similarity of 87% (215/247 bp). WSA2 has been detected in mesophilic anaerobic digesters (Chouari et al., 2005). Interestingly, OTU W-1 was observed in marine denitrifying sludges of MAU, MAD, and MAP at 1.4–1.8% (Fig. 4–3); therefore, WSA2 has the potential to acquire niches in salty environments. The presence of WSA2 in mesophilic methanogenic reactors and enrichment of WSA2 in response to formate or H_2/CO_2 suggested that it is a mesophilic hydrogenotrophic methanogen (Chouari et al.,

2005; Narihiro et al., 2009). However, some metabolic functions of WSA2 are still unknown because no isolates of WSA2 have been observed to date.

Terrestrial Miscellaneous Euryarchaeotal Group (TMEG)

The Terrestrial Miscellaneous Euryarchaeotal Group (TMEG) belonging to *Thermoplasmatales* was detected at RHS (6.5%), RHC (4.0%), and MLU (3.2%), respectively (Fig. 4–3). TMEG is present in water from gold mines, marine waters, hypersaline microbial mats, deep-sea sediments, and methanogenic bioreactors used to treat municipal wastewater (Takai et al., 2001; Teske and Sorensen, 2008; Ionescu et al., 2009; Bandara et al., 2012; Balcazar et al., 2013). A previous study reported that TMEG was distributed in aquatic and terrestrial sites similar to MCG (Thauer et al., 2008); however, the metabolic functions of TMEG remain unknown. The predominant OTU in TMEG was OTU T-1 (Fig. 4–3), which was closely related to *Methanomassiliicoccus luminyensis* B10 (HQ896499) at 95% (231/244 bp) (Fig. 4–4). Members of the genus *Methanomassiliicoccus* produce methane from H_2 and methanol (Dridi et al., 2012a; Iino et al., 2013); therefore, OTU T-1 of TMEG likely produces methane from H_2 in methanogenic bioreactor wastewater.

Miscellaneous Crenarchaeotic Group (MCG)

MCG belonging to phylum *Thaumarchaeota* was observed in samples ASU (3.3%), RHS (2.5%), and AMU (1.7%) (Fig. 4–3). MCG has been detected in deep sea sediments containing organic compounds derived from fossilized organic matter (Parkes et al., 2005). The major OTUs in the MCG were M-1 and -2 (Fig. 4–3), which are distantly related, uncultured archaeal species ('*Candidatus* Nitrosocaldus yellowstonii' HL72, EU239960; 86% sequence similarity; 211/244 bp) belonging to the phylum *Thaumarchaeota* (Fig. 4–4). MCG cells have been evaluated by catalyzed reporter deposition-fluorescence *in situ* hybridization, and the distribution of MCG in marine

sediments suggests that these microbes are anaerobic heterotrophs, which do not participate in methane and sulfur cycles, but likely use organic carbon present in wastewater treatment systems (Kubo et al., 2012).

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Chapter 5

High organic loading treatment for industrial molasses wastewater and microbial community shifts corresponding to system development

Molasses wastewater contains high levels of organic compounds, cations, and anions, causing operational problems for anaerobic biological treatment. To establish a high organic loading treatment system for industrial molasses wastewater, this study designed a combined system comprising an acidification tank, a thermophilic multi-stage (MS)-upflow anaerobic sludge blanket (UASB) reactor, mesophilic UASB reactor, and down-flow hanging sponge reactor. The average total chemical oxygen demand (COD) and biochemical oxygen demand removal rates were 85% ± 3% and 95% ± 2%, respectively, at an organic loading rate of 42 kgCODcr·m−3·d−1 in the MS-UASB reactor. By installation of the acidification tank, the MS-UASB reactor achieved low H2-partial pressure. The abundance of syntrophs such as fatty acid-degrading bacteria increased in the MS-UASB and 2nd-UASB reactors. Thus, the acidification tank contributed to maintaining a favorable environment for syntrophic associations. This study provides new information regarding microbial community composition in a molasses wastewater treatment system.

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5.1 Introduction

Molasses-based wastewater discharged from the sugar industry contains high concentrations of organic and inorganic substances such as sugar, cations, and anions (Onodera et al., 2013; Satyawali & Balakrishnan, 2008), which inhibit growth of methanogenesis (Chen et al., 2008). Until now, this wastewater has been dealt by long-term treatment in an anaerobic lagoon system; however, greenhouse gases are emitted from these systems. Therefore, the development of an effective treatment system is required.

Several studies of effective treatment of high organic loading molasses wastewater have been conducted using an upflow anaerobic sludge blanket (UASB) reactor, which can recover energy in the form of methane from wastewater treatment (Kleerebezem & Macarie, 2003). However, during the operation of a UASB reactor treating high organic loading wastewater, a higher biogas flux often causes biomass washout. Previous studies reported that an upflow staged sludge bed (USSB) reactor can successfully treat molasses-based wastewater because the upflow velocity is reduced by the included multiple gas–solid separators (GSS) (Syutsubo et al., 2013). Until now, several types of molasses wastewater such as diluted molasses and diluted desugared molasses have been investigated by treatment in a laboratory-scale UASB reactor, USSB reactor, membrane bioreactor, and baffled reactor (Bilad et al., 2011; Boopathy & Tilche, 1991; Syutsubo et al., 2013). To complement such synthetic wastewater-based studies, this study investigated treatment of true industrial molasses wastewater because the available information is limited (Hilton & Archer, 1988).

Previous studies conducted 16S rRNA gene-based microbial community analysis in molasses-treatment anaerobic reactors by using polymerase chain reaction (PCR) cloning and denaturing gradient gel electrophoresis analyses (Kongjan et al., 2011; McHugh et al., 2003). Those studies reported that H_2-producing bacteria belonging to

Firmicutes and hydrogenotrophic or aceticlastic methanogens predominated in the thermophilic (55°C) UASB reactor used to treat molasses wastewater. However, to improve understanding of the mechanisms of molasses wastewater treatment, more information regarding the microbial community structure of granular sludge is required, as few studies involving the analysis of microbial communities have been performed.

This study focused on industrial molasses wastewater discharged from the sugar-refining process at thermophilic temperatures (approximately 50°C), which is primary treated in the valuable materials recovery step from molasses. In terms of thermal recycling, thermophilic molasses wastewater treatment is advantageous for energy-effective treatment. In addition, most thermophilic wastewater treatment systems have been applied to alcohol distillery wastewater (Harada et al., 1996; Satyawali & Balakrishnan, 2008) and desugared molasses (Kongjan et al., 2013). Therefore, information relevant to treatment of molasses wastewater from sugar refining under thermophilic conditions is important to develop an effective system. Furthermore, few 16S rRNA-based microbial community analyses of molasses treatment granules using high-resolution DNA sequencing have been conducted. Thus, this study designed a combined system comprising an acidification tank, a thermophilic (55°C) multi-stage (MS)-UASB reactor, a mesophilic (35–40°C) 2nd-UASB reactor, and a down-flow hanging sponge (DHS) reactor for molasses wastewater treatment. The 2nd-UASB reactor and the DHS reactor were installed after the MS-UASB reactor to treat the remaining organics, which have been used for post-treatment of molasses-based wastewater in previous studies (Onodera et al., 2013; Syutsubo et al., 2013). In order to examine the performance of this system for treating molasses wastewater, the removal characteristics were evaluated by means of continuous experiments. In addition, to investigate the relationships between microbial community composition and system development, this study performed 16S rRNA gene sequencing analysis from granular sludges in the MS-UASB and 2nd-UASB reactors.

5.2 Materials and Methods

5.2.1 Characteristics of wastewater

The influent wastewaters were composed of molasses and industrial molasses wastewater during Run 1 (start-up) and Run 2 (Phases 1–6), respectively. During the start-up period, molasses diluted with tap water (diluted molasses) was used as the influent wastewater. The molasses contained ($mg \cdot L^{-1}$): total chemical oxygen demand (COD_{cr}), 1,080,000; total biochemical oxygen demand (BOD), 480,000; acetate, 5,500; NH_4^+, 730; K^+, 77,000; Na^+, 1,400; Mg^{2+}, 6,700; Ca^{2+}, 13,000; and SO_4^{2-}, 16,000. The industrial molasses wastewater contained ($mg \cdot L^{-1}$): total COD_{cr}, 99,000; total BOD, 31,000; acetate, 200; NH_4^+, 1,200; K^+, 6,600; Na^+, 320; Mg^{2+}, 540; Ca^{2+}, 950; and SO_4^{2-}, 1,300. The molasses and industrial molasses wastewater were diluted to adjust COD_{cr} concentration by tap water (Table 5–1). The molasses and industrial molasses wastewater were obtained from a sugar factory in Kagoshima prefecture, Japan.

Table 5–1 Operating conditions of the total system used in this study.

Phase	Operation period (Day)	Feed ($g\text{-}COD_{cr} \cdot L^{-1}$)	HRT* (h)				Supplied $NaHCO_3$ ($g\text{-}NaHCO_3 \cdot g\text{-}COD_{cr}^{-1}$)	OLR[§§] ($kgCOD \cdot m^{-3} \cdot d^{-1}$)
			Acid tank**	MS[¶]-UASB[¶¶]	UASB	DHS[§]		
Start up	0–75	1.5–1.8	-	24	26	15	0.3	1.3–1.9
	76–183	1.9–8.5	-	12	13	8.0	1.0	3.7–19
1	184–226	9.0	N.D.[§§§]	13	14	8.2	0.1	17
2	227–249	10	N.D.	12	12	6.9	0.1	17
3	250–289	13	35	12	11	6.6	0.1	21
4	290–333	23	59	12	28	17	0.1	22
5	334–393	30	55	12	26	16	0.1	33
6	394–456	34	65	12	27	16	0.1	42

*Hydraulic retention time

**Acidification tank

[¶]Multi-stage

[¶¶]Upflow anaerobic sludge blanket

[§]Down-flow hanging sponge

[§§]Organic loading rate of MS-UASB reactor

[§§§]No data

5.2.2 System operating conditions

A schematic diagram of the combined treatment system is shown in Fig. 5–1. This combined treatment system consisted of an acidification tank, an MS-UASB reactor, a 2nd-UASB reactor, and a DHS reactor. The combined system was installed in a temperature-controlled room and the temperature was maintained at 35°C. The acidification tank was installed after the influent tank on the 197th day during phase 1 (Fig. 5–1). From the 290th day, the MS-UASB effluent was circulated to the acidification tank. The recirculation ratio was fixed as 1:1 (MS-UASB effluent: influent ratio of acidification tank). The height of the MS-UASB reactor was 1.0 m, and three GSS were equipped at heights of 0.3, 0.6, and 0.8 m, respectively. The temperature of the MS-UASB reactor was maintained at 55°C using a hot-water jacket. The MS-UASB was seeded with thermophilic granular sludge obtained from the treatment of alcohol distillation wastewater. The height of the 2nd-UASB reactor was 1.0 m, and the seed sludge was mesophilic granular sludge obtained from the treatment of industrial food-processing wastewater. The liquid volumes of the acidification tank, the MS-UASB reactor, the 2nd-UASB reactor, and the DHS reactor were 13, 10, 11, and 13 L, respectively. After the acidification of wastewater, pH was adjusted to approximately 6.0 by using 1M NaOH and pH controller (NPH-680D, NISSIN, Japan). The sponge media for the DHS reactor was a polyurethane sponge cube (33 mm) packed inside a cylindrical plastic net ring (33 mm diameter, 33 mm long). The sponge volume of the DHS reactor was assumed to be 6.5 L (sponge media occupancy of 50%). The DHS reactor was supplied with air from bottom of the reactor using an air pump (APN-085V-1, Iwaki, Japan) at 5 $L \cdot min^{-1}$. The DHS reactor was seeded using an activated sludge. Details of the operating conditions are listed in Table 5–1.

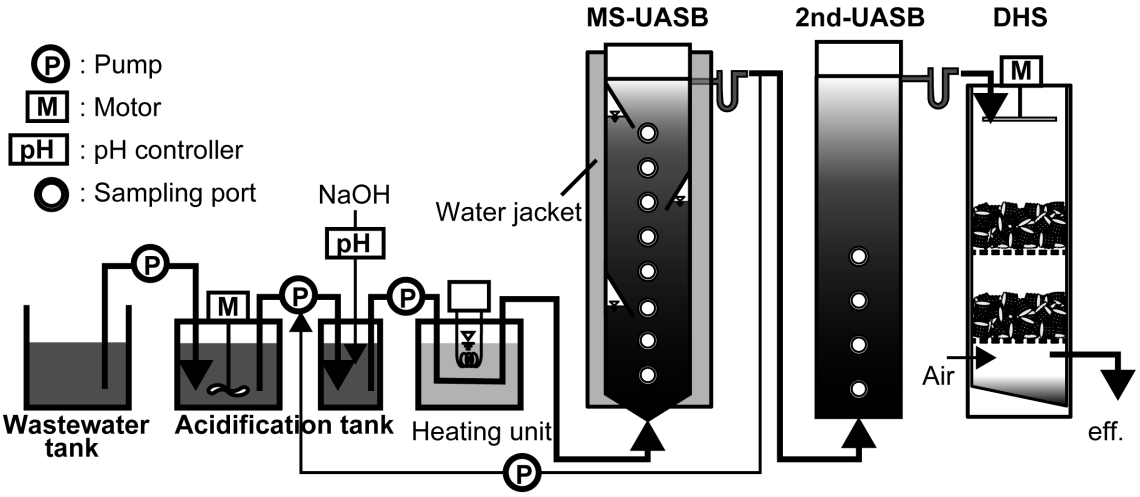

Fig. 5–1 Schematic diagram of the system used in this study

5.2.3 Analytical methods

Water samples were collected from the influent wastewater, the acidification tank effluent, the MS-UASB reactor effluent, the 2nd-UASB reactor effluent, and the DHS reactor effluent for routine analysis. The temperature, pH, oxidation–reduction potential (ORP), and biogas production volume of each reactor were measured on-site. Temperature, pH, and ORP measurements were obtained using a pH/ORP meter (TPX-999Si, TOKO, Japan). The biogas volume was measured using a gas meter (WS-1A, Shinagawa, Japan) equipped on the MS-UASB reactor and the 2nd-UASB reactor. COD_{cr} (COD) and total nitrogen were determined using a HACH water quality analyzer (DR-2500, HACH, US). Volatile fatty acids (VFA) were detected using a flame ionization detector (FID) gas chromatograph (GC-1700, Shimadzu, Japan) fitted with a 30 m × 0.53 mm (ID) glass capillary column (Srabilwax, Bellefonte, USA). The levels of nitrogen compounds, and sulfate, sodium, magnesium, potassium, and calcium ions were determined using high-performance liquid chromatography one or two times a week (LC-10A Tvp, Shim-pack IC-C1, IC-A1, Shimadzu, Japan). Biogas composition was analyzed using a gas chromatograph equipped with a thermal conductivity detector

(GC-8A, Shimadzu, Japan) fitted with a 2 m × 3 mm (ID) stainless steel column with Unibeads-C (60/80 mesh). The suspended solids (SS), volatile suspended solids (VSS), biochemical oxygen demand (BOD), and alkalinity were measured following the procedures of APHA (1998). The COD and BOD were analyzed one or two times a week.

5.2.4 Sample collection and DNA extraction

Sludge samples were obtained from the MS-UASB reactor (at a height of 0.1 m) and the 2nd-UASB reactor (at a height of 0.1 m) on the 179th, 247th, and 357th days. These sludge samples were gently washed and stored at −20°C until DNA was extracted. DNA extraction was performed using a FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, California, USA), following the manufacturer's protocol.

5.2.5 PCR amplification and 16S rRNA gene sequencing

PCR amplification of 16S rRNA genes was performed with the universal forward primer Univ515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the universal reverse primer Univ806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2012). The PCR reaction mixture (20 μL) contained 2.0 μL of template DNA (10 ng·μL^{-1}), 0.5 μM of forward and reverse primers, and 10 μL of Premix Ex Taq Hot Start Version (TaKaRa, Bio, Otsu, Japan). PCR was carried out using a thermal cycler (Veriti200, Applied Biosystems, USA) with the following conditions: initial denaturation at 94°C for 3 min, denaturation at 94°C for 45 s, annealing at 50°C for 60 s, elongation at 72°C for 90 s, and a final extension at 72°C for 10 min. The number of PCR cycles was 25. Purification of PCR products was conducted using a QIAquick PCR purification kit (QIAGEN, CA) following the manufacturer's protocol. The method of Caporaso *et al.* (Caporaso et al., 2012) was used for 16S rRNA gene sequencing. DNA sequencing was performed according to the manufacturer's protocol with a MiSeq reagent Kit v2 (Illumina, USA) of

the MiSeq system.

5.2.6 Data analysis

All raw data analyses were conducted using the QIIME software package, version 1.8.0 (Caporaso et al., 2010). Trimming of low-Phred-quality-score Illumina reads, paired-end assembly, chimera checking, and operational taxonomic unit (OTU) picking at 97% identity were performed according to Kuroda *et al.* (Kuroda et al., 2015). Taxonomies were assigned using the Blast retained on the Greengenes database ver. 13_8 (McDonald et al., 2012), and predominant phylotypes were identified as related species using a web-based Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Principal component analysis (PCA) was performed with STAMP software (Parks & Beiko, 2010). Representative OTUs were selected on the basis of the >2% maximum abundance rate in each granule microbial community.

5.2.7 Deposition of DNA sequence data

The raw 16S rRNA gene sequences in this study have been deposited in the DDBJ Sequence Read Archive database (DRA003505). Representative 16S rRNA gene sequences of OTUs have been deposited in the DDBJ/EMBL/GenBank databases (LC050650–LC050682).

5.3 Results and Desicussion

5.3.1 Performance of the wastewater treatment system

Organic removal

In this chapter, the organic removal characteristics of the proposed combined system were evaluated by increasing the organic loading rate (OLR) of the influent wastewater. During the entire study period, the temperature of the MS-UASB reactor, 2nd-UASB reactor, and DHS reactor were 52 ± 3.7, 37 ± 3.7, and 36 ± 3.9°C, respectively, and the pH values of the MS-UASB, 2nd-UASB, and DHS effluents were 7.3 ± 0.3, 7.7 ± 0.2, and 9.0 ± 0.2, respectively. The ORP values of the influent wastewater, MS-UASB reactor effluent, 2nd-UASB reactor effluent, and DHS reactor effluent were −201 ± 76, −345 ± 61, −250 ± 92, and 8 ± 37 mV, respectively. During the start-up period, the OLR of the MS-UASB reactor was increased to 18.0 kgCOD·m^{-3}·d^{-1}, and the total COD and BOD removal rates for the entire system were 86% ± 6.0% and 97% ± 3.0%, respectively (Fig. 5–2).

During Run 2, the OLR of the MS-UASB reactor increased from 17 ± 0.7 to 43 ± 2.4 kgCOD·m^{-3}·d^{-1} (Fig. 5–2). The organic removal efficiency decreased with an increase in the OLR. On the 179th day (the end of the start-up period), VFAs were produced by port 3 (28 cm) of the MS-UASB reactor, and 97% of the VFAs consisted of *n*-butyrate (1.4 gCOD·L^{-1}), acetate (1.0 gCOD·L^{-1}), and propionate (0.6 gCOD·L^{-1}) (Fig. 5–3). In addition, the pH decreased to 5.3 in the bottom of the MS-UASB reactor with an influent pH of 6.0. The H_2-partial pressure in the MS-UASB reactor was 700 ± 770 Pa during Phase 1 (Fig. 5–4). Thus, this VFA accumulation caused low organic removal efficiency in the MS-UASB reactor. On the other hand, the 2nd-UASB reactor achieved >99% VFA removal. Syntrophic fatty acid degradation requires a low hydrogen partial pressure such as butyrate (<10 Pa) and propionate (< 10^{-10} to 10^{-3} Pa) because these reactions are thermodynamically difficult (Schink & Stams, 2006). Thus, in order to avoid VFA

accumulation in the bottom of the MS-UASB reactor, an acidification tank was installed for the pre-treatment of influent wastewater on the 197th day (Fig. 5–1).

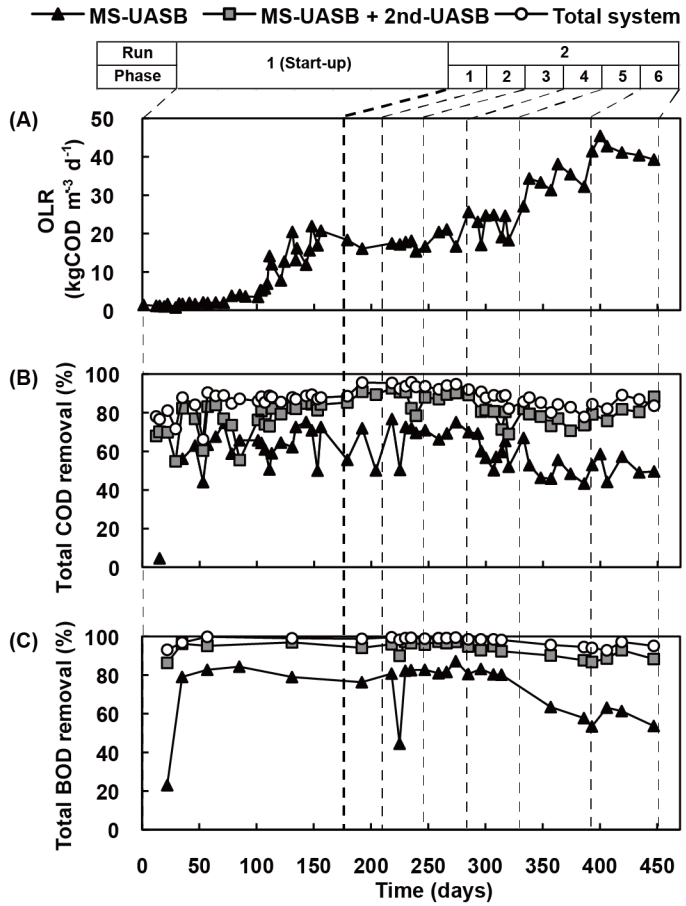

Fig.5–2 Time course of (A) OLR, (B) total COD removal rate and (C) total BOD removal rate in this system

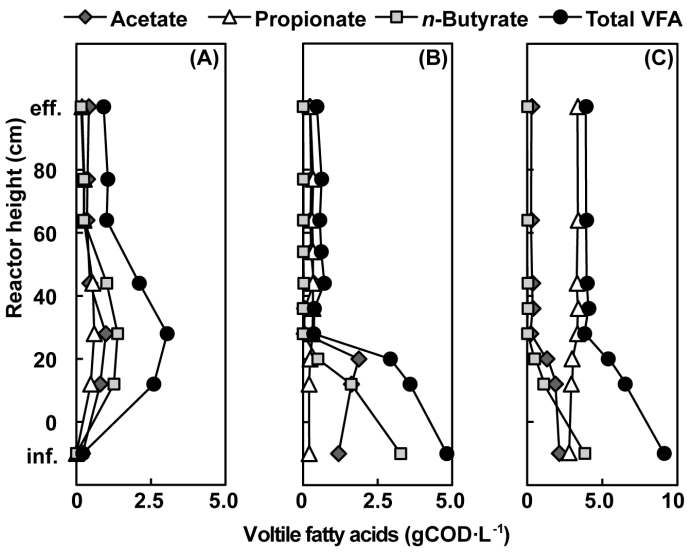

Fig.5–3 MS-UASB profiles of VFAs on days (A) 179, (B) 247, and (C) 357.

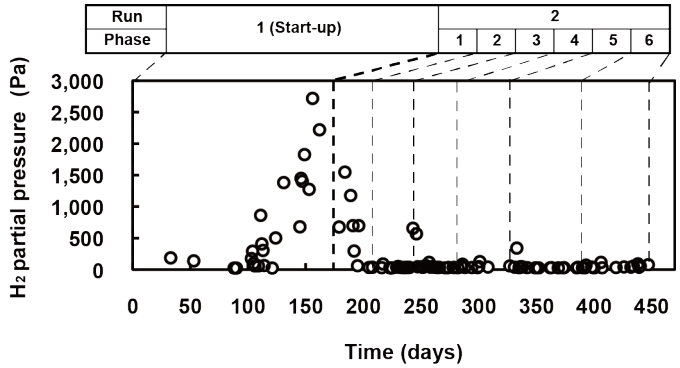

Fig. 5–4 H_2-partial pressure in the MS-UASB reactor during operational periods

Table 5–2 Summary of the COD and BOD concentrations in the system used in this study.

Phase	1		2		3		4		5		6	
Days	184–226		227–249		250–289		290–333		334–393		394–456	
Total COD_{cr} ($mg \cdot L^{-1}$)												
Influent	9100	(200)	10100	(500)	12900	(500)	23000	(1300)	29600	(1300)	34600	(2000)
Acid tank eff.	8700	(600)	8400	(800)	10300	(1200)	10800	(1200)	17100	(1300)	20700	(900)
MS-UASB eff.	3200	(1100)	2400	(200)	3100	(600)	4300	(800)	8500	(500)	10000	(1300)
UASB eff.	800	(100)	1300	(500)	1100	(200)	2400	(400)	3900	(700)	3800	(1100)
DHS eff.	500	(100)	500	(100)	700	(200)	1300	(300)	2700	(500)	3000	(600)
Soluble COD_{cr} ($mg \cdot L^{-1}$)												
Influent	8100	(500)	9300	(800)	11900	(900)	21700	(1000)	27500	(600)	32200	(1500)
Acid tank eff.	8000	(900)	7700	(700)	9900	(1300)	8900	(1600)	13800	(1100)	17100	(1400)
MS-UASB eff.	2200	(1000)	1600	(200)	1600	(500)	2400	(500)	6400	(400)	6400	(500)
UASB eff.	600	(100)	500	(60)	800	(150)	1000	(100)	2000	(100)	2600	(1200)
DHS eff.	400	(80)	400	(40)	600	(110)	1000	(200)	2000	(400)	1500	(200)
Total BOD ($mg \cdot L^{-1}$)												
Influent	4700	(1000)	5100	(500)	6900	(300)	17100	(3000)	27400	(1200)	17800	(3900)
Acid tank eff.	5300	(800)	5000	(500)	6700	(400)	8300	(1800)	10500	(100)	9400	(690)
MS-UASB eff.	1700	(1300)	900	(100)	1200	(300)	1600	(500)	4400	(600)	3800	(230)
UASB eff.	300	(200)	200	(30)	200	(80)	600	(200)	1200	(200)	950	(260)
DHS eff.	60	(40)	40	(10)	70	(30)	100	(40)	550	(90)	470	(240)
Soluble BOD ($mg \cdot L^{-1}$)												
Influent	No Data		5000	(500)	6600	(700)	16500	(2400)	23800	(3000)	16200	(4400)
Acid tank eff.	No Data		4600	(800)	6100	(1100)	8100	(1600)	10000	(600)	8400	(2200)
MS-UASB eff.	No Data		700	(110)	810	(290)	1500	(500)	4200	(900)	3400	(900)
UASB eff.	No Data		120	(40)	200	(70)	340	(170)	690	(90)	450	(230)
DHS eff.	No Data		30	(10)	50	(20)	80	(40)	250	(180)	100	(50)

*effluent

(): standard deviation.

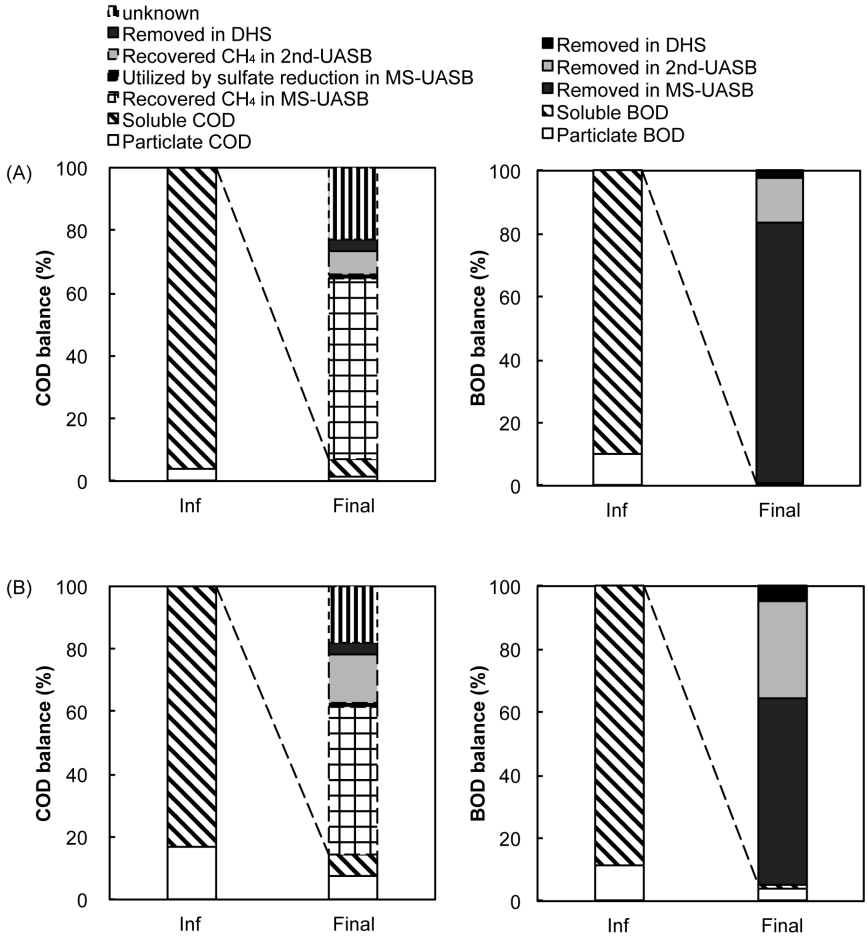

Fig. 5–5 COD and BOD mass balances in the total system during (A) Phase 3 and (B) Phase 6.

On the 247th day, 4.9 gCOD·L^{-1} of VFAs were produced in the acidification tank and 90% of the VFAs were removed in the MS-UASB reactor. However, propionate remained in the MS-UASB effluent. On the other hand, the remaining VFAs, composed of 0.2 gCOD·L^{-1} acetate and 0.2 gCOD·L^{-1} propionate, were completely degraded by port 1 (14 cm) in the 2nd-UASB reactor (Fig. 5–3). The results after acidification tank installation showed that the total COD removal rate of the MS-UASB reactor during the start-up period was 70% ± 3.7%, compared with 62% ± 11% in the VFA accumulation period (146–183 days) (Table 5–2 and Fig. 5–2). The results for the COD mass balance in Phase 3 were as follows: recovered methane in MS-UASB reactor, 44%;

sulfate-reduction in MS-UASB reactor, 3%; recovered methane in 2nd-UASB reactor, 6%; removed COD in DHS reactor, 4%; unknown removed COD, 31%; remaining total COD, 1%; and remaining soluble COD, 11% (Fig. 5–5). The results for the BOD mass balance were as follows: removed BOD in MS-UASB reactor, 82.4%; removed BOD in 2nd-UASB reactor, 14%; removed BOD in DHS reactor, 2.6%; and remaining total and soluble BOD, 0.3% and 0.7%, respectively. Furthermore, VFA accumulation did not occur in the bottom of the MS-UASB reactor (Fig. 5–3), suggesting that the acidification tank was important for stable process performance of high organic loading treatment in the MS-UASB reactor.

On the 290th day (Phase 4), to utilize the alkalinity produced in the MS-UASB reactor, part of the MS-UASB reactor effluent was recirculated to the acidification tank (Fig. 5–1). However, the COD removal rate of the MS-UASB reactor decreased to 52% ± 6.0% (Table 5–2). On the 357th day, 9.1 $gCOD \cdot L^{-1}$ VFAs from the acidification tank were treated by 3.9 $gCOD \cdot L^{-1}$ in the MS-UASB reactor, and most of the remaining VFA was propionate (3.4 $gCOD \cdot L^{-1}$; Fig. 5–3). However, the 2nd-UASB reactor treated 94% of the VFAs from the MS-UASB effluent, indicating that the combined system in this study was capable of maintaining stable organic treatment even though the wastewater treatment efficiency of the MS-UASB reactor decreased. The total COD and BOD removal rates of the entire system were 85% ± 3.2% and 95% ± 2.2%, respectively, during Phase 6 at an OLR of 42 ± 2.4 $kgCOD \cdot m^{-3} \cdot d^{-1}$ for the MS-UASB reactor (Table 5–2 and Fig. 5–5). The COD mass balance during Phase 6 was: recovered methane in MS-UASB reactor, 47%; sulfate-reduction in MS-UASB and 2nd-UASB reactors, 1.1%; recovered methane in the 2nd-UASB reactor, 16%; removed COD in the DHS reactor, 4%; unknown removed COD, 18%; remaining total COD, 7%; and remaining soluble COD, 7% (Fig. 5–5). The BOD mass balance in Phase 6 was: removed BOD in MS-UASB reactor, 60%; removed BOD in 2nd-UASB reactor, 30%; removed BOD in DHS reactor, 5.0%; and remaining total and soluble BOD, 3.9% and 1.1%, respectively.

These results demonstrated that the 2nd-UASB and DHS reactors contributed to removal of the remaining COD and BOD in the MS-UASB effluent after the MS-UASB reactor performance had decreased (Fig. 5–5). Fig. 5–6 shows the linear relationship (R^2 = 0.77) between methane production rate and COD removal rate, indicating that 71% of the removed COD was converted to methane in the MS-UASB reactor.

The maximum organic removal rate during Phase 6 was 27 kgCOD·m^{-3}·d^{-1} for the MS-UASB reactor. Previously reported organic removal rates from treatment of molasses-based wastewater were 5.6 kgCOD·m^{-3}·d^{-1} (mesophilic UASB reactor, OLR 7.1 kgCOD·m^{-3}·d^{-1}), 14 kgCOD·m^{-3}·d^{-1} (mesophilic hybrid anaerobic buffered reactor, 20 kgCOD·m^{-3}·d^{-1}), and 37 kgCOD·m^{-3}·d^{-1} (mesophilic USSB reactor, 43 kgCOD·m^{-3}·d^{-1}) (Boopathy & Tilche, 1991; Gonzalez et al., 1998; Onodera et al., 2012). Therefore, the MS-UASB reactor of this study has an OLR comparable with that of previous studies using mesophilic anaerobic reactors. The MS-UASB reactor of this study showed a lower organic removal rate than that of Onodera *et al.* (2012) but that study used diluted molasses with tap water. As a possibility, the industrial molasses wastewater is thought to be more difficult to biologically treat than the diluted molasses because the industrial wastewater might contain unknown persistent organic materials discharged from the

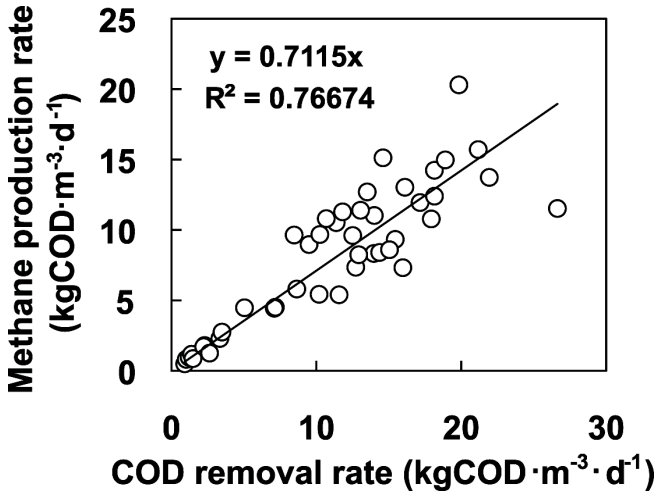

Fig. 5–6 Relationship between methane production rate and COD removal rate in the MS-UASB reactor

sugar-refining process. Thus the BOD: COD ratio of industrial wastewater (32%) is lower than diluted molasses (45%). In addition, propionate accumulation of this study occurred in the MS-UASB reactor when the OLR increased to 42 $kgCOD \cdot m^{-3} \cdot d^{-1}$. Several previous studies have reported such propionate accumulation issues in the thermophilic anaerobic digestion process (Harada et al., 1996; Tagawa et al., 2002). Therefore, the enhancement of propionate degradation might be required to improve the OLR and organic removal rate for this MS-UASB reactor process.

In the entire system, the cation concentration was relatively stable during Phases 1–6 because anaerobic–aerobic biological treatment systems cannot treat cations (Table 5–3). A high sodium concentration was caused by low treatment efficiency due to the element's toxicity for cells and the collapse of granules (Vallero et al., 2003). In addition, with high Ca^{2+} concentration (780–1,560 $mgCa \cdot L^{-1}$), very low methanogenic activity (approximately <0.1 $gCOD \cdot gVSS^{-1} \cdot d^{-1}$) with acetate was demonstrated by calcium precipitation in granules (Van Langerak et al., 2000). Onodera *et al.* (2013) reported that the COD removal rate in a mesophilic (35°C) USSB reactor used to treat diluted molasses decreased from 37 $kgCOD \cdot m^{-3} \cdot d^{-1}$ to 8 $kgCOD \cdot m^{-3} \cdot d^{-1}$ with an increase in the influent cation concentration from approximately 4.0 to 14.3 $g \cdot L^{-1}$ (Onodera et al., 2013). However, a remarkable decrease in organic removal efficiency was not observed because OLR was increased with the monitoring of cation concentration to avoid the inhibition on the anaerobic degradation (Table 5–3) (Onodera et al., 2013). Thus, this study succeeded in treating molasses wastewater at an OLR of 42 $kgCOD \cdot m^{-3} \cdot d^{-1}$ in a MS-UASB reactor with a high cation concentration (>5.0 $g \cdot L^{-1}$).

Table 5–3 Summary of the cations concentration in the system used in this study.

Phase	1		2		3		4		5		6	
Days	184–226		227–249		250–289		290–333		334–393		394–456	
K^+ ($mg{\cdot}L^{-1}$)												
Influent	1051	(391)	679	(23)	996	(132)	2426	(726)	2613	(187)	2860	(174)
Acidification tank eff.*	1121	(431)	584	(16)	1046	(367)	2362	(378)	2593	(167)	2853	(177)
MS-UASB eff.	1046	(305)	637	(14)	900	(63)	2401	(345)	2727	(366)	2941	(133)
2nd-UASB eff.	992	(188)	659	(68)	1041	(313)	2328	(380)	2737	(245)	2969	(209)
DHS eff.	1009	(80)	720	(245)	1023	(335)	2137	(326)	2723	(185)	3161	(222)
Na^+ ($mg{\cdot}L^{-1}$)												
Influent	544	(276)	186	(51)	368	(99)	888	(269)	806	(195)	1301	(253)
Acidification tank eff.	738	(252)	887	(210)	1209	(494)	1333	(280)	1562	(368)	1271	(102)
MS-UASB eff.	812	(79)	808	(264)	1021	(116)	1420	(319)	1739	(379)	1438	(383)
2nd-UASB eff.	746	(102)	721	(261)	1011	(86)	1420	(248)	1734	(345)	1467	(260)
DHS eff.	751	(106)	738	(177)	1078	(147)	1398	(243)	1659	(335)	1547	(189)
Ca^{2+} ($mg{\cdot}L^{-1}$)												
Influent	145	(23)	143	(13)	218	(60)	412	(35)	536	(128)	537	(117)
Acidification tank eff.	126	(24)	94	(13)	178	(37)	219	(35)	338	(41)	509	(105)
MS-UASB eff.	102	(23)	68	(10)	111	(50)	139	(23)	212	(23)	330	(93)
2nd-UASB eff.	111	(11)	83	(21)	124	(58)	135	(20)	175	(19)	302	(84)
DHS eff.	94	(19)	74	(27)	99	(34)	101	(10)	126	(15)	263	(84)
Mg^{2+} ($mg{\cdot}L^{-1}$)												
Influent	77	(27)	48	(5)	82	(16)	328	(119)	324	(123)	447	(119)
Acidification tank eff.	83	(34)	46	(4)	128	(84)	261	(58)	290	(34)	370	(29)
MS-UASB eff.	85	(28)	50	(2)	110	(69)	232	(34)	297	(54)	312	(14)
2nd-UASB eff.	94	(15)	67	(11)	129	(60)	247	(26)	305	(30)	335	(14)
DHS eff.	99	(10)	66	(33)	121	(46)	210	(27)	281	(19)	342	(22)

*effluent

( ) : standard deviation.

Nitrogen and sulfate removal

Table 5–4 lists the nitrogen and sulfate concentrations during Phases 1–6 in each wastewater treatment reactor. During Phases 1–4, most NH_4^+ (50–127 $mgN{\cdot}L^{-1}$) was oxidized to NO_3^- or NO_2^- (74%–90% of removal rate) in the DHS reactor (Table 5–4). On the other hand, 60% and 72% of NH_4^+ were present during Phases 5 and 6,

respectively. In addition, partial nitrification occurred in the DHS reactor because NO_2^- still remained in the DHS effluent, even though the dissolved oxygen concentrations during Phases 5 and 6 were sufficient (4.0 ± 1.4 and 5.2 ± 2.0 $mg \cdot L^{-1}$, respectively). NO_2^--oxidizing bacteria are known to be sensitive to NH_4^+ concentrations compared to ammonia-oxidizing bacteria (Welander et al., 1998). Thus, NO_2^- accumulation might have occurred as a result of the high NH_4^+ concentration in the DHS influent (Table 5–4). Conversely, the total nitrogen concentration in the final effluent was higher than the sum of the NH_4^+, NO_3^-, and NO_2^- concentrations (87–343 $mgN \cdot L^{-1}$) during each phase. Thus, unknown nitrogen compounds that were not NH_4^+, NO_3^-, or NO_2^- were present in the effluent. A previous study reported that molasses wastewater contains high concentrations of melanoidins and alkaline degradation products of caramels, and these compounds contain nitrogen (Arimi et al., 2014). In this study, the molasses wastewater and the effluents from the treatment systems were dark or brown in color (Fig. 5–7). Therefore, in order to increase the nitrogen removal efficiency, decolorization such as ozonation, membrane treatment, and chemical treatment may be required.

Before recirculation of the MS-UASB effluent, SO_4^{2-} was mainly reduced in the MS-UASB reactor during Phases 1 and 2 (Table 5–4). However, after recirculation of the MS-UASB effluent (Phases 4–6), 56%–81% of SO_4^{2-} was removed in the acidification tank. Although it has been reported that thermophilic methanogens are sensitive to inhibition by H_2S (Chen et al., 2008), the MS-UASB performance could maintain stable organic treatment and methane production owing to the contribution of the acidification tank to pre-treatment of molasses wastewater.

Table 5–4 Summary of nitrogen and sulfur components in the system used in this study.

Phase	1		2		3		4		5		6	
Days	184–226		227–249		250–289		290–333		334–393		394–456	
Total nitrogen ($mgN \cdot L^{-1}$)												
Influent	169	(42)	152	(29)	169	(15)	372	(65)	430	(31)	630	(95)
Acidification tank eff.*	157	(42)	138	(21)	143	(17)	291	(34)	396	(25)	598	(109)
MS-UASB eff.	178	(19)	133	(16)	165	(3)	340	(61)	360	(37)	627	(6)
2nd-UASB eff.	143	(18)	149	(30)	127	(23)	289	(70)	411	(38)	508	(68)
DHS eff.	120	(7)	87	(6)	107	(43)	222	(30)	225	(46)	343	(88)
NH_4^+ ($mgN \cdot L^{-1}$)												
Influent	29	(16)	11	(3)	19	(13)	20	(21)	56	(22)	117	(99)
Acidification tank eff.	35	(21)	20	(7)	37	(17)	90	(17)	140	(30)	145	(56)
MS-UASB eff.	46	(11)	32	(7)	48	(13)	98	(13)	162	(31)	193	(28)
2nd-UASB eff.	63	(7)	50	(6)	74	(26)	127	(22)	166	(20)	216	(29)
DHS eff.	6	(7)	3	(3)	4	(3)	8	(10)	23	(14)	54	(20)
NO_2^- ($mgN \cdot L^{-1}$)												
Influent	8	(8)	0	(1)	0	(0)	5	(5)	1	(1)	0	(1)
Acidification tank eff.	1	(1)	0	(0)	0	(0)	1	(2)	0	(0)	0	(0)
MS-UASB eff.	1	(1)	0	(0)	0	(0)	1	(0)	0	(0)	0	(0)
2nd-UASB eff.	0	(0)	0	(0)	1	(3)	1	(1)	1	(1)	0	(0)
DHS eff.	5	(9)	23	(5)	38	(25)	9	(4)	29	(23)	17	(16)
NO_3^- ($mgN \cdot L^{-1}$)												
Influent	5	(8)	0	(0)	0	(0)	3	(3)	0	(0)	0	(0)
Acidification tank eff.	0	(0)	0	(0)	0	(0)	0	(0)	0	(1)	0	(0)
MS-UASB eff.	0	(0)	0	(0)	0	(0)	1	(1)	0	(0)	0	(0)
UASB eff.	0	(0)	0	(0)	0	(0)	2	(2)	0	(0)	0	(0)
DHS eff.	32	(8)	19	(14)	24	(14)	85	(44)	38	(31)	44	(24)
SO_4^{2-} ($mgS \cdot L^{-1}$)												
Influent	142	(10)	136	(3)	200	(30)	507	(86)	553	(91)	558	(94)
Acidification tank eff.	121	(19)	71	(32)	48	(35)	188	(50)	244	(92)	116	(18)
MS-UASB eff.	1	(1)	0	(1)	0	(0)	9	(10)	45	(20)	21	(16)
2nd-UASB eff.	1	(1)	3	(6)	1	(2)	3	(5)	2	(1)	0	(0)
DHS eff.	37	(2)	47	(10)	64	(31)	85	(15)	94	(17)	89	(17)

*effluent

(): standard deviation.

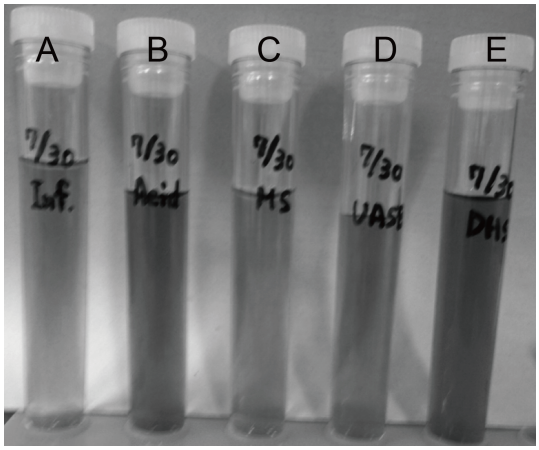

Fig. 5–7 Color of influent or effluent in the total system at 307th day (Phase 4). (A) Molasses wastewater, (B) Acidification tank effluent, (C) MS-UASB effluent, (D) 2nd-UASB effluent and (E) DHS effluent.

5.3.2 Relationships between microbial community composition and wastewater treatment conditions in the MS-UASB and 2nd-UASB reactors

Using 16S rRNA gene sequencing, this study investigated the microbial community compositions in the MS-UASB and 2nd-UASB reactors on the 179th, 247th, and 357th days (Fig. 5–8). In the MS-UASB reactor, the hydrogenotrophic methanogen *Methanothermobacter* (OTUs 3653 and 7739) and the sulfate-reducing bacterium *Thermodesulfovibrio* (OTU 2225) were predominant on all days at 16.8%, 3.1%, and 6.9% (detection rate), respectively. These predominant organisms are consistent with those observed in other thermophilic UASB microbial communities (Kongjan et al., 2013). On the 179th day, H_2-producing bacteria that use poly- or monosaccharides belonging to *Thermoanaerobacterium* (OTUs2470 and 3569), *Caldicellulosiruptor* (OTU3213), and *Coprothermobacter* (OTU1579) predominated in the MS-UASB reactor at 21.5%, 2.9%, 6.4%, and 15.1%, respectively, suggesting that these organisms caused the VFA accumulation and pH decrease in the bottom of the MS-UASB reactor (Fig. 5–3). A recent study has reported that *Coprothermobacter* can syntrophically degrade proteinaceous materials together with *Methanothermobacter thermautotrophicus* (Sasaki

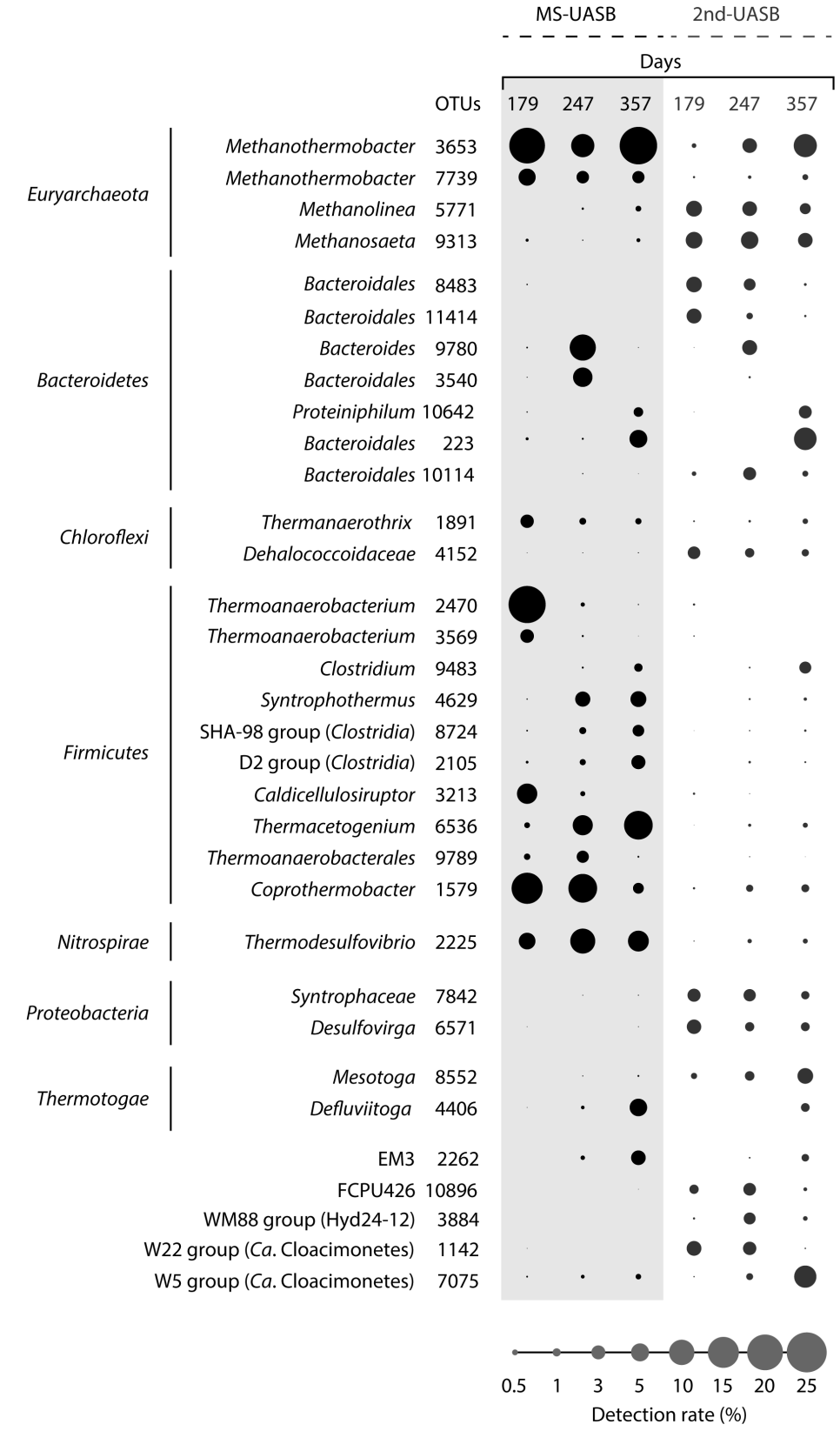

Fig. 5–8 Microbial community compositions of the MS-UASB and 2nd-UASB reactors. *Circle sizes* correspond to abundance rates, as shown at the bottom of the figure.

et al., 2011). Thus, this type of syntrophic association could have been established in the MS-UASB reactor because the genus *Methanothermobacter* predominated. On the 247th day, after setup of the acidification tank, the abundance of different taxa belonging to the phylum *Firmicutes*, such as *Syntrophothermus* (OTU 4629), *Thermacetogenium* (OTU 6536), and *Thermoanaerobacterales* (OTU 9789) of the class *Clostridia*, increased to 3.5%, 6.3%, and 2.3% from <1% on the 179th day (Fig. 5–8). In addition, the abundances of *Thermoacetobacterium* and *Caldicellulosiuptor* decreased to <1%. In the acidification tank, 10.0 $gCOD \cdot L^{-1}$ molasses wastewater was converted to 4.8 $gCOD \cdot L^{-1}$ VFAs at 247th day (Table 5–2 and Fig. 5–3); thus, the MS-UASB environment could be unfavorable for some fermentative hydrogen-producing microbes. Indeed, the H_2-partial pressure in the MS-UASB reactor decreased (Fig. 5–4). On the 357th day, after recirculation of the MS-UASB effluent to the acidification tank, the community of fermentative hydrogen-producing bacteria was reduced compared with the 179th day (Fig. 5–8).

In the 2nd-UASB reactor, *Methanothermobacter* (OTU 5771), *Methanosaeta* (OTU 9313), *Syntrophaceae* (OTU 7842), *Desulfovirga* (OTU 6571), and *Mesotoga* (OTU 8552) were commonly present. These organisms are methanogens, syntrophic fatty acid degraders, sulfate-reducing bacteria, and carbohydrate-degrading bacteria (Boone & Bryant, 1980; Demirel & Scherer, 2008; Mountfort et al., 1984; Tanaka et al., 2000). Therefore, these microorganisms mainly degraded the remaining organics in the MS-UASB effluent. A previous metagenomic and metatranscriptomic study suggested that the genus *Mesotoga* might syntrophically degrade acetate (Nobu et al., 2015). Although the co-existence of *Methanosaeta* and *Mesotoga* as acetate utilizers is unclear, knowledge of microbial ecology is important to elucidate the role of each taxon.

The acidification tank effluent contained a high VFA concentration (Fig. 5–3); therefore, VFA-oxidizing organisms in the MS-UASB reactor are important to maintain the system. To ensure favorable conditions for VFA-oxidizing organisms, a low H_2-partial pressure is required because of thermodynamic difficulties (Schink & Stams,

2006). In general, bacterial VFA oxidation is carried out by H_2-consuming organisms such as hydrogenotrophic methanogens. In this study, VFA accumulation was observed during the start-up period because acid fermentation occurred in the MS-UASB reactor (Fig. 5–3). This result is consisted with the microbial community data discussed above. After the installation of the acidification tank, the H_2-partial pressure decreased (Fig. 5–4) and the levels of syntrophic actetate- or butyrate-oxidizers (*Thermacetogenium* or *Syntrophothermus*) increased (Fig. 5–8). Therefore, these microorganisms mainly utilized acetate and butyrate with *Methanothermobacter* in the MS-UASB reactor. In the 2nd-UASB reactor, conditions of low H_2-partial pressure were maintained, because H_2 gas was not detected over most of the operational period. Therefore, syntrophs such as *Syntrophaceae* were detected on all days.

After recirculation of the MS-UASB effluent, the microbial community composition in the 2nd-UASB reactor became similar to that of the MS-UASB reactor on the PCA plots (Fig. 5–9). One possible reason for this is that washout of granular sludge from the MS-UASB reactor to the 2nd-UASB reactor might have occurred, because the SS concentration was relatively high (1,200 $mg \cdot L^{-1}$) in the MS-UASB effluent during Phase 5 (Table 5–5). The abundances of *Methanothermobacter* (OTU 3653) *Proteiniphilum* (OTU 10642), *Bacteroidales* (OTU 223), *Defluviitoga* (OTU 4406), and candidate division EM3 (OTU 2262) increased to 8.4%, 2.6%, 7.9%, 1.2%, and 0.9%, respectively, with microbial community shifts in the MS-UASB reactor (Fig. 5–8). The roles of OTUs 10642, 223, and 2262 remained unclear because of low 16S rRNA gene sequence similarities with known species (Table 5–9). In the MS-UASB effluent, high propionate concentration (3.3 $gCOD \cdot L^{-1}$) remained; however, the remaining propionate was mostly treated by the 2nd-UASB reactor (Figs. 5–2 and 5–3). This result is consistent with the microbial community compositions of the MS-UASB and 2nd-UASB reactors because no thermophilic propionate-oxidizers, such as the genus *Pelotomaculum*, were observed in the MS-UASB reactor owing to the very strict

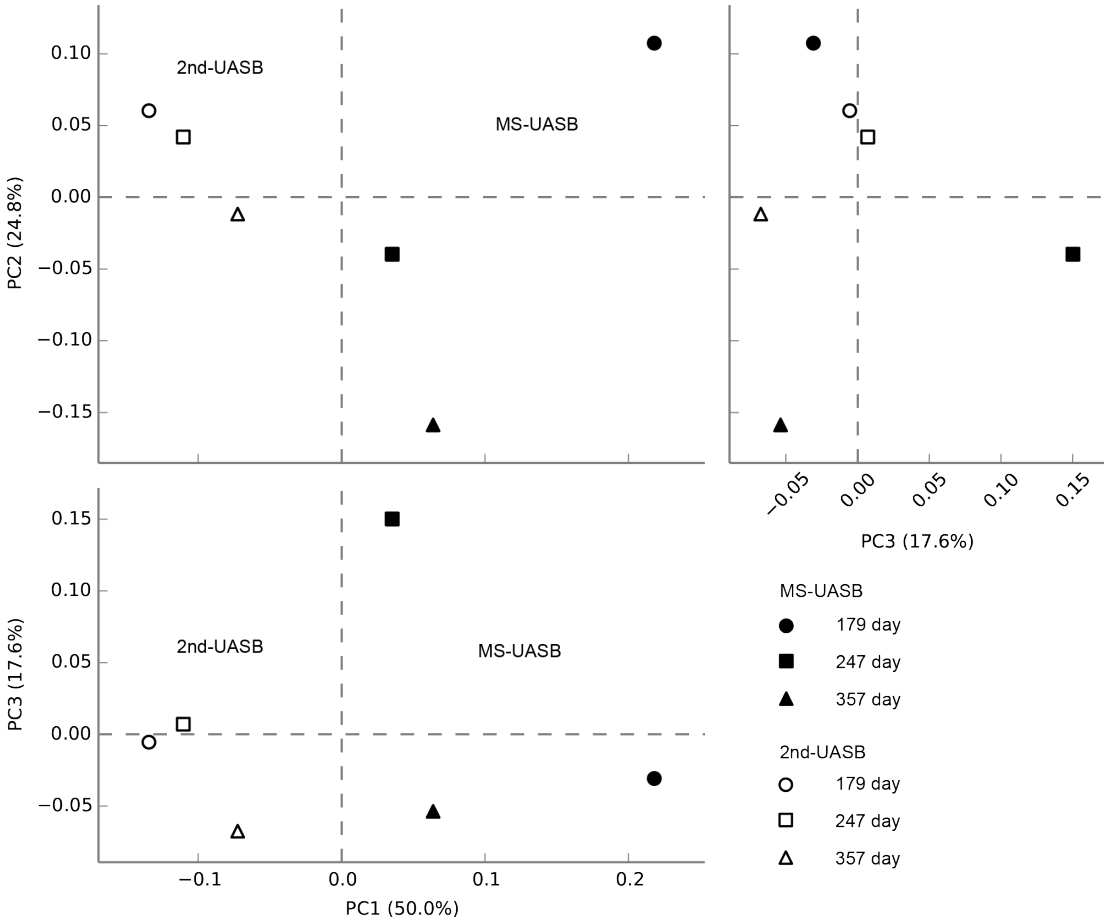

Fig. 5–9 Principal component analysis plots of MS-UASB and 2nd-UASB microbial community at days 179th, 247th and 357th.

requirement for low H_2-partial pressure (Schink & Stams, 2006). In addition, uncultured groups belonging to "*Candidatus* Cloacimonetes" (OTUs 1142 and 7075) predominated in the 2nd-UASB reactor on all days. Previous studies suggested that some taxonomies belonging to "*Ca.* Cloacimonetes" can degrade propionate with H_2-consuming organisms (Nobu et al., 2015); therefore, the propionate in the MS-UASB effluent might have been degraded by the uncultured microbes belonging to "*Ca.* Cloacimonetes" in the 2nd-UASB reactor.

In the *Archaea*, a very low abundance of aceticlastic methanogens (<0.3%) was observed in the MS-UASB reactor on all days (Fig. 5–8); this result is consistent with a

Table 5–5 Summary of the process data in the system used in this study.

Phase	1		2		3		4		5		6	
Days	184-226		227-249		250-289		290-333		334-393		394-456	
pH												
Influent	6.0	(0.8)	4.8	(1.1)	5.6	(0.9)	5.0	(0.9)	5.7	(1.0)	6.2	(0.9)
Acid tank eff. *	5.4	(0.6)	5.8	(0.6)	6.3	(0.8)	6.4	(0.2)	6.3	(0.4)	6.0	(0.3)
MS-UASB eff.	7.4	(0.3)	7.5	(0.2)	7.5	(0.2)	7.5	(0.1)	7.6	(0.1)	7.5	(0.1)
2nd-UASB eff.	7.7	(0.1)	7.7	(0.1)	7.8	(0.1)	7.9	(0.1)	8.0	(0.1)	7.8	(0.1)
DHS eff.	8.9	(0.1)	8.7	(0.1)	9.0	(0.1)	9.1	(0.1)	9.1	(0.2)	9.2	(0.1)
SS (mg/L)												
Influent	-		-		-		-		-		-	
Acid tank eff.	640	(220)	1290	(940)	680	(170)	1420	(600)	1550	(440)	2300	(610)
MS-UASB eff.	450	(120)	970	(350)	1040	(150)	1370	(910)	1200	(280)	2150	(1210)
2nd-UASB eff.	130	(50)	870	(640)	220	(110)	850	(450)	1100	(770)	1280	(550)
DHS eff.	60	(30)	70	(20)	120	(100)	280	(210)	700	(150)	930	(330)
VSS (mg/L)												
Influent	-		-		-		-		-		-	
Acid tank eff.	120	(70)	340	(700)	70	(40)	200	(210)	140	(50)	130	(60)
MS-UASB eff.	70	(10)	340	(200)	150	(70)	240	(310)	110	(40)	200	(230)
2nd-UASB eff.	12	(3)	340	(60)	20	(6)	120	(90)	150	(150)	130	(70)
DHS eff.	7	(3)	13	(12)	9	(2)	30	(30)	80	(60)	200	(100)
Biogas production (NL/d)												
MS-UASB eff.	44.4	(12.2)	54.7	(2.0)	63.9	(6.5)	60.8	(11.6)	67.5	(12.7)	90.5	(13.0)
2nd-UASB eff.	10.6	(6.1)	7.5	(1.5)	8.3	(3.2)	4.6	(1.5)	10.3	(3.4)	13.1	(4.2)
Methane production (NL/d)												
MS-UASB eff.	24.3	(7.2)	39.3	(3.8)	45.6	(6.0)	41.3	(7.5)	46.1	(8.9)	73.2	(13.6)
2nd-UASB eff.	8.4	(4.6)	6.0	(1.3)	6.5	(2.8)	3.3	(1.2)	8.2	(3.6)	8.4	(4.0)

*effluent

( ) : standard deviation.

previous report using an archaeal-specific primer-set (Kuroda et al., 2015). McHugh *et al.* (2003) reported that *Methanosaeta* and *Methanobacterium* predominated at >90% in *Archaea* in thermophilic (55°C) UASB granules used to treat molasses wastewater (influent COD: 10 $gCOD \cdot L^{-1}$; OLR: 48 $kgCOD \cdot m^{-3} \cdot d^{-1}$). A high cation concentration is known to be a causative factor of methanogenic activity inhibition (Chen et al., 2008). Onodera *et al.* (2013) reported that the COD removal rate decreased from 37 $kgCOD \cdot m^{-3} \cdot d^{-1}$ to 8 $kgCOD \cdot m^{-3} \cdot d^{-1}$ while the cation concentration was 14.3 $g \cdot L^{-1}$ (Na^+:

Table 5–6 Taxonomic assignment of representative OTUs of this study.

OTU ID	Greengenes ver. 13_8		Related species	
	Taxon		identities (%)	Taxonomy (Accession No.)
3653	*Euryarchaeota*	*Methanobacteriaceae*	252/252 (100%)	*Methanothermobacter crinale* strain HMD (HQ828065)
7739		*Methanothermobacter*	253/253 (100%)	*Methanothermobacter thermautotrophicus* CaT2 (AP011952)
5771		*Methanomicrobiales*	248/252 (98%)	*Methanolinea tarda* strain NOBI-1 (NR_028163)
9313		*Methanosaeta*	252/252 (100%)	*Methanosaeta concilii* strain X16932 (KM408635)
8483	*Bacteroidtes*	*Bacteroidales*	226/252(90%)	*Labilibacter marina* strain Y11 (KJ093446)
11414		*Bacteroidales*	228/251(91%)	*Mangrovibacterium diazotrophicum* strain SCSIO N0430 (JX983191)
9780		*Bacteroidales*	250/251(99%)	*Bacteroides graminisolvens* strain JCM 15093 (NR_113069)
3540		*Bacteroidales*	235/251(94%)	Candidatus *Bacteroides timonensis* strain AP1 (JX041639)
10642		*Porphyromonadaceae*	251/251(100%)	*Proteiniphilum acetatigenes* (HQ710548)
223		*Bacteroidales*	221/251(88%)	*Microbacter margulisiae* strain ADRI (NR_126216)
10114		*Bacteroidales*	228/251(91%)	*Paludibacter propionicigenes* (AB910740)
1891	*Chloroflexi*	*Anaerolinaceae*	247/251(98%)	*Thermanaerothrix daxensis* strain GNS-1 (NR_117865)
4152		*Dehalococcoidaceae*	226/251(90%)	*Dehalogenimonas alkenigignens* strain IP3-3 (NR_109657)
2470	*Firmicutes*	*Thermoanaerobacterium*	247/251(98%)	*Thermoanaerobacterium thermosaccharolyticum* strain CT6 (JX984971)
3569		*Thermoanaerobacterium*	249/250(99%)	*Thermoanaerobacterium thermosaccharolyticum* strain Y-1 (KM036188)
9483		*Clostridiales*	251/251(100%)	*Clostridium scindens* JCM 10418 (AB971816)
4629		*Syntrophomonadaceae*	251/251(100%)	*Syntrophothermus lipocalidus* strain DSM 12680 (NR_102767)
8724		SHA-98 group	219/249(88%)	*Thermanaeromonas toyohensis* strain ToBE (NR_024777)
2105		D2 group	227/251(90%)	*Caldanaerobacter subterraneus* subsp. yonseiensis (HG970169)
3213		*Caldicellulosiruptor*	241/251(96%)	*Caldicellulosiruptor saccharolyticus* strain DSM 8903 (NR_074845)
6536		*Thermacetogenium*	249/251(99%)	*Thermacetogenium phaeum* strain DSM 12270 (NR_074723)
9789		*Thermoanaerobacterales*	231/251(92%)	*Syntrophaceticus schinkii* strain Sp3 (NR_116297)
1579		*Coprothermobacter*	251/251(100%)	*Coprothermobacter proteolyticus* strain DSM 5265 (NR_074653)
2225	*Nitrospirae*	*Thermodesulfovibrio*	251/251(100%)	*Thermodesulfovibrio aggregans* strain TGE-P1 (NR_040795)
7842	*Proteobacteria*	*Syntrophaceae*	232/251(92%)	*Syntrophus gentianae* strain HQGOe1 (JQ346737)
6571		*Syntrophobacteraceae*	245/251(98%)	*Desulfovirga adipica* strain TsuA1 (NR_036764)
8552	*Thermotogae*	*Thermotogaceae*	251/251(100%)	*Mesotoga infera* strain VNs100 (NR_117646)
4406		S1 group	244/251(97%)	*Defluviitoga tunisiensis* strain SulfLac1 (NR_122085)
2262	EM3	–	206/245(84%)	*Dictyoglomus thermophilum* strain H-6-12 (NR_074876)
10896	FCPU426	–	216/245(88%)	*Clostridium cellulolyticum* strain H10 (NR_102768)
3884	Hyd24-12	Hyd24-12	215/251(86%)	*Caldithrix palaeochoryensis* strain MC (NR_116885)
1142	*Ca.* Cloacimonetes	W22 group	234/251(93%)	*Candidatus* Cloacamonas acidaminovorans str. Evry (NR_102986)
7075		W5 group	224/251(89%)	*Candidatus* Cloacamonas acidaminovorans str. Evry (NR_102986)

approx. 6.8 g·L^{-1}; K^+: 6.0 g·L^{-1}; Ca^{2+}: 1.0 g·L^{-1}; Mg^{2+}: 0.5 g·L^{-1}). In addition, approximately 50% of aceticlastic methanogenic activity was inhibited by K^+ (12 g·L^{-1}) or Na^+ (9 g·L^{-1}). In this study, the cation concentration in the MS-UASB reactor influent was 5.0 g·L^{-1} (Na^+: approx. 2.8 g·L^{-1}; K^+: 1.3 g·L^{-1}; Ca^{2+}: 0.5 g·L^{-1}; Mg^{2+}: 0.4 g·L^{-1}) during Phase 6 (Table 5–3). The concentrations of individual cations in this study are lower than those in previous reports of inhibition (Chen et al., 2008; Kugelman & Chin, 1971; Onodera et al., 2013). In addition, Kugelman and Chin (1971) described antagonism of toxicity by multiple cations with acetate feeding in a laboratory-scale digester, suggesting that cation inhibition of aceticlastic methanogens did not occur significantly in the MS-UASB reactor. Focusing on other factors, the sulfate concentration of the MS-UASB influent might have influenced the archaeal community because 48–244 mgS·L^{-1} sulfate was removed in the MS-UASB reactor (Table 5–4). A previous study reported that the 50% inhibition of unionize H_2S concentration for aceticlastic methanogens is ten times higher than for hydrogenotrophic methanogens under thermophilic conditions (Pender et al., 2004). Furthermore, the VFA concentration may affect the archaeal community because *Methanothermobacter* became the most predominant methanogen (other methanogens were *Methanoculleus* and *Methanosarcina*) in a thermophilic methanogenic reactor when pH decreased from 6.3 to 4.7, and acetate (approx. 3.1 gCOD·L^{-1}) and propionate (> 0.2 gCOD·L^{-1}) accumulation occurred (Hori et al., 2006). Thus, these complicated factors could have caused the low abundance of *Methanosaeta* in the MS-UASB reactor. In addition, correlation of propionate accumulation with shifts in the abundance of *Methanothermobacter* has been observed (Hori et al., 2006). In this study, the abundance of *Methanothermobacter* on the 179th (24.4%) and 357th (24.3%) days were higher than that on the 247th day (10.8%) in the MS-UASB reactor (Fig. 5–8), suggesting that the results of this study are consistent with those of Hori *et al.* (2006) because propionate accumulation was observed on the 179th (0.6 gCOD·L^{-1}) and 357th days (3.4 gCOD·L^{-1}) (Fig. 5–3). Therefore, propionate accumulation could have

influenced thermophilic methanogenic community development.

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Chapter 6

A single-granule-level approach reveals ecological heterogeneity in an upflow anaerobic sludge blanket reactor

In this chapter, to understand the heterogeneity and core composition of the microbial community among individual granules, I collected 300 PTA –degrading granules with small (1–2 mm), medium (2–3 mm), and large (3–4 mm) diameters from a lab-scale UASB reactor (U1) and two full-scale reactors (E and F) at two different bed depths. The microbial community was characterized through MiSeq-based 16S rRNA gene sequencing of DNA extracted from individual granules. The granule microbial communities in U1 are structurally similar based on principal coordinate analysis with weighted UniFrac. In contrast, such analysis on reactors E and F revealed two distinct co-existing granule community structures across all granule sizes. 16S rRNA gene sequence analyses revealed the core members in PTA wastewater treatment such as aromatics-degrading syntrophs; acetate-, methanol-, and H_2- utilizing methanogens; and uncultivated phyla with potentially important functions. Core microorganism and microbial network analyses suggested that syntrophs and methanogens formed substrate-dependent syntrophic partnerships. Besides, distinct OTUs belonging to "Candidatus Aminicenantes" and Methanosaeta were highly correlated in two types of granules. Thus, a single-granule-level approach revealed the veiled ecological heterogeneity in UASB reactors that can potentially be utilized to understand granular microbial communities.

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6.1 Introduction

Upflow anaerobic sludge blanket (UASB) reactor is a promising biotechnology, which can be applied to various wastewaters such as sewage, industrial wastewater, and food-processing wastewater (Kleerebezem and Macarie, 2003). To effectively treat wastewater using a UASB reactor, the formation of anaerobic granular sludge with a healthy microbial community is critical to ensure the biomass settle-ability and the organics degradability. A limited number of important granulation factors have been investigated using morphological, chemical, or physical techniques (e.g. organic loading rate, upflow velocity, reactor hydrodynamics, types and concentrations of metal ions and polymers, as well as nitrogen and phosphorous sources) (Pol et al., 2004; Abbasi and Abbasi, 2012). Various 16S rRNA-based granule microbial community analyses have been conducted using molecular biology techniques such as PCR-cloning and high-resolution DNA sequencing (Sekiguchi et al., 1998; Kuroda et al., 2015; Narihiro et al., 2015b). Several studies that used fluorescence *in situ* hybridization (FISH) with sectioned granular sludge caught a glimpse of predominant patterns of spatial distribution of target microorganisms among all granules (Sekiguchi et al., 1999; Wu et al., 2001; Yamada et al., 2005). However, the microbial and ecological differences between individual granules are important but remain unclear, as previous 16S rRNA-based studies collectively analyze multiple granules and FISH studies only provide information for targeted organisms.

Purified terephthalic acid (PTA) is a key product for manufacture of plastics and polyester. Wastewater from PTA production mainly contains terephthalate (TA), benzoate (BZ), methanol (MT), and acetate (AC) and is commonly treated by anaerobic bioreactors such as an UASB reactor. In this study, I focused on mesophilic UASB reactors treating PTA wastewater to understand the granule heterogeneity and core microorganisms. *Syntrophorhabdus* and *Pelotomaculam* are known as TA-degrading

syntrophs that predominated in TA-degrading hypermesophilic (46–50°C) UASB reactors (Qiu et al., 2006; Wu et al., 2013; Nobu et al., 2014). Genomic and metabolic analyses have demonstrated multiple syntrophic interactions responsible for the degradation of intermediates including butyrate and acetate, which were produced in TA metabolism (Lykidis et al., 2011; Nobu et al., 2015). In addition to the major players of TA-degradation (>90% as carbon), our recent study revealed that such microbial community also harbored scavengers of biomass detritus (up to 10% as carbon), such as putative acetogens and degraders of glycerol, amino acids, branched chain fatty acids, and propionate (Nobu et al., 2015). The ecological heterogeneity of granules is likely to contribute to the exceptional complexity of microbial community in TA-degrading reactors. Thus the information relevant to single-granule microbial micro-community is necessary to further understand TA degradation mechanisms and improve process stability, especially because few studies on mesophilic microbial community treating industrial PTA wastewater have been reported (Wu et al., 2001; Perkins et al., 2011; Kim et al., 2012).

In this chapter, in order to resolve the ecological heterogeneity in UASB reactors, I collected 300 individual granules with small (GSA: 1-2 mm), medium (GSB: 2-3 mm), and large (GSC: 3-4 mm) diameters from a lab-scale UASB reactor (U1) and two full-scale UASB reactors with identical configurations (termed Reactors E and F) at two different depths. The microbial community was characterized through MiSeq-based 16S rRNA gene sequencing of DNA extracted from discrete granules.

6.2 Materials and Methods

6.2.1 Characteristics of PTA wastewaters, reactor operations, and reactor performances

Reactors E and F received PTA wastewater with the following concentrations: TA, 4.5 mM; BZ, 6.0 mM; isophthalic acid (IA), 2.1 mM; orthophthalic acid (OA), 1.0 mM; PT, 0.6 mM; TMA, 1.0 mM; MT, 24.3 mM; AC, 35.7 mM; and methyl acetate (MA), 1.2 mM. The synthetic wastewater of lab-scale reactor U1 contained: TA, 3.6 mM; BZ, 2.5 mM; IA, 0.6 mM; OA, 0.3 mM; PT, 4.4 mM; TMA, 0.3 mM; MT, 4.8 mM; AC, 23.0 mM; and MA, 1.3 mM. The total organic carbon (TOC) concentrations of industrial and synthetic wastewaters were 2,752 and 1,700 mgTOC·L^{-1}, respectively. Total suspended solids (TSS) concentration of full-scale, industrial PTA wastewater was 403 mgSS·L^{-1}.

Temperatures of two full-scale reactors and a lab-scale reactor were maintained at 34°C and 38°C, respectively. The liquid volumes of the full-scale reactors and a lab scale reactor are 4,562 m^3 and 12 L, respectively. Reactors E and F have been operated for eight years and exhibited 95% TOC removal efficiency. The full-scale reactors were seeded with mesophilic sludges. Reactor U1 treated synthetic PTA wastewater for 11 months, and TOC removal rate was approximately 94%. U1 was seeded with mesophilic sludge from a UASB treating PTA wastewater. The effluents of two full-scale reactors and a lab-scale reactor contain 390 and 86 mgSS·L^{-1}, respectively.

6.2.2 Analytical methods

The following parameters were measured daily on influent and effluent of both full-scale reactors: TOC was analyzed using a TOC analyzer (TOC-L CPN Basic System, Shimadzu, Japan). TSS was measured following the procedure of APHA (1998). For U1: Methanol and methyl acetate were detected using gas chromatography with FID on a hp 5890 with a RTX-1 nonpolar column. AC is detected on a hp 5890 Series II with a

DB-Wax polar column; and TA, BZ, IA, OA, PT, and TMA were detected using an Agilent 1200 HPLC System with multiple wavelength detector, or equivalent; the HPLC column was an Agilent SB-C18, 4.6 mm i.d. x 50 mm, 1.8 μm particle diameter (p/n 822975-902). For reactors E and F: TA, BZ, IA, OA, PT, and TMA were aromatic compounds, fatty acids and methyl compounds were detected using a high-performance liquid chromatography (Agilent ZORBAX Eclipse XDB-C18, Rapid Resolution HT 4.6 mm i.d. x50 mm, 1.8 μm particle diameter, operated at 600 Bar).

6.2.3 Sample collection and DNA extraction

This study collected 300 granules with diameters from small (GSA: 1–2 mm), medium (GSB: 2–3 mm), to large (GSC: 3–4 mm) individually at two different depths (height: 1 m and 6 m) from full-scale reactors E and F and from lab-scale reactor U1. These granules were stored at -80°C until extraction of DNA. DNA was extracted from individual 300 granules by using FastDNA Spin Kit for Soil (MP Biomedicals, Carlsbad, CA, USA), according to the manufacturer's protocol.

6.2.4 PCR amplification and 16S rRNA gene sequencing

16S rRNA gene amplification was performed with the universal forward primer (Univ515F: 5'-GTGCCAGCMGCCGCGGTAA-3') and the universal reverse primer (Univ909R: 5'-CCCCGYCAATTCMTTTRAGT-3') (Tamaki et al., 2011; Kozich et al., 2013). The PCR reaction (25 μL) containing 30 ng template DNA, 0.5 μM of forward and reverse primers, and 12.5 μL of Taq DNA polymerase 2.0 mix (Bulls eye, St Louis, MO, USA) was carried out using a thermal cycler (T100™, BIO-RAD, USA) with the following conditions: initial denaturation at 95°C for 3min, denaturation at 94°C for 45 s, annealing at 55°C for 60 s, elongation at 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR cycle numbers were 25 cycles. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA) according to

manufacturer's protocol. The 16S rRNA gene sequencing was conducted using the MiSeq Reagent kit v3 and MiSeq system (illumina, San Diego, CA, USA) at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

6.2.5 Data analysis

Raw 16S rRNA gene sequences were analyzed using QIIME ver. 1.8.0 (Caporaso et al., 2010b). The Phred quality score under 30 was trimmed using a fastx_trimmer tool (http://hannonlab.cshl.edu/fastx_toolkit/) before assembling with the paired-end assembler (Masella et al., 2012). OTUs were selected with ≥97% sequence identity cut-off using the UCLUST (Edgar, 2010). Representative sequences of picked OTUs were aligned by PyNAST (Caporaso et al., 2010a). Chimeric sequences were identified from the alignments by ChimeraSlayer (Haas et al., 2011). Taxonomy was assigned using blast retained on the Greengenes database ver. 13_8 (McDonald et al., 2012). Taxonomic placements of predominant OTUs were confirmed using the web-based Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ARB program package based on Greengenes 16S rRNA gene database (Ludwig et al., 2004; McDonald et al., 2012). The phylogenetic tree of 16S rRNA gene sequences was constructed based on neighbor-joining and parsimony methods in ARB using Greengenes 16S rRNA gene database (Ludwig et al., 2004). The topology of constructed tree was confirmed by 1,000 bootstrap replicates (Felsenstein, 1985). I defined the OTU frequency on basis of its abundance and occurrence in individual granules. If one OTU occurred in ≥70% of sampled granules with >0.5% abundance, regardless of granule sources, I chose it as a predominant OTU.

6.2.6 Statistical analysis

Alpha diversity indices (observed OTUs, Chao1, singles, doubles, phylogenetic diversity, and Good's coverage) and the weighted UniFrac distances were calculated by

QIIME. Chao1, singles, doubles, and phylogenetic diversity were calculated at a sampling depth of 18,000 reads. The weighted UniFrac distances were used for PCoA and jackknife-resampling methods (even sampling at 18,000 reads). Significant differences of alpha diversity indices were calculated using Welch's t-test. The statistical analysis of metagenomic profiles software package was used to determine statistical differences of OTUs abundance (Parks and Beiko, 2010). To confirm the possible OTU interactions, I calculated Spearman's rank correlation coefficients based on predominant and high frequent OTUs in reactor E (Hammer et al., 2001). For reduction of complexity, I chose the thresholds with Spearman's correlation *rs* >0.4 and statistically significant p-value <0.001, respectively. The nodes and edges were used by representative taxa of each OTU and Spearman's correlation, respectively. The node size shifts correspond to average OTU abundance. The OTU networks were visualized by using Cytoscape (Shannon et al., 2003).

6.3 Results and Discussion

This study obtained 32 million 16S rRNA gene reads from a total of 300 individual granules, and the median sequence length of assembled 16S rRNA gene was 374 bp. I analyzed 18,900–158,000 sequences per sample, and 420–1,452 operational taxonomic units (OTUs) per sample were observed based on 97% cut-off of 16S rRNA gene sequence similarity (Table 6–1). Based on Chao1 index, numbers of estimated OTU numbers (625–2,933 per sample) were 1.4–2.2-fold greater than those of observed. The data set in this study can represent the most majority of microbial community in individual granules because Good's coverage value was ≥0.99 under all conditions (Table 6–1).

Table 6–1 Alpha diversity indices in PTA wastewater treatment UASB granules.

Reactor Name	granule	Granular size	No. of granules	Diversity Indices[¶]						
				No. of Seq	No. of OTUs	Chao1[¶¶]	Singles[¶¶]	Doubles[¶¶]	PD[§¶¶]	Coverage
E (Full-scale)	E*a*	GSA[§§]	13	89020	400 ± 38	709 ± 117	172 ± 32	48 ± 6	75 ± 4	1.00
		GSB*	10	87337	452 ± 39	865 ± 113	214 ± 29	56 ± 7	80 ± 5	0.99
		GSC**	4	81910	460 ± 35	855 ± 90	215 ± 20	58 ± 3	81 ± 4	0.99
	E*b*	GSA	27	90118	457 ± 48	821 ± 131	199 ± 36	55 ± 7	80 ± 5	1.00
		GSB	30	86879	495 ± 41	956 ± 132	233 ± 32	59 ± 6	82 ± 4	0.99
		GSC	36	80146	499 ± 46	954 ± 137	235 ± 33	61 ± 6	83 ± 5	0.99
F (Full-scale)	F*c*	GSA	20	72992	440 ± 49	744 ± 130	180 ± 34	54 ± 7	82 ± 5	0.99
		GSB	12	53555	453 ± 58	820 ± 129	202 ± 37	55 ± 10	80 ± 6	0.99
		GSC	13	35635	541 ± 56	1016 ± 120	256 ± 32	69 ± 9	88 ± 6	0.99
	F*d*	GSA	19	78661	451 ± 38	770 ± 118	183 ± 32	53 ± 6	82 ± 4	0.99
		GSB	27	55654	497 ± 41	895 ± 144	217 ± 38	60 ± 7	86 ± 4	0.99
		GSC	25	36616	510 ± 48	922 ± 151	225 ± 38	62 ± 7	87 ± 4	0.99
U1 (Lab-scale)	–	GSA	20	44600	559 ± 53	1027 ± 181	248 ± 44	67 ± 7	94 ± 6	0.99
		GSB	20	42544	600 ± 56	1137 ± 177	282 ± 43	75 ± 7	97 ± 5	0.99
		GSC	20	42819	709 ± 71	1481 ± 224	371 ± 56	89 ± 10	105 ± 6	0.99

[¶]Calculations based on the operational taxonomic units (OTUs) determined at an evolutionary distance of 0.03

[¶¶]Calculation at a sampling depth of 18,000 reads

[§]Phylogenetic diversity

[§§]Range of Granular sludge diameter is 1.0–2.0 mm

*Range of Granular sludge diameter is 2.0–3.0 mm

**Range of Granular sludge diameter is 3.0–4.0 mm

6.3.1 Granule microbial community similarity in UASB reactor

Although a previous study investigated the bulk granule microbial communities (Narihiro et al., 2009), the possibility of the co-existence of multiple granule types has yet to be considered and remains unclear. To investigate the ecological heterogeneity in UASB reactors, I analyzed the phylogenetic similarity using Jackknife-supported principal coordinate analysis (PCoA) with weighted UniFrac (Fig. 6–1 and Fig. 6–2A, B, and C). When I compare all granules, the granule microbial communities in full-scale reactors E and F are distinct from those in lab-scale reactor U1 (Fig. 6–1A). In the full-scale reactor E, the PCoA plots reveals two distinct and co-existing granule types (Reactor E: granule E*a* and E*b*) (Fig. 6–1B), while the lab-scale reactor U1 shows no significant patterns (Fig. 6–1D). Besides, reactor F possesses two clusters of granule microbial communities (Fig. 6–1C). This observation is confirmed by UPGMA algorism with jackknife supporting value that the separation of two clusters in reactor E was more valid (>75%) than reactor F (<25%) (Fig. 6–2A and B), even though operational conditions of these reactors are almost same (e.g. reactor type, influent wastewater, and organic removal efficiency). Notably, the phylogenetic differentiation of granules is independent from granule sizes because different sized granules in each granule type have similar microbial community compositions based on OTU abundance scatter diagrams (R^2>0.85, Table 6–2), suggesting that there are other factors responsible for distinctive granule community and potentially function.

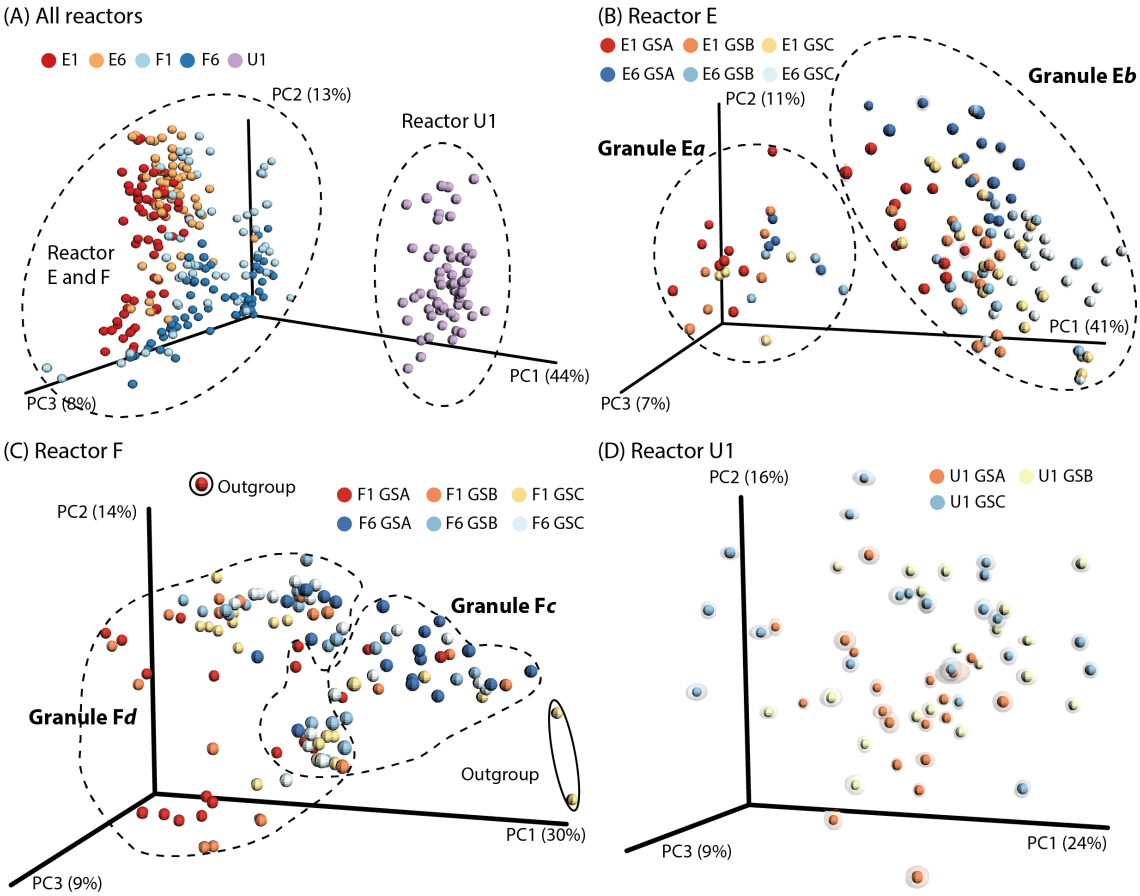
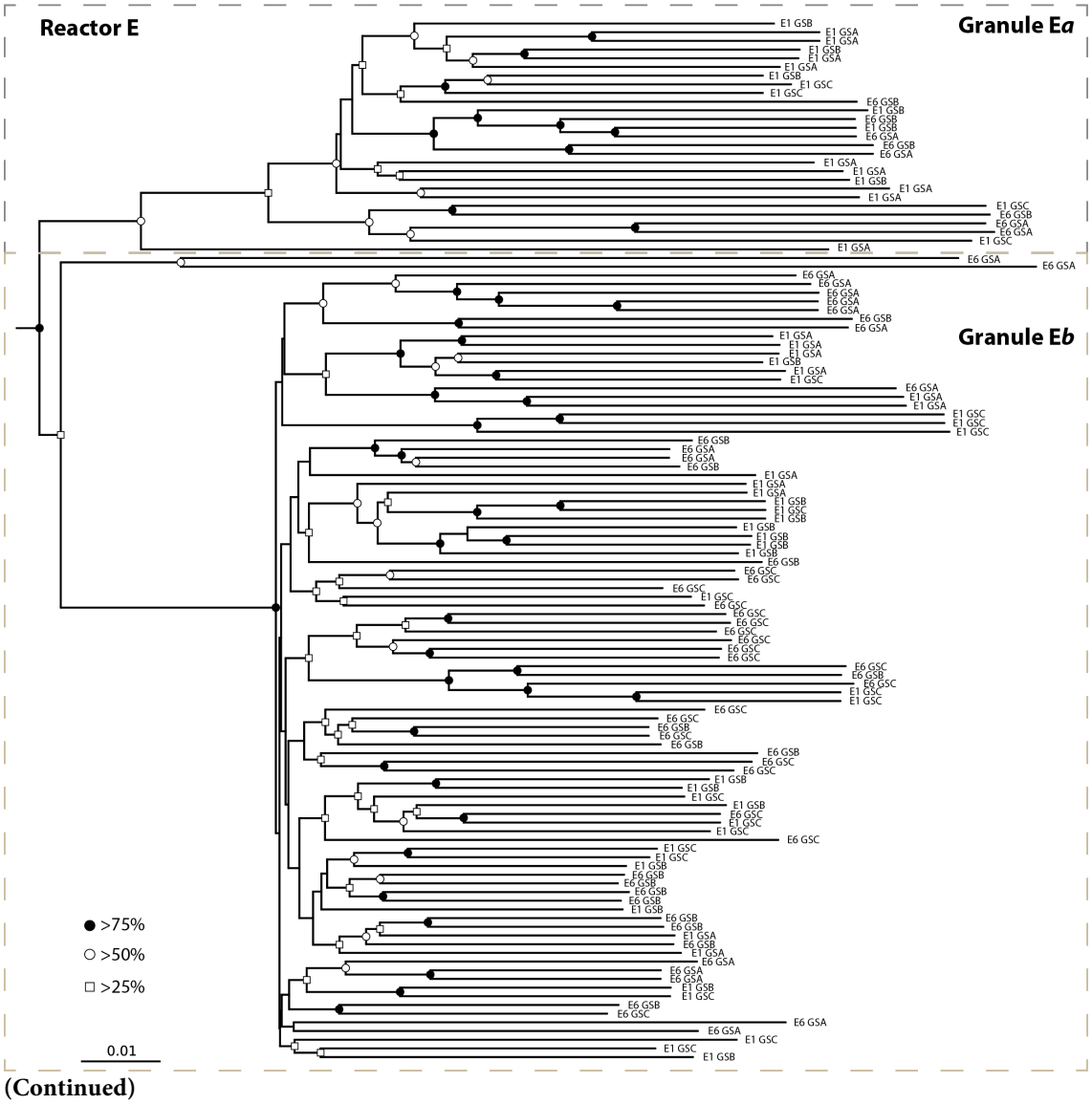


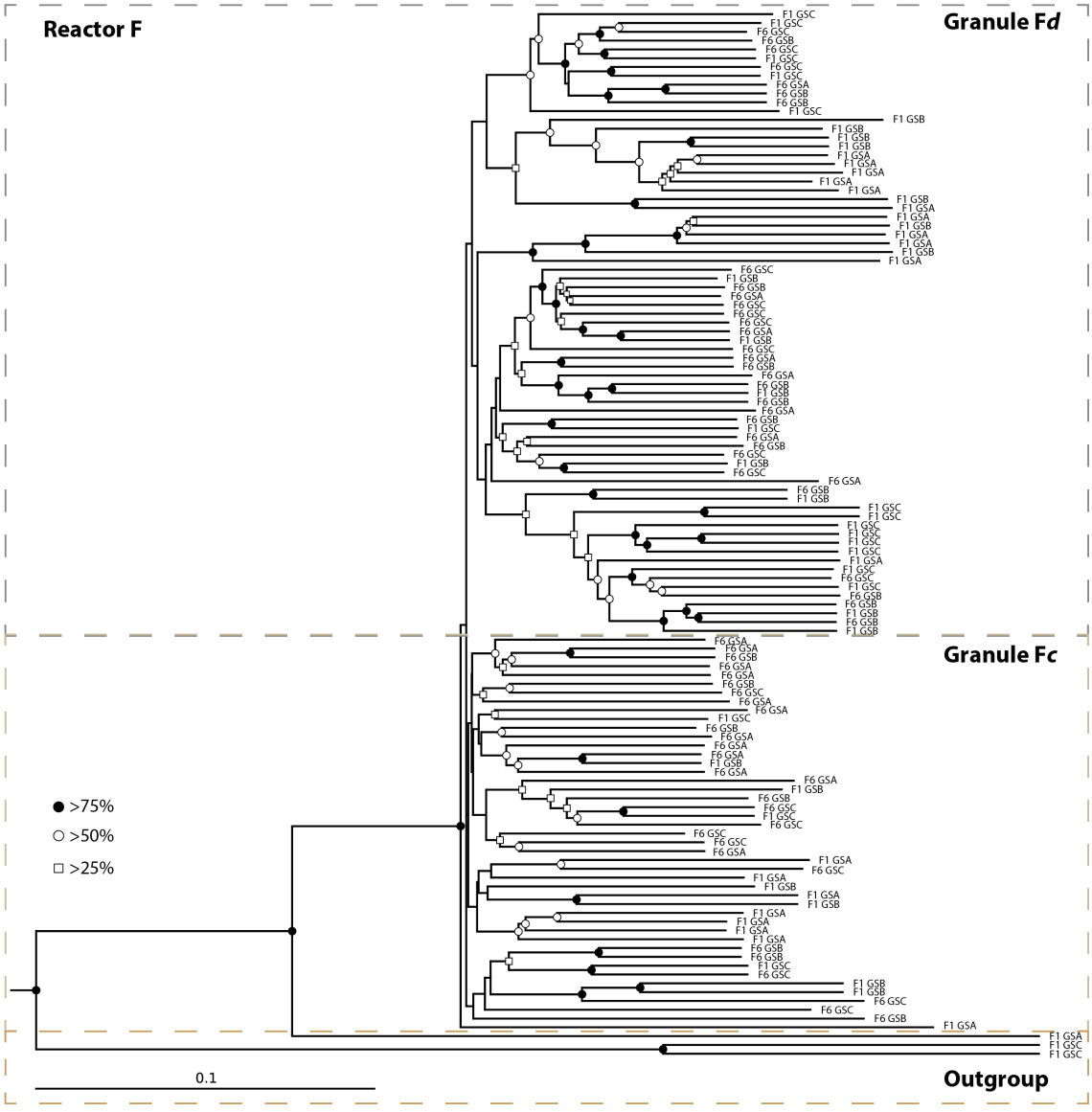
Fig.6–1 Jackknife-supported Principal coordinate analysis plots with weighted UniFrac in (A) All reactors, (B) reactor E, (C) reactor F, and (D) reactor U1. GSA, GSB, and GSC indicate small (1–2 mm), medium (2–3 mm), large (3–4 mm), respectively. For these analyses, 16S rRNA sequence reads were normalized to 18,000 reads per sample. "Cluster" of each granule type is supported by Jackknife-supported weighted UniFrac tree (Fig. 6–2)

(A)


Reactor E
Granule E*a*
Granule E*b*
E1 GSB
E1 GSA
E1 GSC
E6 GSB
E6 GSA
E6 GSC
● >75%
○ >50%
□ >25%
0.01

(Continued)

(B)



(Continued)

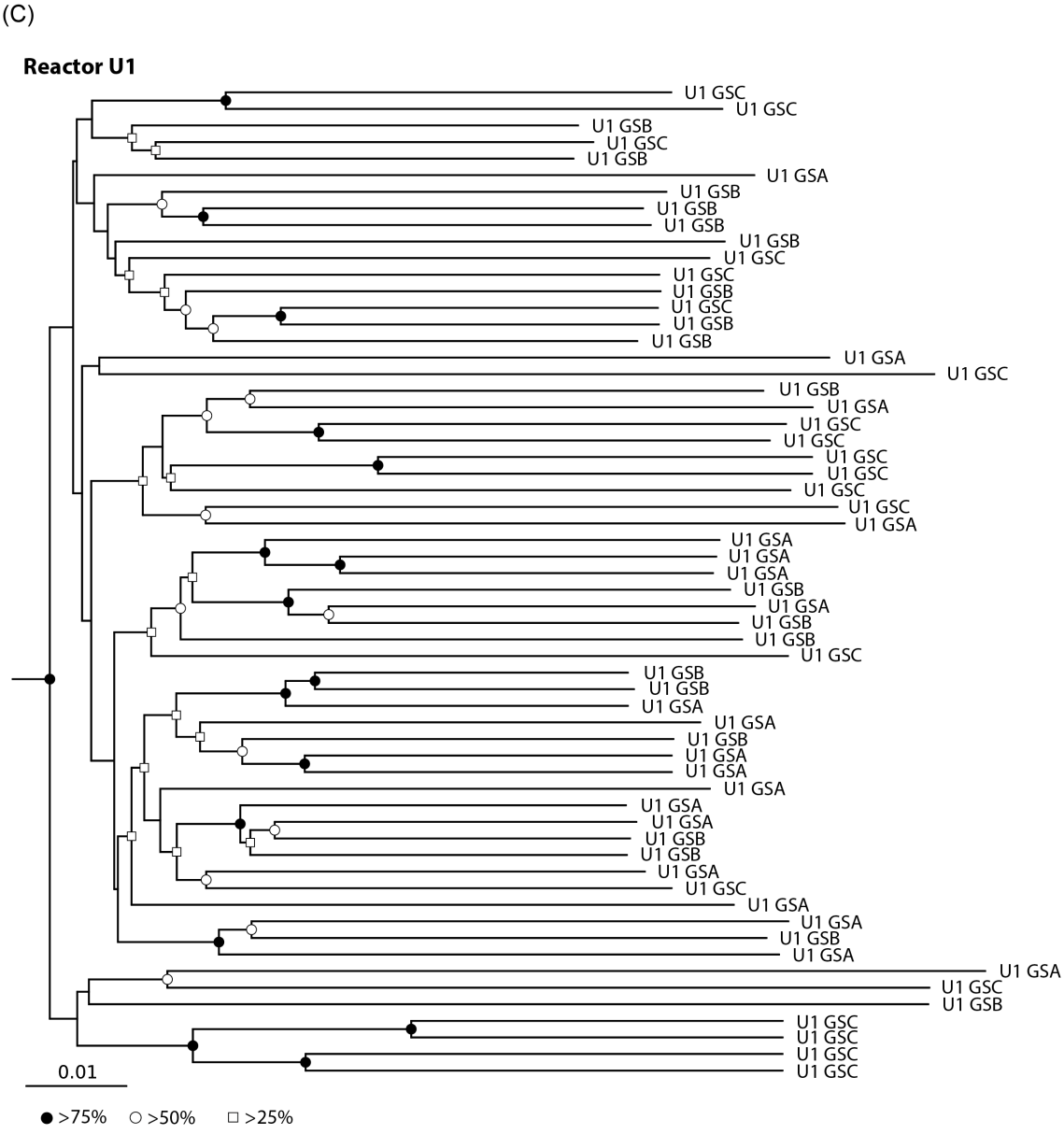


Fig. 6–2 Jackknife-supported weighted UniFrac tree for 16S rRNA gene-based granule community in (A) reactor E, (B) reactor F, and (C) reactor U1. GSA, GSB, and GSC indicate the granule diameter as 1–2 mm, 2–3 mm, and 3–4 mm, respectively. For this analysis, 16S rRNA sequence reads were normalized to 18,000 reads per sample. The solid circle, open circle, and open squera indicate the Jackknife-supported probabilities at >75%, >50%, and >25%, respectively.

Table 6–2 The Coefficient of determination based on OTU scatter diagram of different sized granule in each granule type.

E1*a*	Granule	GSA	GSB	GSC		E6*a*	Granule	GSA	GSB	GSC
	GSA						GSA			
	GSB	0.99					GSB	0.98		
	GSC	0.95	0.95				GSC	–	–	
E1*b*	Granule	GSA	GSB	GSC		E6*b*	Granule	GSA	GSB	GSC
	GSA						GSA			
	GSB	0.95					GSB	0.96		
	GSC	0.94	0.99				GSC	0.89	0.96	
F1*c*	Granule	GSA	GSB	GSC		F6*c*	Granule	GSA	GSB	GSC
	GSA						GSA			
	GSB	0.91					GSB	0.98		
	GSC	0.88	0.98				GSC	0.97	0.98	
F1*d*	Granule	GSA	GSB	GSC		F6*c*	Granule	GSA	GSB	GSC
	GSA						GSA			
	GSB	0.94					GSB	0.98		
	GSC	0.85	0.94				GSC	0.95	0.91	

6.3.2 Core microorganisms in PTA wastewater treating UASB reactors

I define core microorganisms as those that occurred with >0.5% abundance in ≥70% granules regardless of granule sources to confirm main PTA wastewater degrading players in UASB reactors (Fig. 6–3). Based on the observed OTUs frequencies, *Syntrophorhabdus*-related (OTUs 86644 and 23907), *Syntrophus*-related (OTU 57595), *Methanosaeta* (OTU 14738), *Methanomethylovorans* (OTU 70689), and *Methanomassiliicoccus* (OTU 73432) commonly exist in both full- and lab-scale reactors (Fig. 6–4 and Table 6–3), suggesting that they are core degraders of PTA wastewater components (i.e., TA, BZ, AC, and MT). Correspondingly, previous studies on microbial community analyses of mesophilic UASB reactors treating PTA wastewater have demonstrated the dominance of these core microbes (Perkins et al., 2011; Li et al., 2014). Interestingly, the hydrogenotrophic methanogens are different between the full-scale (*Methanolinea* OTU 43922 and *Methanobacterium* OTU 35610) and lab-scale (*Methanoregula* OTU 43922) reactors. A previous 16S rRNA gene analysis of mesophilic (35°C) TA-degrading granular sludge observed a fourth predominant hydrogenotrophic methanogen belonging to *Methanospirillaceae* (Wu et al., 2001). Specific syntrophic partnerships depending on substrates and/or inoculum have been proposed (Narihiro et al., 2015a). While the PTA wastewater of our study contains multiple aromatic compounds and other carbon sources, the wastewater of the previous study included only TA (Wu et al., 2001). And, 66.8% of the *Syntrophorhabdus*-related members in the granule microbial community treating TA (sole-carbon source as substrate) have been observed (Wu et al., 2001). Therefore, it is presumed that hydrogenotrophic methanogen in mesophilic anaerobic TA-degrading microbial community forms specific partnership dependent on the substrate because the granules treating three types of wastewater possess the different syntrophs and hydrogenotrophic methanogens: (1) only TA (Wu et al., 2001), *Syntrophorhabdus*-related members and *Methanospirillum*-related methanogen; (2) synthetic PTA wastewater of U1, *Syntrophorhabdus*-related OTU 13764

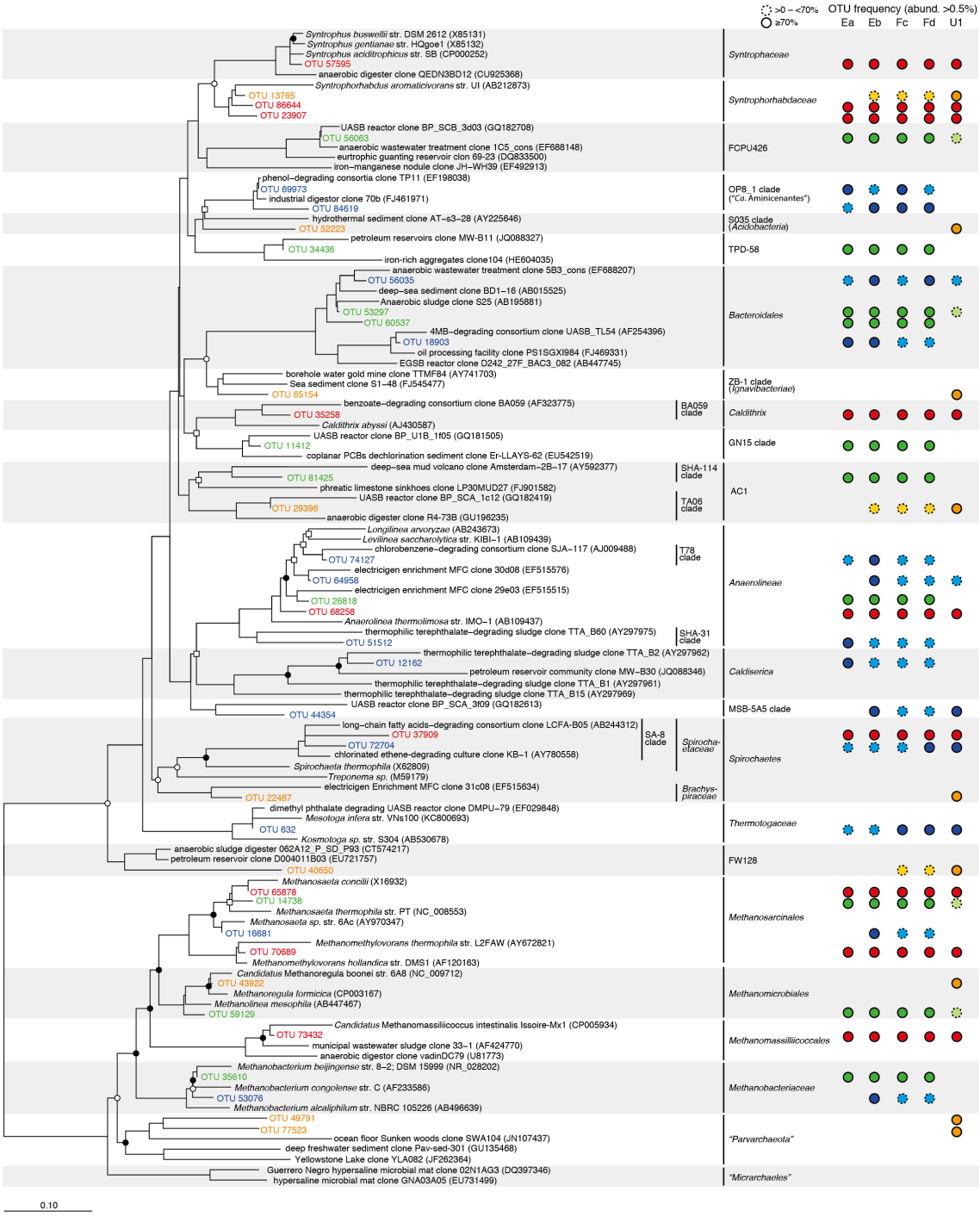


Fig. 6–3 Phylogenetic tree representing predominant OTUs in PTA-wastewater treatment UASB reactor using the neighbor-joining and parsimony methods based on 16S rRNA gene sequences. The solid circle, open circle, and open squera indicate the bootstrap-supported probabilities at >90%, >75%, and >50%, respectively. Circle colors of OTU frequency indicate the OTUs existence patterns such as core OTU in PTA wastewater treatment (red), core in full-scale (green), core in lab-scale (yellow), and others (blue).

with defined core-syntrophs and *Methanoregula*; (3) industrial PTA wastewater from E and F, core-syntrophs and *Methanolinea*/*Methanobacterium* (Fig. 6–4). However, I need further elucidation of these specific partnerships because influence of seed sludge microbial community and whole PTA wastewater degrading mechanisms such as *p*-toluic acid (PT)- and trimellitic acid (TMA)-degradations, are still unknown.

For other core or lab-/full-scale specific microorganisms, several uncultured phyla (TPD-58, FCPU426, GN04, AC1, and FW128) were observed (Figs. 6–3 and 6–4). However, most of their functions are unknown because no isolates or detailed genomic studies of these taxa have been published. Even several omics approaches for capturing the microbial dark matter genomes (Rinke et al., 2013; Gasc et al., 2015; Nobu et al., 2015) have not elucidated the specific role of these uncultured phyla.

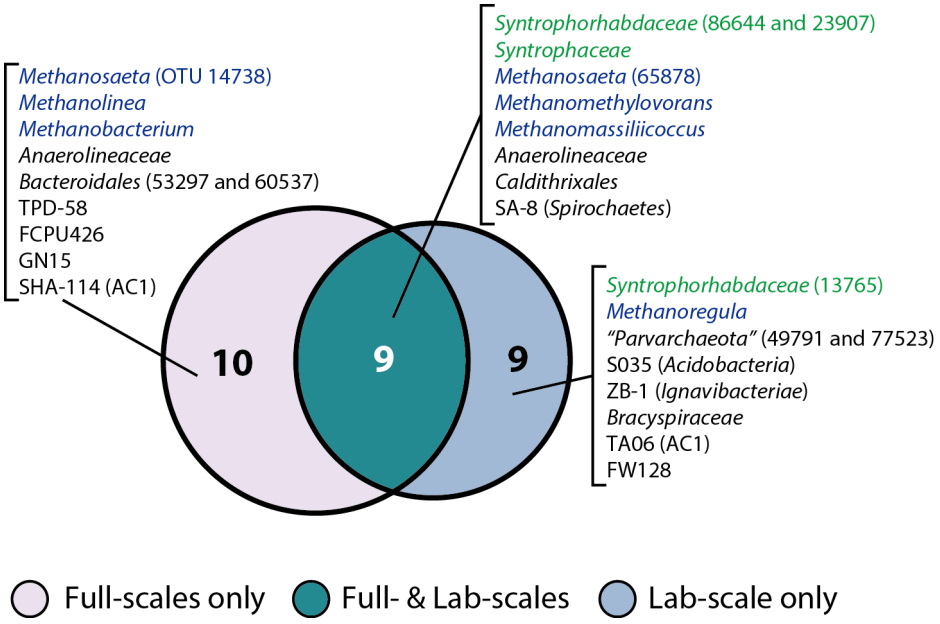


Fig. 6–3 Venn diagram of the shared microorganisms in PTA-watewater treatment UASB granules. Syntrophs and methanogens are highlighted by green and blue, respectively.

Table 6–3 Taxonomic assignment of representative OTUs of this study.

OTU ID	Greengenes ver. 13_8		Related species or clones in NCBI database	
	Taxon		identities (%)	Taxonomy (Accession No.)
49791	*"Parvarchaeota"*	WCHD3-30	319/380(84%)	Uncultured euryarchaeote clone KuA23 (AB077233)
77523		WCHD3-30	245/253(97%)	Uncultured archaeon OTU_6052 (LN775620)
67387	*Crenarchaeota*	pGrfC26	321/380(84%)	*Candidatus* Nitrosocaldus yellowstonii strain HL72 (EU239960)
53076	*Euryarchaeota*	*Methanobacterium*	380/380(100%)	*Methanobacterium subterraneum* strain 9-7 (DQ649330)
35610		*Methanobacterium*	380/380(100%)	*Methanobacterium beijingense* strain 4-1 (AY552778)
43922		*Methanoregula*	376/380(99%)	*Methanoregula boonei* strain 6A8 (NR_074180)
59129		*Methanolinea*	376/380(99%)	*Methanolinea tarda* strain NOBI-1 (NR_028163)
14738		*Methanosaeta*	363/380(96%)	*Methanosaeta thermophila* strain PT (NR_074214)
65878		*Methanosaeta*	380/380(100%)	*Methanosaeta concilii* strain X16932 (KM408635)
16681		*Methanosaeta*	379/380(99%)	*Methanosaeta harundinacea* strain 6Ac (NR_102896)
70689		*Methanomethylovorans*	376/380(99%)	*Methanomethylovorans hollandica* strain DSM 15978 (NR_102454)
73432		*Methanomassiliicoccus*	377/379(99%)	*Candidatus* Methanomassiliicoccus intestinalis Issoire-Mx1 (CP005934)
57595	*Proteobacteria*	*Syntrophaceae*	373/376(99%)	*Syntrophus gentianae* strain HQGOe1 (JQ346737)
86644		*Syntrophorhabdaceae*	365/375(97%)	*Syntrophorhabdus aromaticivorans* UI (NR_041306)
23907		*Syntrophorhabdaceae*	359/375(96%)	*Syntrophorhabdus aromaticivorans* UI (NR_041306)
13765		*Syntrophorhabdaceae*	369/375(98%)	*Syntrophorhabdus aromaticivorans* UI (NR_041306)
52223	*Acidobacteria*	unassigned group	324/376(86%)	*Thermoanaerobaculum aquaticum* strain MP-01 (NR_109681)
53297	*Bacteroidetes*	*Bacteroidales*	335/372(90%)	*Prolixibacter bellariivorans* strain JCM 13498 (LC015091)
60537		*Bacteroidales*	328/372(88%)	*Ruminofilibacter xylanolyticum* strain S1 (DQ141183)
56035		*Bacteroidales*	341/374(91%)	*Prolixibacter bellariivorans* strain JCM 13498 (LC015091)
18903		*Rikenellaceae*	313/373(84%)	*Pontibacter korlensis* strain AG6 (KJ949605)
12162	*Caldiserica*	TTA-B1	311/376(83%)	*Caldisericum exile* strain AZM16c01 (NR_075015)
35258	*Caldithrix*	BA059	337/379(89%)	*Caldithrix palaeochoryensis* strain MC (NR_116885)
65154	*Ignavibacteriae*	*Ignavibacteriaceae*	341/374(91%)	*Melioribacter roseus* strain P3M-2 (NR_074796)
68258	*Chloroflexi*	*Anaerolineaceae*	352/375(94%)	*Leptolinea tardivitalis* strain YMTK-2 (NR_040971)
64958		*Anaerolineaceae*	350/375(93%)	*Longilinea arvoryzae* strain KOME-1 (NR_041355)
74127		T78	340/375(91%)	*Leptolinea tardivitalis* strain YMTK-2 (NR_040971)
26818		GCA004	348/375(93%)	*Leptolinea tardivitalis* strain YMTK-2 (NR_040971)
51512		SHA-31	314/378(83%)	*Anaerolinea thermolimosa* strain IMO-1 (NR_040970)
22487	*Spirochaetes*	*Brachyspiraceae*	313/374(84%)	*Exilispira thermophila* strain RASEN (NR_041644)
37909		*Treponema*	322/372(87%)	*Treponema zuelzerae* strain DSM (NR_104797)
72704		*Treponema*	316/372(85%)	*Treponema zuelzerae* strain DSM (NR_104797)
632	*Thremotogae*	*Kosmotoga*	375/375(100%)	*Mesotoga infera* strain VNs100 (NR_117646)
81425	AC1	SHA-114	361/368(98%)	Uncultured bacterium clone 6E1_cons (EF688249)
29396		TA-06	376/376(100%)	Uncultured bacterium clone BP_SCC_2c10 (GQ182963)
56063	FCPU426		376/376(100%)	Uncultured bacterium clone BP_SCC_2a10 (GQ182861)
44354	GN04	unassigned group	377/377(100%)	Uncultured bacterium clone BP_SCA_3d05 (GQ182494)
11412		GN15	376/376(100%)	Bacterium enrichment culture clone L11_2_64 (JX473550)
40650	OD1	unassigned group	370/376(98%)	Uncultured bacterium clone B16 (JX100399)
84619	"*Ca.* Aminicenantes"	OP8_1	374/374(100%)	Bacterium enrichment culture clone L55B-115 (JF947100)
69973		OPB95	374/374(100%)	Uncultured bacterium clone HMTAb196 (KM373094)
34436	TPD-58	unassigned group	376/376(100%)	Uncultured bacterium clone MW-B11 (JQ088327)

6.3.3 Comparison of predominant microorganisms in different types of granules

Full-scale UASB reactors E and F

Microbial community compositions of granule E*a* and E*b* in reactor E, and granule F*c* and F*d* in reactor F reveal different patterns (Fig. 6–5). The granules E*a* and E*b* surprisingly have statistically distinct abundances of syntrophs (dominated by *Syntrophorhabdus*-related OTU 86644 or Syntrophus-related OTU 57595 in E*a* and E*b*), hydrogenotrophic methanogens (*Methanobacterium* OTUs 35610 and 53076 in E*a* and E*b*), methylotrophic methanogens (*Methanomassiliicoccus* OTU 73432 *Ea* and *Methanomethylovorans* OTU 70689 E*b*), aceticlastic methanogens (*Methanosaeta* OTUs 65878 E*a* and 16681 E*b*), and uncharacterized organisms ("*Ca.* Aminicenantes" OTUs 84619 and 69973) (Figs. 6–5, 6–6 and 6–7). Besides, reactor F shows similar abundance rate with reactor E between *Methanomethylovorans* and *Methanomassiliicoccus*, and *Syntrophus*-related and *Syntrophorhabdus*-related OTUs. However, the shift of microorganisms in their niches in reactor F is less distinct compared with reactor E because microbial community compositions of granules F*c* and F*d* (R^2=0.799) are more similar than granule *Ea* and *Eb* (R^2=0.674) based on scatter diagram of OTU abundance (Table 6–4). In addition, the less abundant granule in terms of microbial composition (E*a* and F*c)* has more similar microbial community compositions (R^2=0.906) than the more abundant granules (E*b* and F*d*) (R^2=0.768) (Table 6–4). Although the replicate reactors E and F were presumed to have similar microbial community composition due to similar operation as well as the fact that they are the same configuration and receive feed from the same feed tank, these results indicate different types of microbial communities in each reactor. Therefore, slightly different operational conditions may have significant impacts on granule microbial community development.

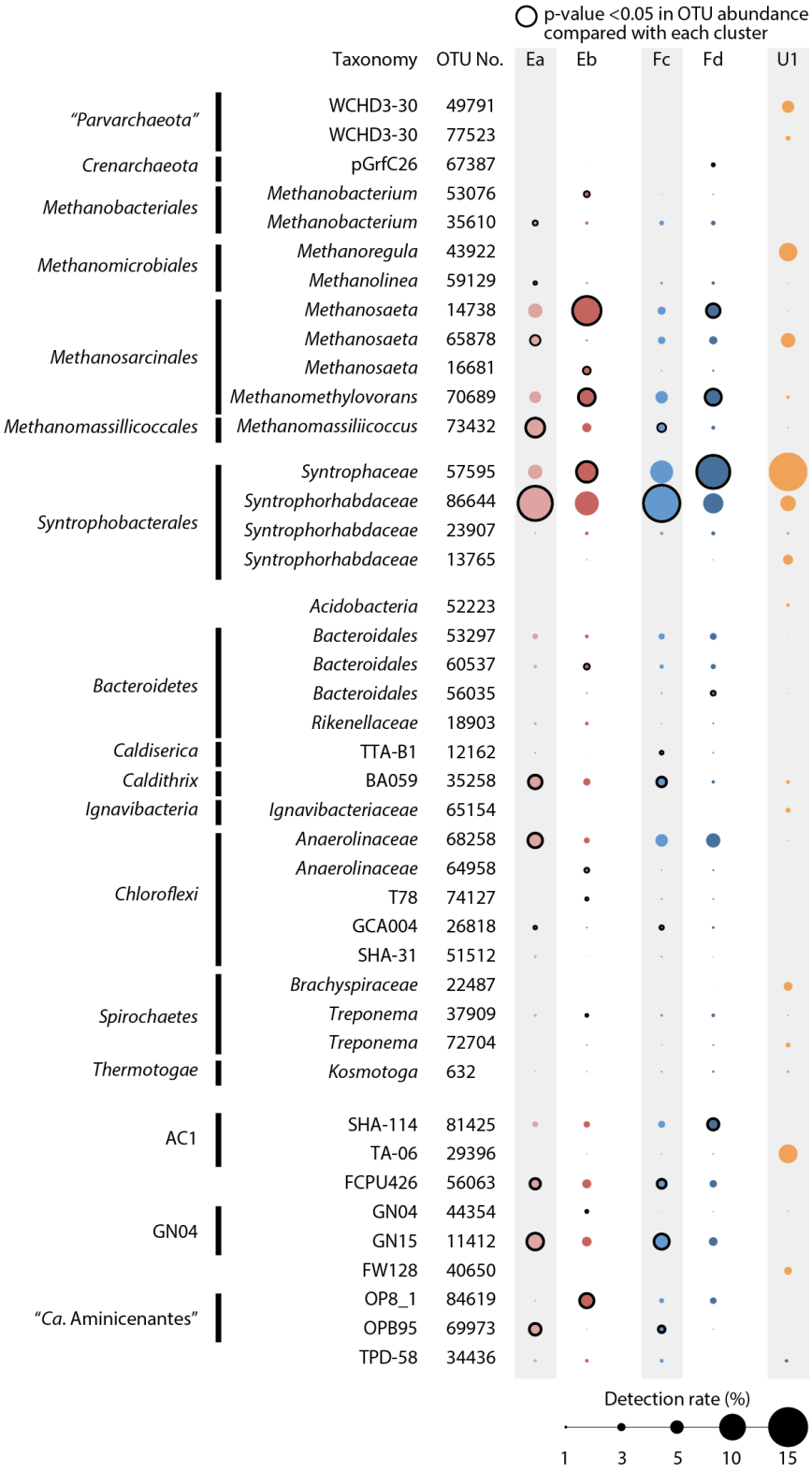
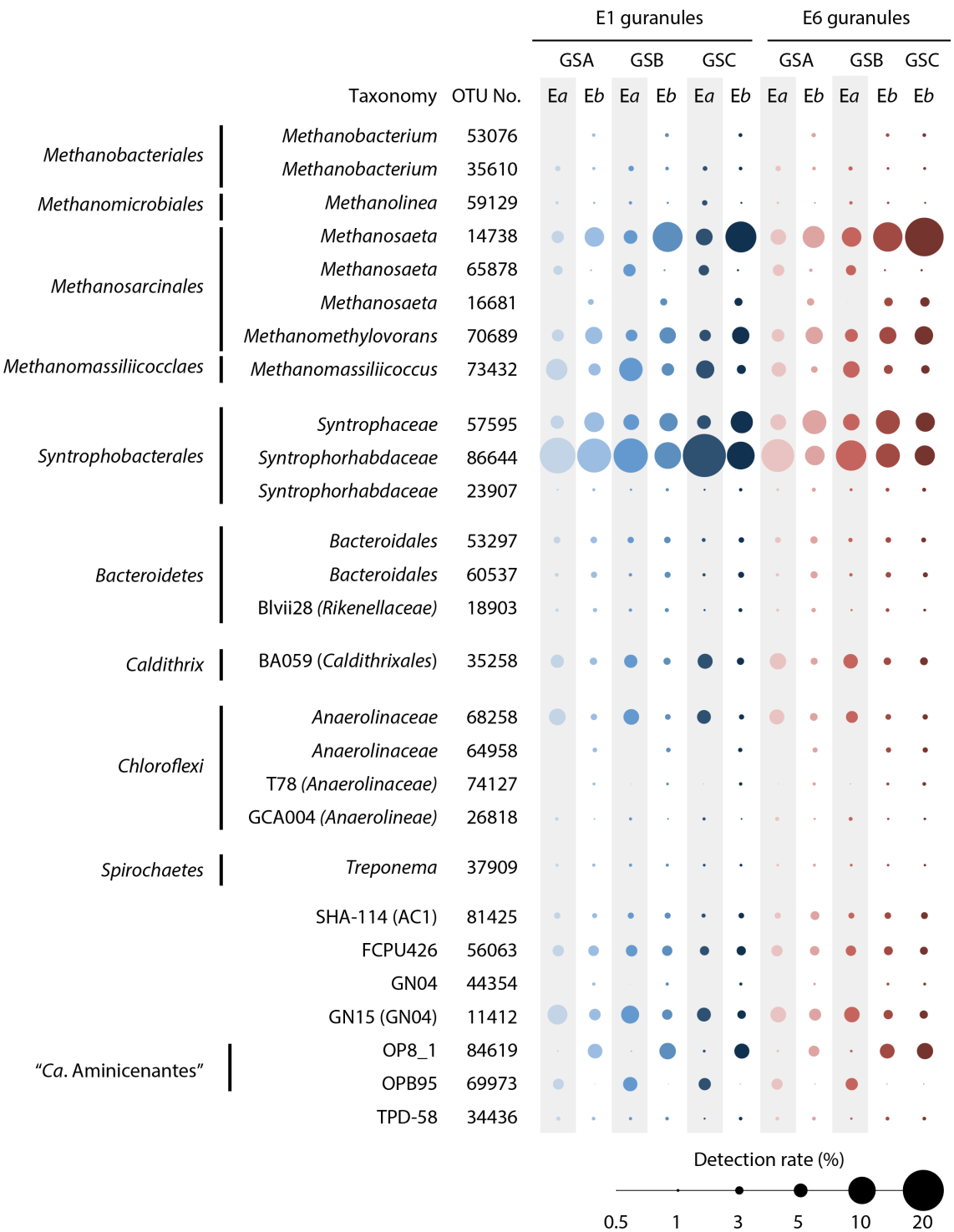


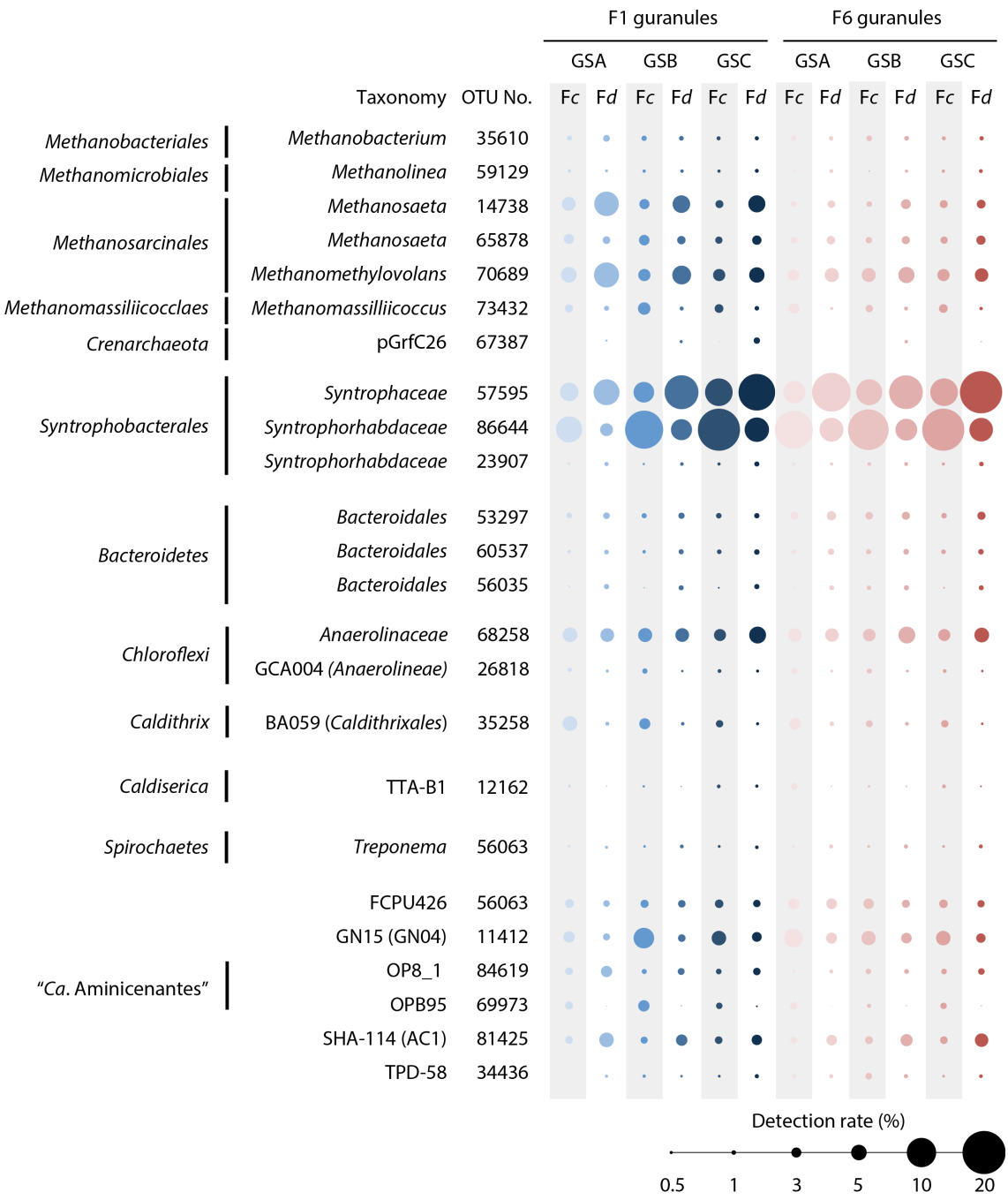
Fig. 6–5 Abundance of predominant OTUs in reactors E, F and U1 using bubble plots. *Circle sizes* correspond to abundance rate, as shown at the bottom of the figure. *Circle lines* indicate the statistical differences of OTU abundance between different granule types based on Welch's t-test (p<0.05).

(A)


E1 guranules
E6 guranules
GSA
GSB
GSC
GSA
GSB
GSC
Taxonomy
OTU No.
Ea
Eb
Ea
Eb
Ea
Eb
Ea
Eb
Ea
Eb
Eb
Methanobacteriales
Methanobacterium 53076
Methanobacterium 35610
Methanomicrobiales
Methanolinea 59129
Methanosarcinales
Methanosaeta 14738
Methanosaeta 65878
Methanosaeta 16681
Methanomethylovorans 70689
Methanomassiliicocclaes
Methanomassiliicoccus 73432
Syntrophobacterales
Syntrophaceae 57595
Syntrophorhabdaceae 86644
Syntrophorhabdaceae 23907
Bacteroidetes
Bacteroidales 53297
Bacteroidales 60537
Blvii28 (Rikenellaceae) 18903
Caldithrix
BA059 (Caldithrixales) 35258
Chloroflexi
Anaerolinaceae 68258
Anaerolinaceae 64958
T78 (Anaerolinaceae) 74127
GCA004 (Anaerolineae) 26818
Spirochaetes
Treponema 37909
SHA-114 (AC1) 81425
FCPU426 56063
GN04 44354
GN15 (GN04) 11412
"Ca. Aminicenantes"
OP8_1 84619
OPB95 69973
TPD-58 34436
Detection rate (%)
0.5
1
3
5
10
20

(Continued)

(B)


F1 guranules
F6 guranules
GSA
GSB
GSC
GSA
GSB
GSC
Taxonomy
OTU No.
Fc Fd Fc Fd Fc Fd Fc Fd Fc Fd Fc Fd
Methanobacteriales
Methanobacterium 35610
Methanomicrobiales
Methanolinea 59129
Methanosarcinales
Methanosaeta 14738
Methanosaeta 65878
Methanomethylovolans 70689
Methanomassiliicocclaes
Methanomassilliicoccus 73432
Crenarchaeota
pGrfC26 67387
Syntrophobacterales
Syntrophaceae 57595
Syntrophorhabdaceae 86644
Syntrophorhabdaceae 23907
Bacteroidetes
Bacteroidales 53297
Bacteroidales 60537
Bacteroidales 56035
Chloroflexi
Anaerolinaceae 68258
GCA004 (Anaerolineae) 26818
Caldithrix
BA059 (Caldithrixales) 35258
Caldiserica
TTA-B1 12162
Spirochaetes
Treponema 56063
"Ca. Aminicenantes"
FCPU426 56063
GN15 (GN04) 11412
OP8_1 84619
OPB95 69973
SHA-114 (AC1) 81425
TPD-58 34436
Detection rate (%)
0.5 1 3 5 10 20

(Continued)

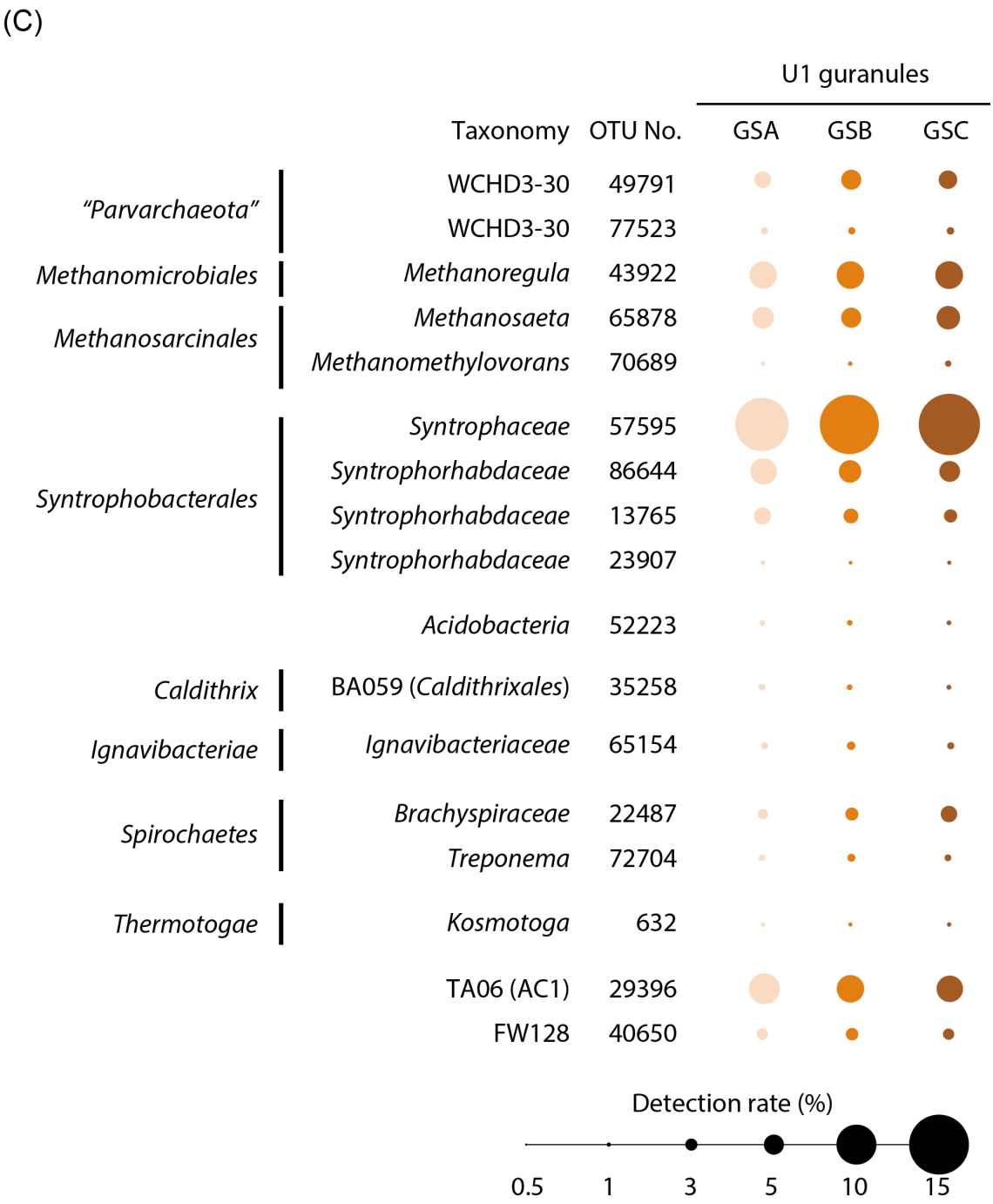


Fig. 6–6 Abundance of predominant OTUs in (A) reactors E, (B) reactor F, and (C) reactor U1 with different sized granules using bubble plots. GSA, GSB, and GSC show the granule diameter as 1–2 mm, 2–3 mm, and 3–4 mm, respectively. *Circle sizes* correspond to abundance rate, as shown at the bottom of the figure.

Table 6–4 The Coefficient of determination based on OTU scatter diagram of each granule type.

Scatter R^2		E		F	
	Granlule	*a*	*b*	*c*	*d*
E	*a*		0.674	0.906	0.64
	b	0.674		0.712	0.768
F	*c*	0.906	0.712		0.799
	d	0.64	0.768	0.799	

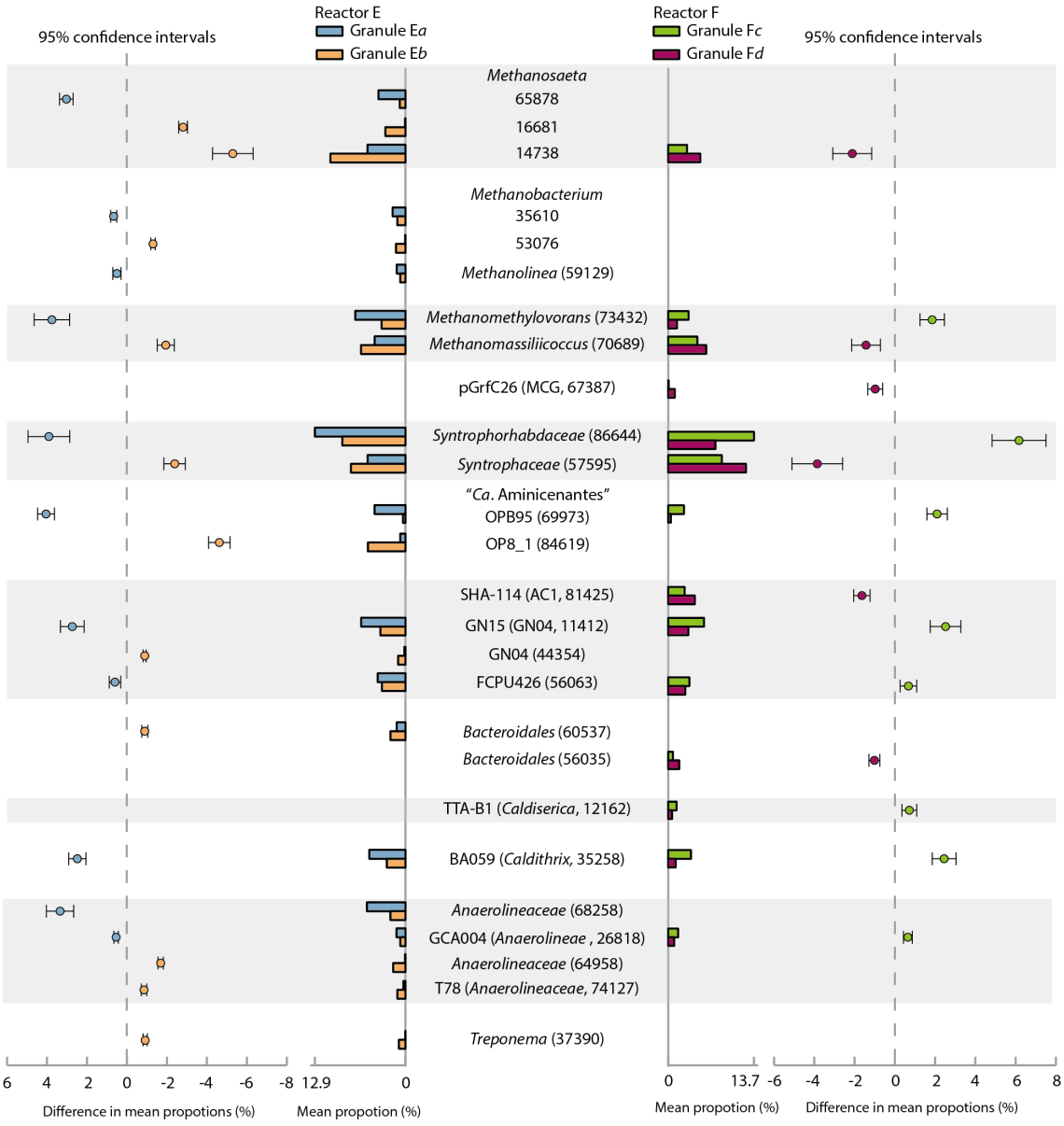


Fig. 6–7 Extended error bar plot with significant different OTUs abundances ($p<0.05$) in reactor E and F.

Lab-scale UASB reactor U1

In reactor U1, quite unique community compositions were observed compared with full-scale reactors (Fig. 6–5). The influent wastewater of U1 contained higher PT concentration (4.4 mM) than full-scale reactors (0.6 mM). It has been reported that PT-degradation is inhibited when AC and BZ concentrations were high (Fajardo et al., 1997). However, even though the influent wastewater contained 2.5 mM BZ and 23 mM AC, PT was degraded from 4.4 mM to 0.8 mM (data not shown). Although detailed information about PT-degraders and its mechanism is still unavailable, PT concentrations may contribute to the differences in the microbial community compositions between full-scales and lab-scale reactors.

In *Archaea*, "*Parvarchaeota*" (uncultured phylum), *Methanoregula* (hydrogenotrophic methanogen), and *Methanosaeta* (aceticlastic methanogen) predominated (Fig. 6–5). MT-utilizing methanogens (*Methanomassiliicoccus* and *Methanomethylovorans*) were less abundant (<0.5%) compared with full-scale reactors, likely due to the relatively low MT concentration (4.8mM) compared to that of the full-scale reactors (24.3 mM). Although aerobic acidophilic "*Ca.* Parvarchaeota" has been characterized genomically (Baker et al., 2006; Baker et al., 2010), little information is available for anaerobic members of this phylum. Therefore, to further understand the functions of anaerobic "*Parvarchaeota*", omics-approaches such as metagenomics and metatranscriptomics need to be applied.

As for syntrophs, *Syntrophus*-related OTU (14.5%) and two *Syntrophorhabdus*-related OTUs (OTUs 86644, 5.8% and 13765, 3.7%) are predominant (Fig. 6–5) in U1. The ratio of the predominant *Syntrophus*:*Syntrophorhabdus* (OTUs 57595:86644, 2.5) is much higher in U1 than those in microbial communities from full-scale granules (0.42–1.6). It is well known that the hydrogen-utilizing organisms are important for the syntrophic association as discussed above. While full- and lab-scale reactors have predominant hydrogenotrophic methanogens such as *Methanolinea*,

Methanobacterium, and *Methanoregula* as discussed above (Fig. 6–4), the abundance of *Methanomassiliicoccus* is quite different (Fig. 6–3; full-scales, 3.2%; lab-scale, 0.7%). *Methanomassiliicoccus* reduces MT with H_2 as electron donor (Dridi et al., 2012; Iino et al., 2013), which can be a syntrophic partner. Therefore, the different characteristics of syntrophic partners might affect abundance of predominant syntrophs in UASB reactors.

6.3.4 Biodiversity across all granule sizes

It is widely accepted that granular size increases when granule maturation occurs (Abbasi and Abbasi, 2012). However, the relationship between granule microbial community complexity and granule size remains unclear. Therefore, to evaluate the different sized granules, I compared the granule biodiversity based on alpha diversity indices across all granules (except for out-group granules) (Fig. 6–8 and Table 6–1). The average numbers of observed OTUs and Chao1 in each type of granule were 400–709 and 709–1481, respectively (Table 6–1). As for comparison across different sized granules, the alpha diversity increased with granules size (Fig. 6–8). An inactive layer or a well-defined hollow core (in >2.0 mm sized granule) has been observed inside granules using a FISH or scanning electron microscopy (Sekiguchi, 2006; Del Nery et al., 2008). It has been proposed that the hollow core or inactive layer is a feature of over-matured granule due to substrate limitation inside of granule (Schmidt and Ahring, 1996) where cell extracts will be released during bacterial or archaeal decay (Yan and Tay, 1997). Because the abundance of syntrophs and methanogens and whole microbial community compositions are stable among different sized granules in same granule type (Fig. 6–6A, B, and C and Table 6–2), microbes with functions irrelevant to TA metabolism, such as degradation of biomass detritus, might emerge during the granule maturation process and contribute to the increasing biodiversity (Nobu et al., 2015).

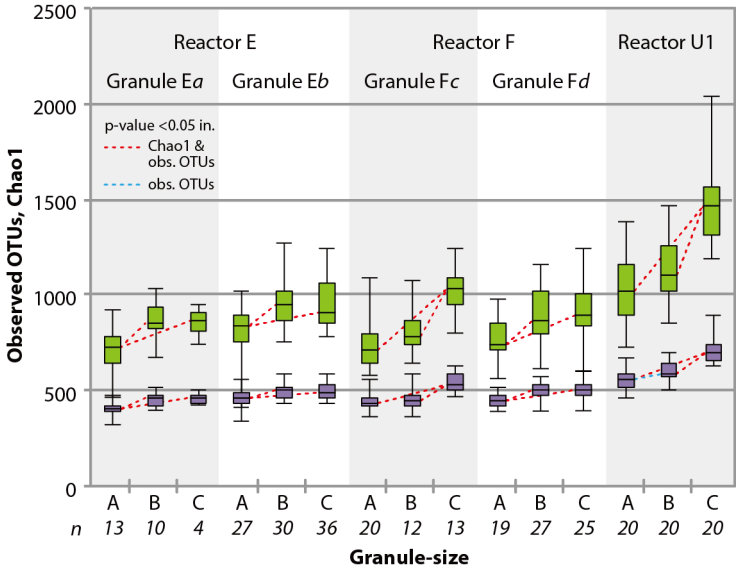


Fig. 6–8 Boxplots of observed OTUs (lower box plots) and Chao1 (upper box plots). The dash lines indicate the statistical differences based on unpaired Welch's t-test. The gray and black lines are p-value <0.05 in Chao1 and observed OTUs and p-value <0.05 in observed OTUs.

6.3.5 Co-existing microorganisms in PTA wastewater treating granules

To deeply understand the relationship across predominant microorganisms, I analyzed the microbial network in each reactor based on Spearman's rank correlation test (Fig. 6–9). The network clearly exhibited the existence of two OTU-groups in reactor E, which are mostly reflected as the microbial community compositions of granule E*a* and E*b* (Figs. 6–5 and 6–9). The most predominant *Syntrophorhabdus*-related OTU 86644 is highly positive-correlated with *Methanomassiliicoccus* (OTU 73432), *Methanolinea* (OTU 59129), and one *Methanosaeta* (OTU 65878), and indirectly positive-correlated with *Methanobacterium* (OTU 35610) through the *Methanosaeta* in granule E*a*. In the granule E*b*, predominant *Syntrophus*-related OTU 57595 had strong positive correlations with minor *Syntrophorhabdus*-related OTU 23907 and *Methanobacterium* (OTU 53076) and had indirect positive correlations with *Methanomethylovorans* and two *Methanosaeta* (OTUs 70689 and 16681). On the other hand, few strong positive correlations were observed in reactor F and U1 (data not

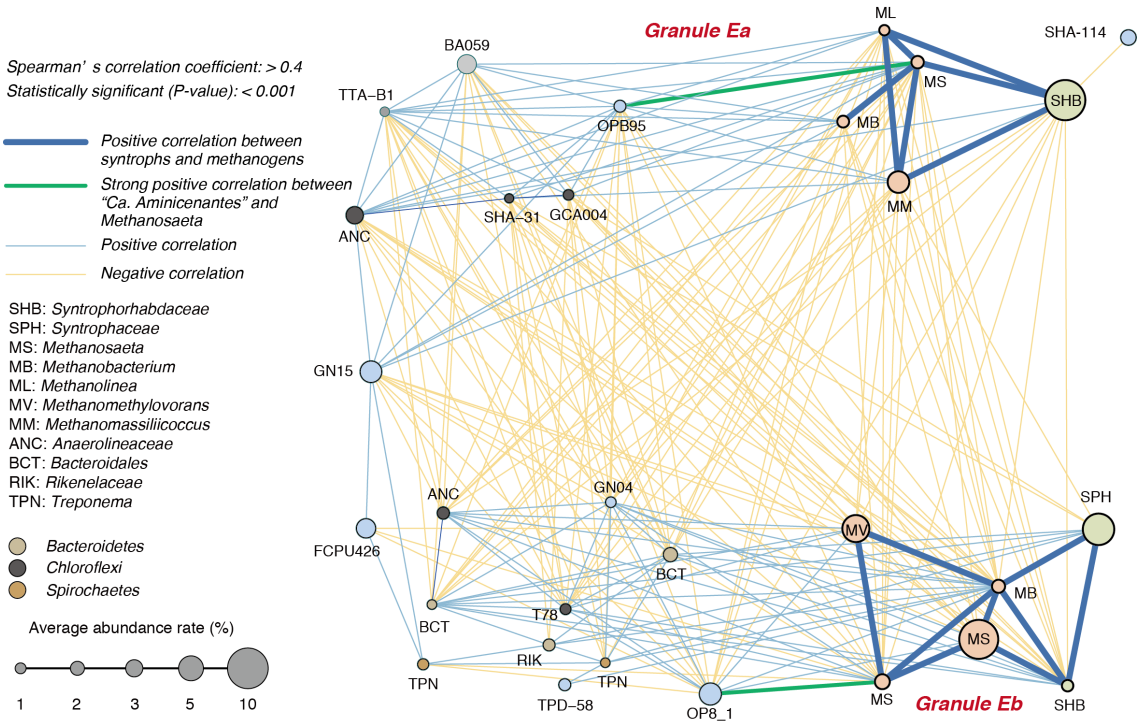


Fig. 6–9 Network of predominant microorganisms in reactor E based on Spearman's correlation analysis(Spearman's *rs* < 0.4 and p-value < 0.001). Highlighted blue and green lines indicate the positive correlations between methanogens and syntrophs and strong positive correlations between OP8 and *Methanosaeta*, respectively. Light blue and light orange lines indicate the positive and negative correlations, respectively. *Circle sizes* correspond to average abundance rate, as shown at the left bottom of the figure. Orange circle color shows the methanogens nodes including *Methanosaeta* (MS), *Methanomethylovorans* (MV), *Methanobacterium* (MB), *Methanolinea* (ML), and *Methanomassiliicoccus* (MM). Light green circle color shows the syntrophs nodes including *Syntrophaceae* (SPH) and *Syntrophorhabdacea* (SHB). *Bacteroidales* (BCT), *Rikenelaceae* (RIK), and *Treponema* (TPN) were indicated with light brown, dark gray, and brown circle colors, respectively.

shown) because the individual granule microbial community compositions are more similar than reactor E (Fig. 6–1 and Table 6–3).

By core microorganism analysis, *Methanomassiliicoccus* is a core member for PTA wastewater treatment (Fig. 6–4). The network analysis indicated the positive-correlation of *Methanomassiliicoccus* with the most predominant *Syntrophorhabdus*-related 86644 OTU and *Methanolinea* (Fig. 6–9). A previous study presumed that *Methanolinea* could have higher affinity for H_2 (Sakai et al., 2009). Although the level of H_2 affinity of

Methanomassiliicoccus is unknown, the meaning of co-existing of *Methanomassiliicoccus* and *Methanolinea* can be explained the H_2 affinity to avoid substrate competition for methanogenesis. Therefore, *Syntrophorhabdus*-related OTU might form syntrophic association with *Methanomassiliicoccus* and *Methanolinea* to maintain the favorable conditions for TA-degradation. The predominant *Syntrophorhabdus*-related OTU and *Syntrophus*-related OTU are positively correlated with *Methanosaeta* because these organisms produce AC as a catabolism by-product. Intriguingly, the minor *Syntrophorhabdus*-related OTU 23907 is positive-correlated with *Syntrophus*-related OTU 57595. *Syntrophus* uses BZ and butyrate, which can be obtained from influent wastewater or metabolites of *Syntrophorhabdus* (Jackson et al., 1999; Qiu et al., 2008; Nobu et al., 2014). Therefore, it is possible that the population of *Syntrophus*-related OTU increased in the major granule *Eb* due to the *Syntrophorhabdus*-related OTUs served benzoate to *Syntrophus*-related OTU.

Distinct "*Ca*. Aminicenantes" (OTUs 84619 and 69973) predominated in reactor E (Fig. 6–5). Both "*Ca*. Aminicenantes" had strong positive correlations with distinct *Methanosaeta* (Fig. 6–5; OTUs 69973 and 65878, *rs*=0.72; OTUs 84619 and 16681, *rs*=0.82). Metagenomic or 16S rRNA gene-based approach suggested that "*Ca*. Aminicenans sakinawicola" can degrade amino acids, and the members of this phylum exist in diverse environments (Rinke et al., 2013; Farag et al., 2014). However, "*Ca*. Aminicenantes" have eight clades at order- or class- level based on 16S rRNA gene analysis (Farag et al., 2014). Therefore, most of the "*Ca*. Aminicenantes" functions remain unclear. On the other hand, the information of this study might be helpful to understand their unknown ecology because it can be speculate the partner relationship between *Methanosaeta* and "*Ca*. Aminicenantes". Despite the fact that OTUs 84619 (OP8_1) and 69973 (OPB95) are taxonomically different at order level, they are strongly correlated with distinct *Methanosaeta* (Fig. 6–5 and Fig. 6–9), suggesting that these "*Ca*. Aminicenantes" play similar roles in different granule types within reactor E.

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Chapter 7

Summary

Summary

Anaerobic wastewater treatment involves complicated biological interactions, in which various microorganisms play important roles in the degradation of wastewater components. Anaerobic bioreactors are complex systems that contain many uncultured organisms about which little is known. By accessing the global network available through my laboratory (http://ecolab.nagaokaut.ac.jp/e/project), I was able to obtain several different kinds of anaerobic wastewater treatment sludge from various countries. For this dissertation, I performed comparative 16S rRNA-based microbial community analyses on different anaerobic bioreactor samples. Although the actual roles of some microorganisms in anaerobic wastewater treatment systems remain unclear, through this work, I uncovered important information that will allow us to better understand the ecology and diversity of uncultured and known microorganisms, patterns in microbial community composition and its changes over the course of the development of anaerobic systems, and ecological heterogeneity in industrial upflow anaerobic sludge blanket (UASB) reactors; all of which can help elucidate anaerobic processes. A brief summary of the conclusions of each chapter is given below.

Chapter 3 Patterns of uncultured *Bacteria* phyla in different wastewater treatment sludges

To investigate the ecology of predominant microorganisms and putative habitats of uncultured bacterial phyla in wastewater treatment sludge, I performed massive parallel 16S rRNA gene sequencing of aerobic, anoxic, and anaerobic wastewater treatment sludge. I detected several candidate phyla, including WWE1, GN04, WS3, GN02, FCPU426, OD1, Hyd24-12, OP8, OP9, and unclassified phylotypes at the phylum level in the sludge sample. Additionally, I was able to estimate the putative habitats and environmental conditions of these uncultured phyla by examining the distribution patterns in each wastewater treatment sludge sample.

Chapter 4 Community composition of known and uncultured archaeal lineages in anaerobic or anoxic wastewater treatment sludge

I used 16S rRNA gene sequencing to identify the diversity of known and uncultured archaeal lineages in the microbial communities of 12 different types of sludge. Comprehensive phylogenetic analysis indicated that the predominant phylotypes and uncultured lineages in each sample belonged to Deep-sea Hydrothermal Vent Euryarchaeotic Group 6 (DHVEG-6), WSA2, Terrestrial Miscellaneous Euryarchaeotic Group, and Miscellaneous Crenarchaeota group; thus, several uncultured lineages were present in anaerobic and anoxic wastewater treatment sludge. I also observed that DHVEG-6 was only predominant in nitrogen/phosphorus removal sludge, indicating that an unknown divergence of uncultured archaea occurs in anaerobic wastewater treatment sludge. Further studies of additional types of sludge using metagenomics, metatranscriptomics, single-cell genomics, and cultivation methods can provide further information on microbial community composition that can be used to develop more effective strategies for the management of sludge and the minimization of associated environmental impacts.

Chapter 5 High organic loading treatment for industrial molasses wastewater and microbial community shifts corresponding to system development

The multi-staged- (MS-) upflow anaerobic sludge blanket (UASB)-UASB-down-flow hanging sponge (DHS) system achieved 85% ± 3.2% total COD removal and 95% ± 2.2% total BOD removal of industrial molasses wastewater during Phase 6 at an OLR of 42 ± 2.4 $kgCOD \cdot m^{-3} \cdot d^{-1}$ in the MS-UASB reactor. Installation of an acidification tank allowed a low partial pressure of H_2 to be maintained in the MS-UASB reactor. Microbial community analysis showed that multiple syntrophic associations contributed to the degradation of organic compounds in

molasses wastewater. These results demonstrate the necessity of maintaining favorable environments for syntrophic associations. Overall, this study provides new insights into a high-organic-loading molasses wastewater treatment system and the composition of its microbial community.

Chapter 6 A single-granule-level approach reveals ecological heterogeneity in an upflow anaerobic sludge blanket reactor

In this chapter, I attempted to understand the ecological heterogeneity of UASB granular sludge through analysis at the single-granule level. Such analysis could indicate the existence of different types of granule microbial community compositions, the core microorganisms present in purified terephthalic acid wastewater treatment systems, and the relationships among predominant microorganisms. I believe that the single-granule-level approach can provide information on general microbial community compositions, and also on unpredicted microbial relationships in UASB granular sludge.

The analyses I used in this study allowed me to evaluate not only the ecology of uncultured and known microorganisms, but also microbial community development in biological wastewater treatment systems. Although the roles of many of the uncultured microoganisms and the mechanisms responsible for sludge development in anaerobic bioreactors remain unknown, the results of this dissertation can be applied in the evaluation and design of wastewater treatment systems. These results will also contribute to elucidating the "*black box*" in anaerobic wastewater treatment systems.

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