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Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester

Herto Dwi Ariesyady[a], *Tsukasa Ito*[b], *Satoshi Okabe*[a,*]

[a]*Department of Urban and Environmental Engineering, Graduate School of Engineering, Hokkaido University, North-13, West-8, Kita-ku, Sapporo 060-8628, Japan*

[b]*Department of Civil Engineering, Faculty of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu 376-8515, Japan*

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ABSTRACT

Functional *Bacteria* and *Archaea* community structures of a full-scale anaerobic sludge digester were investigated by using a full-cycle 16S rRNA approach followed by microautoradiography (MAR)–fluorescent in situ hybridization (FISH) technique and micromanipulation. FISH analysis with a comprehensive set of 16S and 23S rRNA-targeted oligonucleotide probes based on 16S rRNA clone libraries revealed that the Gram-positive bacteria represented by probe HGC69A-hybridized *Actinobacteria* (8.5±1.4% of total 4′, 6-diamidino-2-phenylindole (DAPI)-stained cells) and probe LGC354-hybridized *Firmicutes* (3.8±0.8%) were the major phylogenetic bacterial phyla, followed by *Bacteroidetes* (4.0±1.2%) and *Chloroflexi* (3.7±0.8%). The probe MX825-hybridized *Methanosaeta* (7.6±0.8%) was the most abundant archaeal group, followed by *Methanomicrobiales* (2.8±0.6%) and *Methanobacteriaceae* (2.7±0.4%). The functional community structures (diversity and relative abundance) of major trophic groups were quantitatively analyzed by MAR–FISH. The results revealed that glucose-degrading microbial community had higher abundance (ca. 10.6±4.9% of total DAPI-stained cells) and diversity (at least seven phylogenetic groups) as compared with fatty acid-utilizing microbial communities, which were more specialized to a few phylogenetic groups. Despite the dominance of *Betaproteobacteria*, members of *Chloroflexi*, *Smithella*, *Syntrophomonas* and *Methanosaeta* groups dominated the [^{14}C]glucose-, [^{14}C]propionate-, [^{14}C]butyrate- and [^{14}C]acetate-utilizing microorganism community, and accounted for 27.7±4.3%, 29.6±7.0%, 34.5±7.6% and 18.2±9.5%, respectively. In spite of low abundance (ca. 1%), the hitherto unknown metabolic functions of *Spirochaeta* and candidate phylum of TM7 as well as *Synergistes* were found to be glucose and acetate utilization, respectively.

1. Introduction

Anaerobic digestion is the biological decomposition process regulated by mutual metabolic interactions among at least three functional groups of microorganisms in the absence of oxygen. The first community of microorganisms hydrolyzes complex polymeric substances mainly lipids, cellulose and protein to fundamental structural building blocks such as glucose and amino acids. The second microbial community subsequently ferments these products

*Corresponding author. Tel.: +81 11 706 6266; fax: +81 11 707 6266.
E-mail address: sokabe@eng.hokudai.ac.jp (S. Okabe).
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(e.g. glucose and amino acids) to fatty acids, acetate and hydrogen. This acidogenesis process was reported to be most important and a critical step among decomposition processes in anaerobic digesters (Mawson et al., 1991). Finally, the third community converts acetate and hydrogen to methane and carbon dioxide (methanogenesis). Stable operation of anaerobic digester requires that these functional microbial communities to be in dynamic equilibrium.

The most susceptible members in anaerobic sludge digester are known to be the propionate-, butyrate- or acetate-utilizing microorganisms. Accumulation of these organic acids will cause pH depletion, which has a drastically adverse impact upon the entire microbial consortia and methane production. Moreover, the diversities and distributions of these functional groups and their contributions to the overall anaerobic digesting processes are still poorly understood. A better understanding of diversities and population sizes of those functional microbial communities is required to enhance the performance and stability of anaerobic sludge digester.

Some studies have depicted the microbial community structures of anaerobic digester sludge (Godon et al., 1997; Chouari et al., 2005) and UASB granules (Sekiguchi et al., 1998) using a 16S rRNA approach. Due to the limitation of 16S rRNA gene-cloning analysis in quantifying the system (Head et al., 1998), fluorescent in situ hybridization (FISH) (Harmsen et al., 1996; Sekiguchi et al., 1999) and dot blot hybridization (Chouari et al., 2005) techniques have been used to quantify those community structures. However, no study has comprehensively described functional community structures of the major trophic groups in a full-scale anaerobic sludge digester yet. To analyze the functional microbial community structure, appropriate analytical techniques that can distinguish the in situ metabolic function without isolation and enrichment must be sought and applied. The microautoradiography combined with FISH technique (MAR–FISH) is currently the most widely applied tool for directly linking phylogenetic identification with in situ metabolic activity of microorganisms within a complex microbial community at a single-cell resolution (Lee et al., 1999; Okabe et al., 2004). MAR–FISH is, however, generally limited by the lack of taxonomic resolution of FISH probes and availability of appropriate FISH probes. Therefore, additional technique such as micromanipulation followed by 16S rRNA sequencing analysis is also of necessity to identify intriguing cells that cannot be identified by MAR–FISH (Thomsen et al., 2004).

The objective of this study was, therefore, to characterize and quantify functional community structures of major trophic groups: glucose-, propionate-, butyrate- and acetate-utilizing microbial communities in a full-scale anaerobic sludge digester. First, phylogenetic differentiation (identification) of bacterial and archaeal communities in anaerobic digester sludge was performed by 16S rRNA gene-cloning analysis and FISH with a comprehensive set of 16S and 23S rRNA-targeted oligonucleotide probes. Second, the community structures (diversity and relative abundance) of these major trophic groups were quantitatively analyzed by MAR–FISH.

2. Materials and methods

2.1. Sludge samples

Sludge samples were collected from a real full-scale anaerobic digester plant in Ebetsu City (Hokkaido, Japan), which treats the excess sludge of domestic wastewater treatment facility served for 120,000 population equivalents. This plant is a mesophilic two-phase anaerobic digester, which is operated at 40 °C with an organic loading rate of 2.5 $kg\,m^{-3}\,day^{-1}$ and solid retention time (SRT) of 33 days. The pH is maintained around 7.5 during the operation. The gas production of this full-scale plant is ca. $1.3 \times 10^6\,m^3\,year^{-1}$, containing 55% methane. No electron acceptors were detected in the supernatant of the sludge. However, phosphate (ca. 4 mM) was only detected to be important anion in the sludge.

2.2. Extraction, PCR amplification, cloning and sequencing of 16S rRNA gene

One milliliter of slurry was sampled and then immediately subjected to three cycles of freezing in liquid N_2 and thawing in a 65 °C water bath (3 min at each temperature). After the freeze–thaw cycles, 0.2 ml of the slurry samples were subjected to DNA extraction. DNA was extracted using Fast DNA Spin Kit (BIO101) as described in the manufacturer's instructions. Amplification of 16S rRNA genes from purified genomic DNA was carried out using two primer sets of B8f-U1492r and B63f-B1387r for bacterial community and three primer sets of A25f-A1391r, A25f-U1492r and A109f-U1492r for archaeal community (Table 1). To minimize nonspecific annealing of the primers to nontarget DNA, a hot-start PCR program was used for all amplifications. The conditions used for the PCR were as follows: 5 min of initial denaturation at 94 °C and 20 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. Final extension was carried out for 8 min at 72 °C. The PCR products were evaluated on a 1% (w/v) agarose gel and purified using S. N. A. P.™ UV-Free Gel Purification Kit (Invitrogen). One microliter of the purified PCR products was directly ligated into the pGEM-T vector cloning system (Promega) and transformed into competent cells (high efficiency *E. coli* JM109 (Promega)) as described in the manufacturer's instructions. Plasmids were extracted and purified from clones with the Wizard Plus Minipreps DNA purification system (Promega) in accordance with the manufacturer's instructions. The sequencing 16S rRNA inserts was performed with an automatic sequencer (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems). All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (RDP) (Maidak et al., 1997) and compared with similar sequences of the reference organisms by BLAST search (Altschul et al., 1990). Sequence data were aligned with the CLUSTAL W package (Thompson et al., 1994). Phylogenetic trees were generated by using neighbor-joining algorithms (Saito and Nei, 1987). Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of tree topologies.

Table 1 – Sequences and target positions of the primers used in this study

Name	Sequence (5′–3′)	Target	16S rRNA target site (*E. coli* numbering)	Reference
B8f	AGRGTTTGATCCTGGCTCAG	SSU rRNA bacteria	8–27	Lane (1991)
A25f	CYGGTYGATYCTGCCRG	SSU rRNA archaea	25–41	Dojka et al. (1998)
B63f	CAGGCCTAACACATGCAAGTC	SSU rRNA bacteria	63–83	Marchesi et al. (1998); Osborn et al. (2000)
A109f	ACKGCTCAGTAACACGT	SSU rRNA archaea	109–125	Whitehead and Cotta (1999)
B341f	CCTACGGGAGGCAGCAG	SSU rRNA bacteria	341–357	Muyzer et al. (1993)
U530f	GTGCCAGCMGCCGCGG	SSU rRNA universal	530–545	Lane (1991)
U926f	AAACTYAAAKGAATTGACGG	SSU rRNA universal	926–945	Lane (1991)
B1387r	GGGCGGWGTGTACAAGGC	SSU rRNA bacteria	1387–1404	Marchesi et al. (1998)
A1391r	GACGGGCGGTGTGTRCA	SSU rRNA archaea	1391–1407	Barns et al. (1994)
U1492r	GGTTACCTTGTTACGACTT	SSU rRNA universal	1492–1510	Lane (1991)

3. MAR–FISH

3.1. *Incubation with radiolabeled substrates*

Four anaerobic batch cultures of sludge samples with different organic substrates (i.e. glucose, propionate, butyrate and acetate) were prepared in duplicate. The substrate concentration used for each culture was 5 mM supplemented with mineral solution (Sekiguchi et al. (1998) with some modifications) containing the following compositions (in $mg\,l^{-1}$): $(NH_4)_2HPO_4$ (28), KCl (2.8), NH_4Cl (32), $FeCl_3 \cdot 6H_2O$ (16), $MgCl_2 \cdot 6H_2O$ (32), $MnSO_4 \cdot 6H_2O$ (0.2), $CuSO_4 \cdot 5H_2O$ (0.2), $CoCl_2 \cdot 6H_2O$ (0.2), $NiCl_2 \cdot 6H_2O$ (0.2) and Zn powder (0.2). Anaerobic digester sludge taken from Ebetsu City anaerobic digester plant was washed with the mineral solution using sequential centrifugation (5 min at 2500*g*) and resuspension. The sludge and mineral solution were gently mixed, and then 3 ml of the mixtures were transferred to 5-ml serum bottles.

The cultures were flushed and evacuated with oxygen-free N_2 gas to remove the oxygen and then preincubated for 1 h at 37 °C to ensure that oxygen was completely depleted. Subsequently, the samples were supplemented with radioactively labeled substrates (final radioactivity, 50, 125, 125 and 150 μCi per 3-ml culture of glucose, propionate, butyrate and acetate, respectively) by injecting into the serum bottles anaerobically with sterile syringes. The following radioactively labeled substrates (American Radiolabeled Chemicals Inc.) were used: (i) [U-^{14}C]glucose (287 mCi $mmol^{-1}$), (ii) sodium [1-^{14}C]propionate (56.7 mCi $mmol^{-1}$), (iii) sodium [1-^{14}C]butyrate (56 mCi $mmol^{-1}$) and (iv) sodium [1-^{14}C]acetate (specific activity, 57 mCi $mmol^{-1}$). The vials were then anaerobically incubated by shaking at 30 rpm and 37 °C for 9 h. Controls were prepared by pasteurizing the sludge at 70 °C for 30 min and run in parallel for all substrates.

To determine an appropriate incubation times in our MAR–FISH set-up, in which the interference of substrate cross-feeding can be minimized, time course MAR–FISH experiments were conducted in duplicate for each radiolabeled substrate in the similar manner as described above. Initial substrate concentrations were set at 5 mM. Liquor subsamples were withdrawn at 3, 9, 12 and 24 h of incubation period from the duplicated serum incubation bottles for each substrate. These subsamples were then fixed and washed for in situ hybridization using all probes listed in Table 2 and autoradiographic developing procedures as described below. The MAR-positive cells that were simultaneously hybridized with each probe were directly counted as described below. Based on the result of this time course MAR–FISH experiments, the incubation time was determined to be 9 h for each substrate in this study.

3.2. *Liquid scintillation counting*

The uptake of radioactive substrates by biomass was measured by liquid scintillation counting in all cultures before the FISH and microautoradiographic procedures. The ^{14}C contents were measured directly in the culture samples (biomass plus culture medium). A 0.2 ml portion of aliquots were withdrawn from each culture and centrifuged at 13,000*g* for 5 min. The harvested biomass was washed three times with tap water and transferred to 3 ml of scintillation liquid (Ultima Gold XR; Packard Instrument Co., Meriden, CT). After completion of mixing and storing at room temperature for 24 h, the radioactivity of the biomass was quantified with a Packard model 1600 TR liquid scintillation analyzer. Another 0.2 ml portion of aliquots withdrawn from each culture were directly transferred to 3 ml of scintillation liquid to quantify total radioactivity of the culture. Finally, the percentage of

Table 2 – FISH oligonucleotide probes used in this study

Probe	Sequence (5′–3′)	rRNA target site (*E. coli* numbering)	Specificity	% FA[a]	Reference or source
EUB338	GCTGCCTCCCGTAGGAGT	16S (338–355)	Most but not all *Bacteria*	—[b]	Amann et al. (1990)
EUB338-II	GCAGCCACCCGTAGGTGT	16S (338–355)	Bacterial groups not covered by EUB338 and EUB338-III	—[b]	Daims et al. (1999)
EUB338-III	GCTGCCACCCGTAGGTGT	16S (338–355)	Bacterial groups not covered by EUB338 and EUB338-II	—[b]	Daims et al. (1999)
HGC69A	TATAGTTACCACCGCCGT	23S (1901–1918)	*Actinobacteria* (high-G+C Gram-positive bacteria)	25	Roller et al. (1994)
LGC354A[c]	TGGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes* (low-G+C Gram-positive bacteria) not including *Syntrophomonas* group	35	Meier et al. (1999)
LGC354B[c]	CGGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes* (low-G+C Gram-positive bacteria) not including *Syntrophomonas* group	35	Meier et al. (1999)
LGC354C[c]	CCGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes* (low-G+C Gram-positive bacteria) not including *Syntrophomonas* group	35	Meier et al. (1999)
BET42a[d]	GCCTTCCCACTTCGTTT	23S (1027–1043)	*Betaproteobacteria*	35	Manz et al. (1992)
GAM42a[e]	GCCTTCCCACATCGTTT	23S (1027–1043)	*Gammaproteobacteria*	35	Manz et al. (1992)
ALF968	GGTAAGGTTCTGCGCGTT	16S (968–985)	*Alphaproteobacteria*, except of *Rickettsiales*	20	Neef (1997)
SmiSR354	CGCAATATTCCTCACTGC	16S (354–371)	*Syntrophus* group including *Smithella propionica*	10	This study
Synbac824	GTACCCGCTACACCTAGT	16S (824–841)	*Syntrophobacter* group including *Syntrophobacter wolinii*	10	This study
Syn773	CTAGCTTTCGCACATGAG	16S (756–773)	*Synergistes* group	15	Chouari et al. (2005)
CFB719	AGCTGCCTTCGCAATCGG	16S (719–736)	*Bacteroidetes*	30	Weller et al. (2000)
CTE	TTCCATCCCCCTCTGCCG	16S (659–676)	*Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.*	20	Schleifer et al. (1992)
GNSB-941	AAACCACACGCTCCGCT	16S (941–957)	*Chloroflexi*	35	Gich et al. (2001)
Synm700	ACTGGTRTTCCTCCTGATTTCTA	16S (700–722)	*Syntrophomonas* group	30	Hansen et al. (1999)
TM7905	CCGTCAATTCCTTTATGTTTTA	16S (905–926)	TM 7 candidate phylum	20	Hugenholtz et al. (2001)
Spiro1400	CTCGGATGGTGTGACGGGCG	16S (1400–1419)	*Spirochaeta*	35	Daly and Shirazy-Beechey (2003)
ARC915	GTGCTCCCCCGCCAATTCCT	16S (915–934)	*Archaea*	35	Raskin et al. (1994)
MSMX860	GGCTCGCTTCACGGCTTCCCT	16S (860–880)	*Methanosarcinales* (all *Methanosarcina* and *Methanosaeta*)	45	Raskin et al. (1994)
MX825	TCGCACCGTGGCCGACACCTAGC	16S (825–847)	Some *Methanosaetaceae*	50	Raskin et al. (1994)
MS821	CGCCATGCCTGACACCTAGCGAGC	16S (821–844)	*Methanosarcina*	40	Raskin et al. (1994)
MS1414	CTCACCCATACCTCACTCGGG	16S (1414–1434)	Genus I, II, IV and V of *Methanosarcinaceae*	50	Raskin et al. (1994)
MB1174	TACCGTCGTCCACTCCTTCCTC	16S (1175–1196)	*Methanobacteriaceae*	45	Raskin et al. (1994)
MG1200	CGGATAATTCGGGGCATGCTG	16S (1200–1220)	Family I, II and III of *Methanomicrobiales*	30	Raskin et al. (1994)

[a] FA, formamide concentration in the hybridization buffer.
[b] The probe can be used at any formamide concentrations.
[c] Mixture of LGC354A, LGC354B and LGC354C, further termed as LGC354 was used to identify and enumerate *Firmicutes*/low-G+C Gram-positive bacteria.
[d] Unlabeled probe GAM42a was used as a competitor to enhance the specificity.
[e] Unlabeled probe BET42a was used as a competitor to enhance the specificity.

radioactivity incorporated into the biomass was calculated to evaluate the uptake of radioactive substrates.

3.3. Sample fixation and washing

After 9-h incubation with radioactive substrates, the samples were fixed for 3 h at 4 °C by adding 3 ml of 8% paraformaldehyde, which resulted in a final concentration of 4%. Subsequently, the samples were centrifuged at 13,000*g* for 5 min and washed three times with 2 ml of phosphate-buffered saline (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) to remove excess soluble radioactive substrates. After the fixation and washing steps, the samples were homogenized with a microtube grinder (Funakoshi Co., Ltd., Tokyo) for 5 min, spotted on a gelatin-coated cover glass in duplicate and air dried (Ito et al., 2002).

3.4. Fluorescent in situ hybridization

Dehydration with ethanol and in situ hybridization were conducted according to the procedure described by Amann et al. (1995) and Okabe et al. (1999). Some probes were newly designed in this study by using the PROBE_DESIGN tool of the ARB software package (http://www.arb-home.de/) according to the current version of the 16S rRNA sequence database. The probes were labeled with fluorescein isothiocyanate (FITC), tetramethylrhodamine 5-isothiocyanate (TRITC) or the sulfoindocyanine dye Cy5 at the 5′ end. The sequences, formamide (FA) concentrations and specificities of the probes used in this study were listed in Table 2. The specificities of the newly designed probes were verified using ARB program. The optimal hybridization conditions of those newly designed probes and some previously reported oligonucleotide probes were experimentally determined and evaluated using reference strains (e.g. *Smithella propionica* (OCM-661), *Syntrophus gentianae* (DSM-8423), *Syntrophobacter wolinii* (DSM-2805), *Syntrophomonas sapovorans* (DSM-3441), *Spirochaeta stenostrepta* (DSM-2028) and *Synergistes jonesii* (ATCC-49833)). Simultaneous hybridizations with the probes requiring different stringency conditions were performed by using a successive hybridization procedure; hybridization with the probe requiring higher stringency was performed first and then hybridization with the probe requiring lower stringency was performed. After the completion of in situ hybridization procedure, some samples were stained with DAPI to enumerate total cell numbers by the direct counting method (Hobbie et al., 1977).

3.5. Autoradiographic developing procedure

Following FISH, the autoradiographic procedure was performed directly on the cover glasses by using liquid film emulsion (LM1; Amersham Pharmacia Biotech) (Lee et al., 1999). The optimum exposure time was adjusted to 5 days.

3.6. Microscopy and enumeration by MAR–FISH

A model LSM510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Oberkochen, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (450–514 nm) and two HeNe lasers (543 and 633 nm) was used for microscopic observation and enumeration. The formation of silver grains in the autoradiographic film was observed by using the transmission mode of the system. To prevent miscounting of MAR-positive cells by the appearance of background silver grains, a cell with at least five silver grains was considered as MAR positive. The numbers of MAR-positive cells and total probe-hybridized cells (or total MAR-positive cells) were determined by directly counting at least 1000 silver grain-covered cells in randomly chosen microscopic fields of 3–5 slides prepared for each sample and each probe. Furthermore, this counting procedure was repeated four times. The average values were calculated from these duplicate measurements. All image combining, processing and analysis were performed with the standard software package provided by Zeiss. Processed images were printed out by using the software package Adobe Photoshop 3.0J (Adobe Systems Incorporated, Mountain View, CA).

3.7. Micromanipulation

To identify *Betaproteobacteria* cells that are dominant in glucose-, propionate-, butyrate- and acetate-utilizing communities, FISH using BET42a probe was performed on the slurry samples dispersed on a cover glass. FISH protocol was the same as described by Amann et al. (1995) except the use of a cover glass instead of a slideglass. One unique *Betaproteobacteria* cell, which showed significant MAR positive after incubation with each of [^{14}C]glucose, [^{14}C]propionate, [^{14}C]butyrate and [^{14}C]acetate, was scraped off the cover glass with a micromanipulator (TransferMan NK 2; Eppendorf) equipped with a flexible flange microcapillary (TransferTip®-F, inner/outer diameters; 4/7 μm) under an inverse microscope (Axiovert 200, Carl Zeiss, Germany). The cell-held tip of the microcapillary was inserted into a PCR tube containing PCR reaction mixture solution (TaKaRa Bio), then snapped off in the PCR tube and left in the tube as the template DNA for PCR reaction. After repeating the cycle of freezing and thawing three times, a primer pair (B8f-U1492r) was added into the PCR tube. The hot-start PCR with EX Taq (TaKaRa Bio) was carried out as follows: after initial denaturation at 94 °C for 10 min, 40 cycles consisting of 94 °C for 1 min; 55 °C for 1 min and 72 °C for 2 min, followed by final extension at 72 °C for 10 min. The PCR product was purified and cloned as described above. This micromanipulation was repeated several times for other BET42a probe-hybridized cells.

4. Results

4.1. Overall phylogenetic analysis

In total, 521 clones were obtained from the anaerobic digester sludge: among which 393 (75%) and 128 (25%) clones were belonging to the domain *Bacteria* and *Archaea*, respectively. These clones were grouped into 103 OTUs; 90 OTUs for the domain *Bacteria* and 13 OTUs for *Archaea*, on the basis of having more than 97% sequence similarity within an OTU (Table 3). FISH analysis with bacterial-domain probe (EUB338, EUB338-II and EUB338-III) and archaeal-domain probe

Table 3 – Distribution of 16S rRNA clones detected in the full-scale anaerobic sludge digester

Group	No. of OTUs[a]	No. of clones	Percentage of clones[b]	No. of OTUs matched with specific probe[c]	Percentage of clones matched with specific probe[d]
Bacteria					
Bacteroidetes	17	82	21	10	49
Firmicutes	25	84	21	15	52
Chloroflexi/GNSB	6	55	14	4	75
Alphaproteobacteria	7	16	4	4	50
Betaproteobacteria	4	27	7	—[e]	—[e]
Deltaproteobacteria	5	28	7	4[f]	71[f]
Gammaproteobacteria	4	23	6	—[e]	—[e]
Spirochaeta	5	20	5	3	55
Actinobacteria	7	23	6	—[e]	—[e]
TM7	2	9	2	2	100
Others	8	26	7	—[g]	—[g]
Total *Bacteria*	90	393	100		
Archaea					
Methanosaeta	5	101	79	3	51
Methanospirillum	4	19	15	2	63
Methanoculleus	1	4	3	1	100
Methanobacterium	2	3	2	1	67
Methanobrevibacter	1	1	1	1	100
Total *Archaea*	13	128	100		

[a] Operational taxonomic units.
[b] Percentages of each phylum-related clones compared to total clones of *Bacteria* and *Archaea*, respectively.
[c] The probes were listed in Table 2.
[d] Percentages of clones matched with specific probe compared to total clones in each phylum/subphylum.
[e] Not applicable because 23S rRNA-targeted probe was used for this group.
[f] Probes SmiSR354 and Synbac824 were used for quantification of *Deltaproteobacteria*.
[g] No probe was used for identification and quantification.

(ARC915) revealed that *Bacteria* and *Archaea* accounted for 55.4±8.2% and 26.9±7.5% of total DAPI-stained cells in this anaerobic digester sludge. Using a comprehensive set of 16S and 23S rRNA-targeted oligonucleotide probes for *Bacteria* and *Archaea*, we could detect ca. 50% of total DAPI-stained cells (Fig. 1). The total DAPI count of the anaerobic digester sludge was 1.2×10^{10} cells g-VSS^{-1} (standard deviation of triplicate measurements (SD); <5%).

4.2. *Diversity and abundance of bacteria domain*

The distribution of the 393 bacterial clones (90 OTUs) into the major bacterial phyla is as follows: 24% to the *Proteobacteria* (*Alpha*, *Beta*, *Gamma* and *Delta*), 21% to the *Firmicutes*, 21% to the *Bacteroidetes*, 14% to the *Chloroflexi* and 20% to the minor phyla (*Actinobacteria* (6%), *Spirochaeta* (5%), the candidate phylum of TM7 (2%) and others (7%)) (Table 3).

Based on this result, we performed FISH counting using a major bacterial phylum-specific probe set (Table 2) to quantify each phylogenetic group and could detect ca. 37% of the total DAPI-stained cells (Fig. 1). The Gram-positive bacteria were the major phylogenetic group in the sludge (Table 3). The high-G+C Gram-positive bacteria (*Actinobacteria*) that were hybridized with probe HGC69A represented the predominant phylogenetic group (8.5±1.4% of the total cells). The clones belonging to the low-G+C Gram-positive bacteria (*Firmicutes*) were more frequently detected (25 OTUs, 84 clones) than those belonging to *Actinobacteria* (7 OTUs, 23 clones). However, based on FISH counting, the abundance of the probe LGC354-hybridized *Firmicutes* group accounted for 3.8±0.8% of the total cells, which was lower than that of probe HGC69A-hybridized *Actinobacteria* group. This is perhaps because only 14 OTUs (besides one OTU matched with Synm700 probe) out of 25 OTUs belonging to the *Firmicutes* were matched with LGC354 probe sequence (Table 3). Two OTUs out of 25 OTUs belonging to the *Firmicutes* phylum were affiliated with hitherto-described butyrate-oxidizing bacteria, *Syntrophomonas flectens* and *Syntrophomonas sapovorans* (Zhang et al., 2004), with 93–96% similarity. The probe Synm700-hybridized *Syntrophomonas* accounted for 1.5±0.5% of the total cells (Fig. 1).

The phyla *Bacteroidetes* and *Chloroflexi* represented the second major phylogenetic groups in this sludge, accounting for 4.0±1.2% and 3.7±0.8% of total DAPI-stained bacteria using CFB719 (10/17 OTUs-matched) and GNSB-941 (4/6 OTUs-matched) probes, respectively (Table 3 and Fig. 1). The *Proteobacteria* group also represented the second major phylogenetic groups in this sludge, among which the distribution of *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria* were 4.1±0.7%, 3.9±0.6%, 3.2±0.4% and 2.5±0.5% (sum of the probes SmiSR354- and Synbac824-hybridized cells) of total DAPI-stained bacteria, respectively (Fig. 1). Three out of five OTUs belonging to the *Deltaproteobacteria* were related to a well-known propionate-oxidizing bacterium, i.e. *Smithella propionica* (Liu et al., 1999) with 95–97% similarity (Table 3). FISH quantification using probe SmiSR354 showed that this group accounted for 2±0.7% of the total DAPI-stained cells.

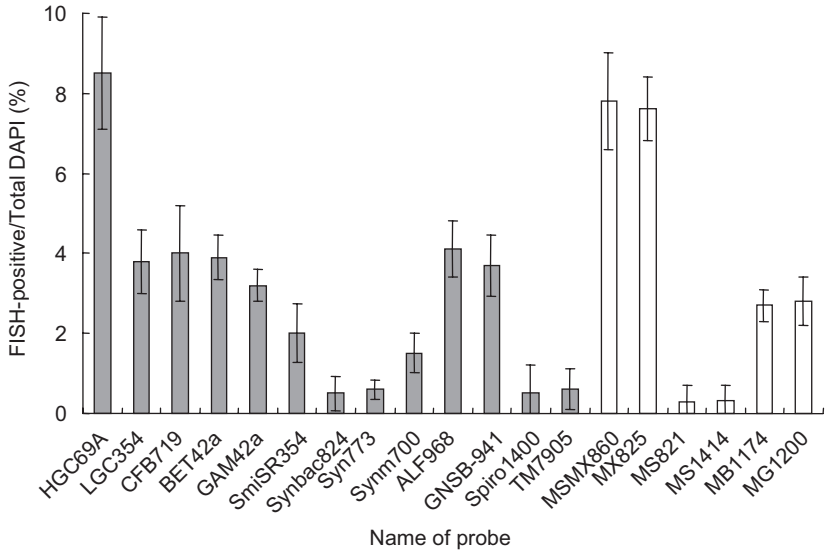

Fig. 1 – Relative abundance of microbial community structure in anaerobic sludge digester analyzed by fluorescent in situ hybridization using specific probes and DAPI staining as reference. Specific probes are for *Actinobacteria*/high-G+C Gram-positive bacteria (HGC69A), *Firmicutes*/low-G+C Gram-positive bacteria (LGC354), *Bacteroidetes* (CFB719), *Betaproteobacteria* (BET42a), *Gammaproteobacteria* (GAM42a), *Smithella* group (SmiSR354) and *Syntrophobacter* group (Synbac824) of *Deltaproteobacteria*, *Synergistes* (Syn773), *Syntrophomonas* group of low-G+C Gram-positive bacteria (Synm700), *Alphaproteobacteria* (ALF968), *Chloroflexi* (GNSB-941), *Spirochaeta* (Spiro1400), TM7 (TM7905), *Methanosarcinaceae* (MSMX860), *Methanosaeta* of *Methanosarcinaceae* (MX825), *Methanosarcina* of *Methanosarcinaceace* (MS821), genus I, II, IV and V of *Methanosarcinaceae* (MS1414), *Methanobacteriaceae* (MB1174) and family I, II and III of *Methanomicrobiales* (MG1200). Bars with dark color indicate the results of *Bacteria*-targeted probes, while colorless bars indicate the results of *Archaea*-targeted probes. The error bars indicate the standard deviation of duplicate measurements.

Probe Synbac824-hybridized *Syntrophobacter* group of *Deltaproteobacteria* accounted for 0.5±0.4% of the total cells (Fig. 1).

In addition, 5 OTUs (20 clones, 5% of the total clones) were affiliated with the *Spirochaeta*. Quantification by FISH with Spiro1400 probe showed that this group accounted for less than 1% (Fig. 1). Two OTUs (nine clones) belonging to the candidate phylum TM7 were identified (Table 3) and its abundance was quantified to be less than 1% of the total cells with TM7905 probe (Fig. 1).

4.3. *Diversity and abundance of Archaea domain*

The diversity of archaeal community within anaerobic digester sludge was shown in Table 3. Among the archaeal clones, 101 clones (five OTUs), corresponding to 79% of the total clones, were closely related to already known acetoclastic methanogenic genus of *Methanothrix* and *Methanosaeta*. The probe MX825-hybridized *Methanosaeta* and probe MS821-hybridized *Methanosarcina* accounted for 7.6±0.8% and less than 1% of the total DAPI-stained cells, respectively (Fig. 1). Three OTUs out of five *Methanosaeta*-related OTUs were matched with this MX825 probe (Table 3). The remaining eight archaeal OTUs (27 clones), corresponding to 21% of the total clones, were related to hydrogenotrophic methanogens such as *Methanobacterium*, *Methanobrevibacter*, *Methanospirillum* and *Methanoculleus* genus. The probe MB1174-hybridized *Methanobacteriaceae* (including *Methanobacterium* and *Methanobrevibacter* genus) and probe MG1200-hybridized *Methanomicrobiales* (including *Methanospirillum* and *Methanoculleus* genus) accounted for 2.7±0.4% and 2.8±0.6% of the total DAPI-stained cells, respectively (Fig. 1). Based on these FISH results, the ratio of acetoclastic methanogens to hydrogenotrophic methanogens was approximately 4:3.

4.4. *Time course MAR–FISH analysis*

To determine an appropriate incubation times in our MAR–FISH set-up, in which the interference of substrate cross-feeding can be minimized, MAR–FISH analyses at different incubation periods (i.e. 3, 9, 12 and 24 h) were conducted using all probes used in this study (Table 2). For MAR–FISH with [U-^{14}C]glucose, the probes HGC69A-, LGC354-, CFB719-, BET42a-, GAM42a-, GNSB-941, Spiro1400- and TM7905-hybridized cells were already MAR positive after 3-h incubation (Table 4). When we incubated longer (12 h), the probes SmiSR354- and Synbac824-hybridized cells (probably propionate-utilizing bacteria) gradually became MAR positive, but no methanogens were MAR positive yet. However, almost all the probes-hybridized cells became MAR positive after 24-h incubation, indicating that the starting substrate, glucose, was degraded down to CH_4 and CO_2. These results clearly indicated that substrate cross-feeding became evident when the cultures were incubated more than 12 h. For MAR–FISH with [1-^{14}C]propionate, the probes BET42a-, SmiSR354- and Synbac824-hybridized cells represented MAR positive after 3-h incubation. After 12-h incubation, additional probes Synm700- and LGC354-, MS821-, MS1414-, MB1174- and MG1200-hybridized cells became MAR positive. After 24-h

Table 4 – The relative abundance of MAR-positive cells that are simultaneously hybridized with specific FISH probes at different incubation times with radiolabeled glucose, propionate, butyrate and acetate

Probes	Incubation time (h)															
	[U-^{14}C]glucose				[1-^{14}C]propionate				[1-^{14}C]butyrate				[1-^{14}C]acetate			
	3	9	12	24	3	9	12	24	3	9	12	24	3	9	12	24
HGC69A	+	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–
LGC354	+	++	++	++	–	–	+	++	+	++	++	++	+	++	++	++
CFB719	+	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–
BET42a	+	++	++	++	+	++	++	++	+	++	++	++	+	++	++	++
GAM42a	+	++	++	++	–	–	–	++	–	–	+	++	+	++	++	++
SmiSR354	–	–	+	++	+	++	++	++	–	–	–	–	–	–	–	–
Synbac824	–	–	+	++	+	++	++	++	–	–	–	–	–	–	–	–
Syn773	–	–	–	+	–	–	–	+	–	–	+	+	+	+	+	+
Synm700	–	–	–	++	–	–	+	++	+	++	++	++	–	–	–	–
ALF968	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GNSB-941	+	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–
Spiro1400	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
TM7905	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
MSMX860	–	–	–	++	–	–	–	++	–	–	+	++	+	++	++	++
MX825	–	–	–	++	–	–	–	++	–	–	+	++	+	++	++	++
MS821	–	–	–	+	–	–	+	+	–	–	+	+	+	+	+	+
MS1414	–	–	–	+	–	–	+	+	–	–	+	+	+	+	+	+
MB1174	–	–	–	+	–	–	+	++	–	–	+	++	–	–	+	++
MG1200	–	–	–	+	–	–	+	++	–	–	+	++	–	–	+	++

Notes:
–: None.
+: <5% of the total MAR-positive cells.
++: ⩾5% of the total MAR-positive cells.

incubation, most of the probe-hybridized cells, except HGC69A-, CFB719-, ALF968-, GNSB-941-, Spiro1400- and TM7905-hybridized cells that were probably glucose-utilizing bacteria (see the column of MAR–FISH with glucose), became MAR positive. For MAR–FISH with butyrate and acetate, the similar successive composition changes of MAR-positive cells were observed. Based on these experimental data, we have chosen the incubation time of 9 h in all the following MAR–FISH experiments.

4.5. *Diversities and abundances of glucose-, propionate-, butyrate- and acetate-utilizing microbial communities*

Among total microbial community, we first determined the population sizes of glucose-, propionate-, butyrate- and acetate-utilizing microbial community by directly counting MAR-positive cells that simultaneously stained with DAPI after 9-h incubation with each substrate. As a result, the glucose-, propionate-, butyrate- and acetate-utilizing bacteria accounted for 10.6±4.9%, 3.9±2.1%, 3.4±1.9% and 6.0±2.1% of total DAPI-stained cells, respectively. These 24% of the total cells were then further characterized by MAR–FISH using a comprehensive set of group-specific FISH probes (Figs. 2 and 3).

4.5.1. *Glucose-utilizing microbial community*

The majority of glucose-utilizing bacteria was *Betaproteobacteria*, *Chloroflexi* and high-G+C Gram-positive bacteria accounting for 39.3±5.6%, 27.7±4.3% and 13.3±4.2% of the total glucose-utilizing MAR-positive cells, respectively (Fig. 3A). However, accumulation of silver grains around *Betaproteobacteria* was very low as compared with other MAR-positive bacteria, suggesting that *Betaproteobacteria* did not actively utilize glucose. In contrast, the probe GNSB-941-hybridized *Chloroflexi* cells were heavily covered with dense silver grains, indicating active utilization of glucose (Fig. 2A). Other minor groups such as *Spirochaeta* and TM7 groups were clearly shown to be MAR positive and accounted for 0.5±1.0% and 1.4±1.9% of the total glucose-utilizing MAR-positive cells, respectively.

4.5.2. *Propionate-utilizing microbial community*

The propionate-utilizing microbial community consisted of only a few phylogenetic groups: *Betaproteobacteria* (48.4±7.6%), the probe SmiSR354-hybridized *Smithella* group in *Deltaproteobacteria* (29.6±7.0%) (Fig. 2B) and the probe Synbac824-hybridized *Syntrophobacter* (12.1±4.2%) (Fig. 3B). The diversity of propionate-utilizing bacterial populations was smaller than that of glucose-utilizing bacterial populations.

4.5.3. *Butyrate-utilizing microbial community*

The butyrate-utilizing microbial community was composed of three phylogenetic groups; *Betaproteobacteria* (47.6±8.7%) (Fig. 2C), the probe Synm700-hybridized *Syntrophomonas* group

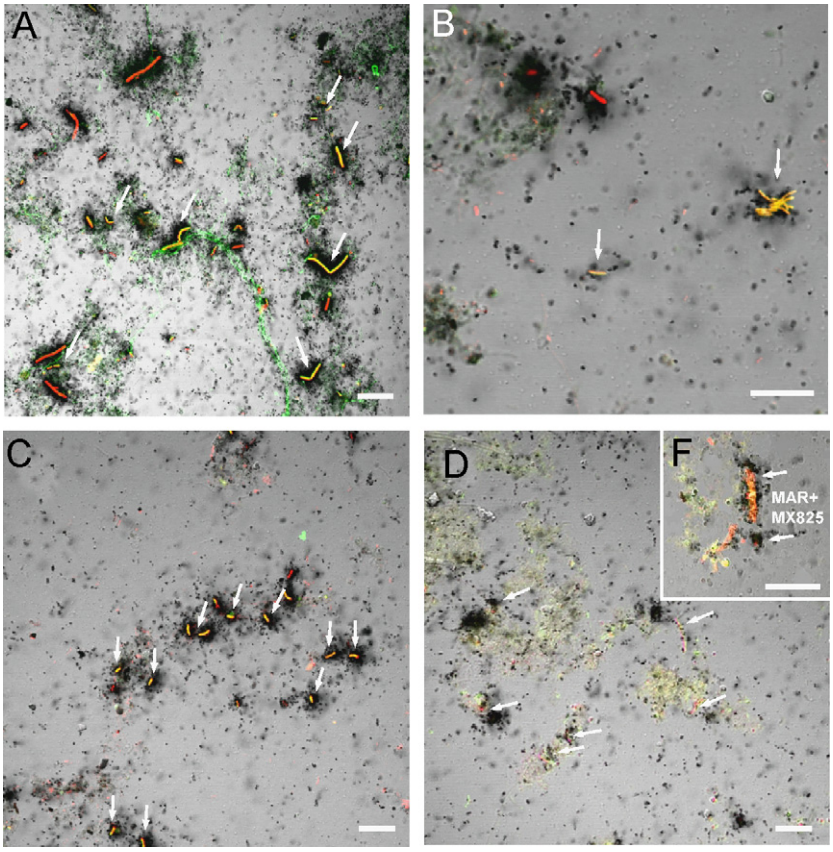

Fig. 2 – Substrate uptake patterns of bacterial and archaeal communities in anaerobic digester sludge using MAR–FISH technique. (A) MAR-positive FITC-labeled probe GNSB941-hybridized *Chloroflexi* cells (arrows), (B) MAR-positive FITC-labeled probe SmiSR354-hybridized *Smithella* cells (arrows), (C) MAR-positive FITC-labeled probe BET42a-hybridized cells (arrows) and (D and F) MAR-positive FITC-labeled probe MX825-hybridized cells (arrows). [^{14}C]glucose (A), [^{14}C]propionate (B), [^{14}C]butyrate (C) and [^{14}C]acetate (D and F) were used as a sole substrate and tracer. Double staining were conducted using mixture of TRITC-labeled EUB338, EUB338-II and EUB338-III probes for A, B and C, thus, the target cells were stained in yellow. For D and F, double staining were conducted using TRITC-labeled ARC915 probe. All bars represent 10 μm of length.

(well-known butyrate-oxidizing bacteria) (34.5±7.6%) and the probe LGC354-hybridized *Firmicutes* (11.1±3.6%) (Fig. 3C).

4.5.4. *Acetate-utilizing microbial community*

Acetate was utilized by both bacterial and archaeal communities: *Bacteria* and *Archaea* contributed to ca. 75% and 25% of the total MAR-positive cells, respectively (Fig. 3D). For bacterial community, the *Betaproteobacteria* dominated the MAR-positive cells (55.3±6.3% of the total MAR-positive cells), followed by the probe LGC354-stained *Firmicutes* (10.2±3.6% of the total MAR-positive cells), the probe GAM42a-stained *Gammaproteobacteria* (5.2±2.1% of the total MAR-positive cells) and the probe Syn773-stained *Synergistes* group (1.3±1.8% of the total MAR-positive cells). For archaeal community, the probe MX825-hybridized *Methanosaeta*, a well-known acetoclastic methanogen, was the predominant acetate-utilizing archaea in this digester sludge (18.2±9.5% of the total MAR-positive cells) (Fig. 2D and F). The probe MS821-hybridized *Methanosarcina* was the second major archaeal group and accounted for 1.4±2.2% of the total MAR-positive cells.

4.6. *Identification of the most abundant functional group, Betaproteobacteria*

The MAR–FISH analysis indicated that *Betaproteobacteria* was the most dominant group in propionate-, butyrate- and acetate-utilizing microbial communities. To acquire the phylogenetic identity of *Betaproteobacteria*, MAR-positive *Betaproteobacteria* cells in each propionate-, butyrate- and acetate-incubated sample were collected by means of a micromanipulation and then the 16S rRNA gene clone sequencing was performed after PCR amplification with a primer set of B8f-U1492r (Table 1). The phylogenetic analysis showed that a half of the clones retrieved from propionate-, butyrate- and acetate-incubated samples were phylogenetically closest to the genus *Variovorax* (18 out of 33 clones), which had 99% sequence similarity with *Variovorax paradoxus* (D88006) (Fig. 4). Moreover, about 90% of the total MAR-positive *Betaproteobacteria* cells in each sample were simultaneously hybridized with CTE probe that is specific for the subgroup I of *Betaproteobacteria* including *Variovorax* spp. These results indicated that most of the propionate-, butyrate- and acetate-utilizing MAR-positive *Betaproteobacteria* were

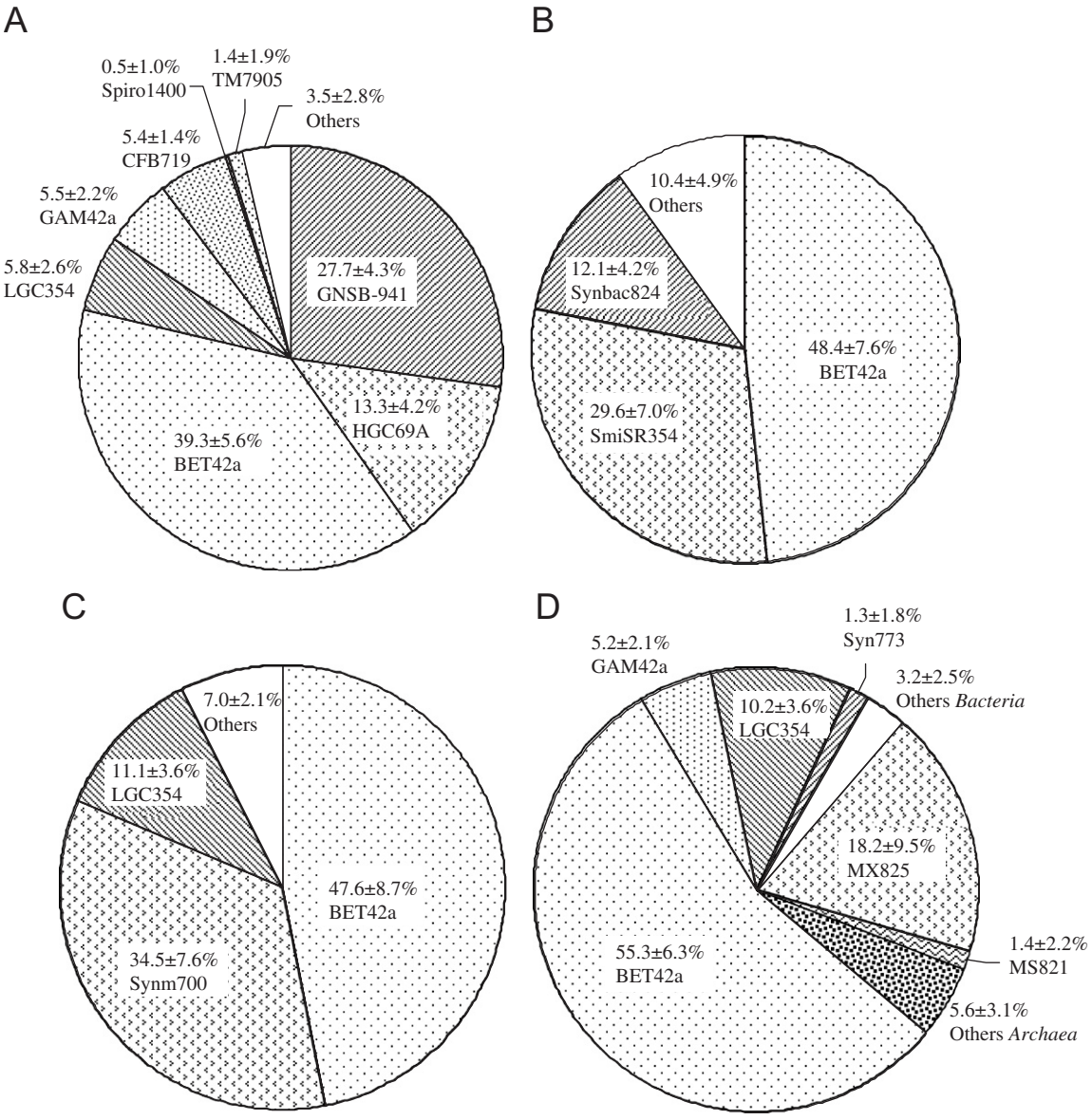

Fig. 3 – Community compositions of (A) glucose-, (B) propionate-, (C) butyrate- and (D) acetate-utilizing microorganisms based on direct counting the MAR-positive cells that were simultaneously hybridized with specific FISH probe listed in Table 2. The values represent the percentages of each specific probe hybridized MAR-positive cells to total MAR-positive cells ± standard deviations of duplicate measurements.

affiliated with *Comamonadaceae*. Furthermore, these MAR-positive *Betaproteobacteria* cells were stained in bright yellow with highly concentrated DAPI solution, indicating that these cells contained polyphosphate or lipid granules (Streichen et al., 1990).

5. Discussion

5.1. *General overview of microbial communities*

In this study, we have comprehensively characterized bacterial and archaeal community structures within a full-scale anaerobic sludge digester, including their diversities, abundances and functions. The *Bacteria* domain was extremely diverse and we detected 90 OTUs distributed among at least seven major phylogenetic groups. This high diversity was attributed to complex input of the anaerobic digester that originated from domestic wastewater sludge having a wide spectrum of substances (Chouari et al., 2005). FISH analysis revealed that the Gram-positive bacteria (including *Actinobacteria* and *Firmicutes*), *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* phyla represented the dominant phylogenetic groups. This is in accordance with previous study reported by Chouari et al. (2005).

In contrast, the archaeal community was composed of less phylogenetically diverse groups represented by 13 OTUs. This is probably because only two types of methanogens are known within the trophic chain: acetoclastic and hydrogenotrophic methanogens. The two major archaeal OTUs were acetoclastic *Methanosaeta* (representing 79% of the total

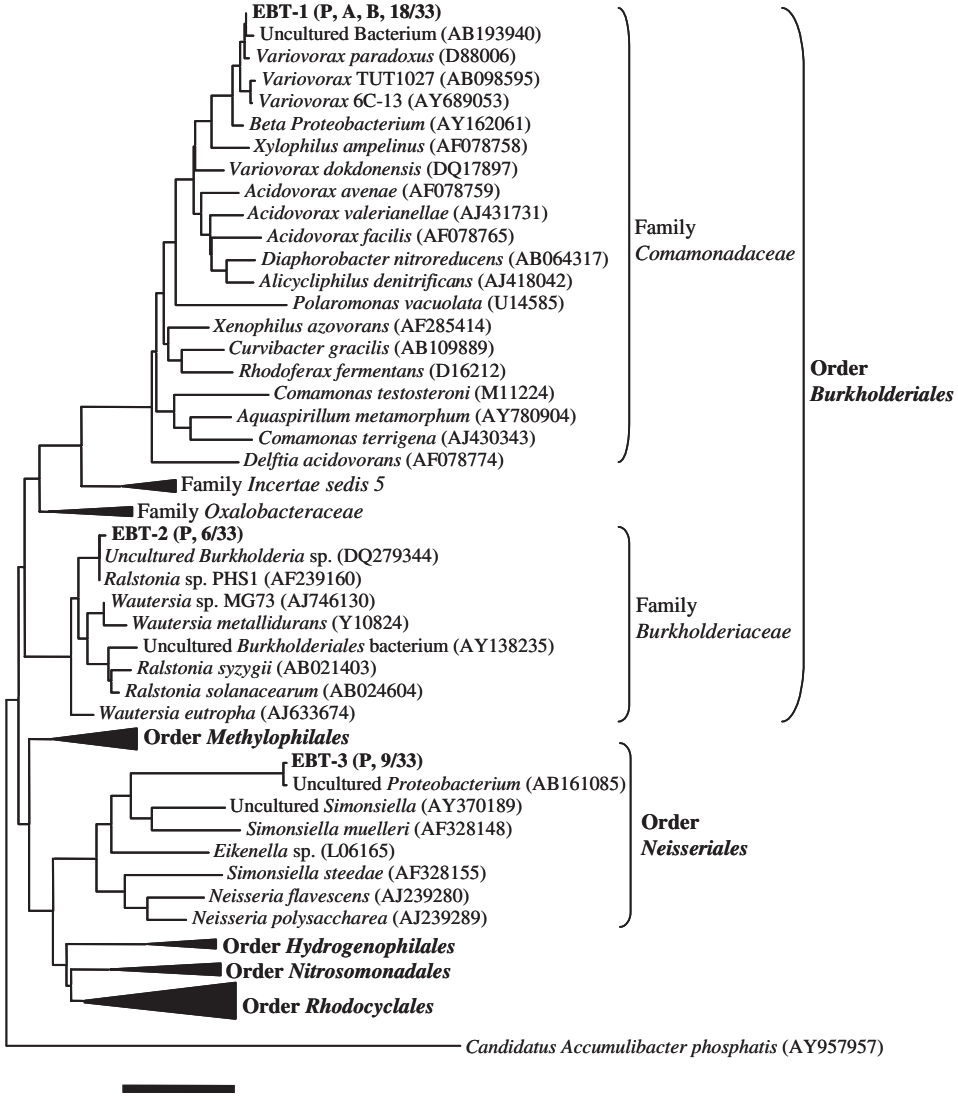

Fig. 4 – Phylogenetic affiliation of three propionate-, butyrate- and acetate-utilizing *Betaproteobacteria* OTUs (EBT-1, EBT-2 and EBT-3) among the members of *Betaproteobacteria* after micromanipulation followed by 16S rRNA gene-cloning analysis. The tree was constructed by using 1408 bp of the 16S rRNA genes and the neighbor-joining method. Numbers in parentheses represent accession numbers. Our OTUs represent the source of clones (P = propionate, B = butyrate and A = acetate) and the frequencies of appearance of identical clones in the clones analyzed. Family *Comamonadaceae* is the target of CTE probe. Scale bar = 5% sequence divergence.

archaeal clones) and hydrogenotrophic *Methanospirillum* (representing 15% of the total archaeal clones). This result is in agreement with previous studies by Raskin et al. (1994) and Godon et al. (1997). The FISH analysis also revealed that probe MX825-hybridized *Methanosaeta* and probe MG1200-hybridized *Methanomicrobiales* were two major archaeal groups, but the population size of both groups was comparable (Fig. 1). The discrepancy between cloning analysis and FISH is explained by non-quantitative nature of PCR-based clone libraries and low coverage of FISH probe (i.e. MX825).

5.2. *Community structure of major trophic groups*

The glucose-degrading microbial community had higher diversity represented by at least seven different phylogenetic groups (Fig. 3A) and abundance (ca. 10.6±4.9% of total DAPI-stained cells) as compared with other fatty acid-utilizing microbial communities, which were more specialized to a few phylogenetic groups. This is also probably one of the reasons why the fatty acid-utilizing microbial communities are often more susceptible to environmental changes than other groups.

For the propionate-utilizing microbial community, the probe SmiSR354-hybridized *Smithella* group dominated over the probe Synbac824-hybridized *Syntrophobacter* group (Fig. 3B). This is perhaps because no sulfate was present in the seed sludge. The *Syntrophobacter* group oxidizes propionate in the presence of sulfate as electron acceptor (Wallrabenstein et al., 1994). In contrast, *Smithella* group does not require sulfate as electron acceptor to oxidize propionate (Liu et al., 1999).

The MAR–FISH analysis indicated that *Betaproteobacteria* was the most dominant group in propionate-, butyrate- and acetate-utilizing microbial communities. There was no detectable electron acceptor in the digester sludge. However, phosphate (ca. 4 mM) was present. Furthermore, these MAR-positive *Betaproteobacteria* cells were stained in bright yellow with highly concentrated DAPI solution. Based on these results, we speculated that the uptake of these fatty acids by *Betaproteobacteria* is related to phosphate uptake. They might be either polyphosphate-accumulating organisms (PAOs) or glycogen-accumulating organisms (GAOs), both of which have an ability to accumulate fatty acids in anoxic conditions (Levantesi et al., 2002). In the literatures, *Rhodocyclus* group and *Candidatus Accumulibacter phosphates* have been reported as major PAOs and *Gammaproteobacteria* members and *Candidatus Competibacter phosphates* have been reported as major GAOs, respectively (Kong et al., 2004; Seviour et al., 2003; Levantesi et al., 2002). The result of micromanipulation followed by 16S rRNA gene sequence analysis showed that most of the propionate-, butyrate- and acetate-utilizing *Betaproteobacteria* were, however, affiliated with the *Variovorax* group, which had rod morphology (Fig. 2C) in single or pairs. Best to our knowledge, *Variovorax* has not been reported to be PAOs or GAOs previously. The microbial diversity of these groups is considered to be phylogenetically diverse (Seviour et al., 2003). The yellow color resulted from DAPI staining is not sufficient enough to indicate that our *Variovorax* as a member of PAO or GAO groups due to its nonspecific staining target. Further investigation is apparently required to identify their phylogeny, metabolic function and contributions to anaerobic digestion processes.

5.3. *Metabolic functions of minor phylogenetic groups with few cultivated representatives*

We also have identified the hitherto unknown functions of microorganisms affiliated with phylogenetic phyla represented by only few cultivated representatives, such as *Chloroflexi*, *Spirochaeta*, *Synergistes* and TM7. The sequences affiliated with these phyla were ubiquitously retrieved from various natural or engineered environments. The *Chloroflexi* group was often found in various wastewater treatments such as anaerobic digester (Sekiguchi et al., 2001, 2003) and biological nutrient removal processes (Björnsson et al., 2002). However, ecophysiological roles of *Chloroflexi* in such systems were not clearly identified to date. To our knowledge, it is the first time to demonstrate that *Chloroflexi* was one of the numerically important glucose-degrading bacterial groups in anaerobic sludge digester. In addition, the probe TM7905-hybridized bacteria, belonging to the candidate phylum TM7 with no cultivated representatives (Hugenholtz et al., 2001), was identified as glucose-utilizing bacteria. The inventory of this candidate phylum obtained from natural environments (Rheims et al., 1996) and engineered ecosystems (Hugenholtz et al., 2001) proved that this candidate phylum is ubiquitous and has wide diversity. The probe Spiro1400-hybridized *Spirochaeta* was also identified as glucose-utilizing bacteria. Although their relative abundances were low (both were less than 1% of total DAPI-stained cells), the existence of these phylogenetic groups in the anaerobic sludge digester system should not be ignored. This is also the first time to report the metabolic function of *Synergistes* group that was often found in anaerobic digesters (Godon et al., 1997, 2005; Sekiguchi et al., 1998). The MAR–FISH analysis clearly demonstrated that the probe Syn773-hybridized *Synergistes* utilized acetate under methanogenic environments (Fig. 3D).

All of the molecular-based techniques used in this study have some inherent biases and limitations, which must be recognized and minimized properly. To minimize the biases and limitations, we used different approaches to perceive a more realistic microbial community structure of anaerobic sludge digester. Different sets of primers were used to comprehensively retrieve 16S rRNA genes from the anaerobic sludge digester and 16S rRNA genes were amplified in triplicate PCR tubes, resulting in more representative clone libraries. Based on the results of clone library analysis, appropriate group-specific or OTU-specific FISH probes were selected and used to determine the OTU distribution in the anaerobic digester sludge. Using both the domain probes, EUB338-mixed and ARC915, 82.3% of the total DAPI-stained cells were detected (EUB-mixed probes: $55.4 \pm 8.2\%$ and ARC915: $26.9 \pm 7.5\%$). Using a set of 16S and 23S rRNA-targeted probes for the bacterial and archaeal populations (Table 2), only 50% of the total DAPI-stained cells were detected and characterized by FISH. DAPI targets DNA, which is present in the cells regardless of their activity and may even be preserved after cell death. Thus, the DAPI count generally targets a greater overall population than the rRNA-based FISH, which depends on the ribosome content within the cells that varies with the cell's activity levels.

Furthermore, the bacterial FISH probe set could cover about 67% of the bacterial populations detected with the EUB338-mixed probes, whereas the archaeal FISH probe set could cover 50% of the archaeal populations detected with ARC915 probe. Although more than half of the OTUs for each phylum was covered by group-specific FISH probes used in this study (Table 3), several phylotypes were still remaining uncovered and uncounted. The coverage of bacterial and archaeal community could be improved by using additional FISH probes to some extent. However, a part of cells present in the anaerobic digester are difficult to be detected by FISH due to low levels of rRNA content.

The probes of SmiSR354 and Synbac824 targeting for *Syntrophus* and *Syntrophobacter*, respectively, were newly designed in this study. Based on the result of the probe-match analysis by ARB, the Synbac824 probe had one mismatch with the genus *Syntrophobacter* and at least one mismatch with non-targets, whereas the SmiSR354 probe had one mismatch with some non-target microbial groups (*Nitrospira marina*, *Leptospirillum* spp., *Chlorobium* spp. and *Desulfobulbus* spp.). However, all of these non-target microbial groups were not detected in the digester sludge by 16S rRNA clone analysis. Furthermore, the abundance of *Desulfobulbus*-specific 660 probe-hybridized cells was negligible in the digester sludge (data not shown), and the SmiSR354-hybridized cells were not hybridized with the *Desulfobulbus*-specific 660 probe. Thus, the designed two probes, SmiSR354 and Synbac824, specifically hybridized with *Syntrophus* and *Syntrophobacter*, respectively, in this study.

For MAR–FISH analysis, substrate cross-feeding is of particular importance at prolonged incubation times (Okabe et al., 2004, 2005), which may lead to misinterpretation of the data. Therefore, we carefully designed our MAR–FISH experimental set-up (e.g. radiolabeled substrate concentrations, 9-h incubation and 5-day exposure) and confirmed that there was no significant interference caused by substrate cross-feeding for all substrates tested in this study (Table 4). For example, the probe SmiSR354-hybridized *Smithella*, probe Synbac824-hybridized *Syntrophobacter* and probe Synm700-hybridized *Syntrophomonas*, well-known propionate- and butyrate-utilizing bacteria, respectively, were identified as MAR negative during 9-h incubation with radiolabeled glucose. Moreover, the probe MX825-hybridized *Methanosaeta*, a well-known acetoclastic methanogen, was still MAR negative. These results clearly indicated that our MAR–FISH experimental set-up (9-h incubation and 5-day exposure) was appropriate to exclude the interference of possible substrate cross-feeding.

6. Conclusions

We have quantitatively characterized bacterial and archaeal community structures within a full-scale anaerobic sludge digester by combining 16S rRNA gene-cloning analysis, FISH and MAR–FISH. Based on the experimental results, the following conclusions were drawn:

1. The *Bacterial* community was composed of extremely diverse groups (at least seven major phylogenetic groups) represented by 90 OTUs, whereas, the *Archaea* community was composed of less phylogenetically diverse groups represented by 13 OTUs (acetoclastic *Methanosaeta* (79% of the total archaeal clones) and hydrogenotrophic *Methanospirillum* (15% of the total archaeal clones)).
2. *Chloroflexi* was one of the numerically important glucose-degrading bacterial groups in anaerobic sludge digester.
3. The hitherto unknown metabolic functions of *Spirochaeta* and candidate phylum of TM7 as well as *Synergistes* were found to be glucose- and acetate-utilizing bacteria, respectively.

A better understanding of the fundamental roles of *Bacteria* and *Archaea* including minor phylogenetic groups in anaerobic sludge digestion processes should aid in the evaluation and control of process operation.

Acknowledgment

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