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ORIGINAL ARTICLE

Identification of a novel acetate-utilizing bacterium belonging to *Synergistes* group 4 in anaerobic digester sludge

Tsukasa Ito[1], Kazumi Yoshiguchi[2], Herto Dwi Ariesyady[2] and Satoshi Okabe[2,3]
[1]*Department of Civil and Environmental Engineering, Graduate School of Engineering, Gunma University, Kiryu, Japan;* [2]*Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, Sapporo, Hokkaido, Japan and* [3]*Japan Science and Technology Agency, CREST, Tokyo, Japan*

Major acetate-utilizing bacterial and archaeal populations in methanogenic anaerobic digester sludge were identified and quantified by radioisotope- and stable-isotope-based functional analyses, microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) and stable-isotope probing of 16S rRNA (RNA-SIP) that can directly link 16S rRNA phylogeny with *in situ* metabolic function. First, MAR-FISH with ^{14}C-acetate indicated the significant utilization of acetate by only two major groups, unidentified bacterial cells and *Methanosaeta*-like filamentous archaeal cells, in the digester sludge. To identify the acetate-utilizing unidentified bacteria, RNA-SIP was conducted with $^{13}C_6$-glucose and $^{13}C_3$-propionate as sole carbon source, which were followed by phylogenetic analysis of 16S rRNA. We found that bacteria belonging to *Synergistes* group 4 were commonly detected in both 16S rRNA clone libraries derived from the sludge incubated with ^{13}C-glucose and ^{13}C-propionate. To confirm that this bacterial group can utilize acetate, specific FISH probe targeting for *Synergistes* group 4 was newly designed and applied to the sludge incubated with ^{14}C-acetate for MAR-FISH. The MAR-FISH result showed that bacteria belonging to *Synergistes* group 4 significantly took up acetate and their active population size was comparable to that of *Methanosaeta* in this sludge. In addition, as bacteria belonging to *Synergistes* group 4 had high K_m for acetate and maximum utilization rate, they are more competitive for acetate over *Methanosaeta* at high acetate concentrations (2.5–10 mM). To our knowledge, it is the first time to report the acetate-utilizing activity of uncultured bacteria belonging to *Synergistes* group 4 and its competitive significance to acetoclastic methanogen, *Methanosaeta*.
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Introduction

Different groups of anaerobic microorganisms decompose organic matter in a series of steps, which ultimately produce methane and carbon dioxide as terminal products. Methane is formed from two primary substrates, acetate and H_2/CO_2 (or formate). Acetate is an important intermediate of anaerobic decomposition of organic matter, as about two-third of methane is produced from acetate in anaerobic digestion reactors (McCarty and Smith, 1986). Thus, acetoclastic methanogens including mainly *Methanosaeta* and *Methanosarcina* have been well studied (Zinder, 1998). Methanogenic acetate degradation is carried out by either the methanogenic archaea or some anaerobic acetate-oxidizing bacteria. When inorganic electron acceptors other than CO_2 are absent, acetate is degraded by syntrophic acetate oxidation coupled to hydrogen-consuming process, for example, hydrogenotrophic methanogenesis. Otherwise, anaerobic acetate oxidation reaction is energetically extremely unfavorable. Only a few syntrophic acetate-oxidizing bacteria have been successfully cultivated to date owing to their slow growth rates and difficulty of reproducing their growth conditions (Schink and Stams, 2006; Hattori, 2008). Therefore, information on the population and diversity of anaerobic acetate-oxidizing bacteria and the competition for acetate with acetate-utilizing methanogen in anaerobic digester sludge is limited.

Microautoradiography combined with fluorescence *in situ* hybridization (MAR-FISH) is a powerful technique to simultaneously determine the phylogenetic identity and *in situ* specific metabolic function of microorganisms in complex microbial

Correspondence: S Okabe, Department of Urban and Environmental Engineering, Graduate School of Engineering, Hokkaido University, Kita 13 Nishi 8, Kita-ku, Sapporo 060-8628, Japan.
E-mail: sokabe@eng.hokudai.ac.jp
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community at a single-cell resolution (Andreasen and Nielsen, 1997; Okabe *et al.*, 2004). Thus, applying this technique, it is possible to identify and quantify slow-growing acetate-utilizing microorganisms in anaerobic digester sludge without the need to isolate them in culture. However, selection and design of specific FISH probes requires phylogenic information (16S rRNA gene sequences) of cultivated and yet-uncultivated microorganisms, which are actively involved in acetate utilization in advance. For this reason, ribosomal RNA-based stable-isotope probing (RNA-SIP) combined with full-cycle 16S rRNA analysis is useful to identify active acetate-utilizing microorganisms under methanogenic conditions and to design specific FISH probes for the following MAR-FISH analysis. RNA-SIP is a powerful approach to directly identify microbial populations active in a defined metabolic process and has recently been applied to identify acetate-utilizing microbial populations in methanogenic lake sediments (Schwarz *et al.*, 2007). It was found that acetate was predominantly consumed by acetoclastic methanogens in the lake sediment. However, this study did not use FISH or MAR-FISH to quantitatively determine the population sizes of acetate-utilizing methanogens and bacteria.

In this study, first, RNA-SIP combined with full-cycle 16S rRNA analysis was performed to identify active acetate-utilizing bacteria in anaerobic digester sludge, and then MAR-FISH with newly designed FISH probes was conducted to quantitatively investigate their acetate-utilizing activity and competitive relationship with acetoclastic methanogens at different concentrations of acetate. The results of RNA-SIP and MAR-FISH showed that bacteria belonging to the *Synergistes* group 4 were only dominant acetate-utilizing bacteria in the anaerobic digestersludge and they had lower affinity to acetate and higher utilization rate than *Methanosaeta* like acetoclastic methanogen at high acetate concentrations.

Materials and methods

Anaerobic sludge samples

Anaerobic sludge samples were taken from the mesophilic anaerobic fed-batch reactors that were operated stably for more than 2 years in our laboratory. The seed sludge for the anaerobic digesters was obtained from an anaerobic mesophilic digester at the Ebetsu municipal wastewater treatment plant located at Ebetsu city, Hokkaido, Japan. Powdered whole-milk (Meiji Dairies Corporation, Tokyo, Japan) composed of carbohydrate (57%), lipid (25%) and protein (13%) was fed every 2 days at a loading rate of 1.5 g COD l^{-1} day^{-1}. Mineral solution was supplemented with the powdered whole-milk. Other details of the reactor operation and the composition of the mineral solution were described elsewhere (Ariesyady *et al.*, 2007b).

Analytical measurements

CH_4, CO_2 and H_2 were analyzed by gas chromatography (Shimazu, Kyoto, Japan) equipped with a thermal conductivity detector and a 6-m, 2-mm i.d. SHINCARBON T column (Shinwa Chemical, Kyoto, Japan). Volatile fatty acids were determined with an ion chromatograph equipped with an ICE-AS1 column (DX-100, Dionex, Sunnyvale, CA, USA).

MAR-FISH

Incubation with radiolabeled substrates. In all, 10 ml slurry samples were taken from the anaerobic digester and centrifuged at 2500 *g* for 5 min. Of these, 8 ml of the supernatant was replaced with the mineral solution (without powdered milk). The sludge and mineral solution were gently mixed, and then 3 ml of the mixtures were transferred to 5-ml serum bottles. The serum bottles were sealed with gas-tight rubber stoppers and anaerobically incubated with shaking at 30 r.p.m. at 37 °C with [U-^{14}C]glucose for 1 h, [1-^{14}C]propionate for 2 h or [2-^{14}C]acetate for 5 h. [U-^{14}C]Glucose was added with unlabeled glucose ($^{14}C/(^{12}C + ^{14}C)$; 1%) to give a final concentration of 2.5 mM. [1-^{14}C]Propionate was added with unlabeled propionate ($^{14}C/(^{14}C + ^{12}C)$; 8%) to give a final concentration of 1.5 mM. [2-^{14}C]Acetate was added with unlabeled acetate ($^{14}C/(^{12}C + ^{14}C)$; 25%) to give final concentrations of 0.5, 1.0, 2.5, 5 and 10 mM. Controls were prepared by pasteurizing the sludge at 70 °C for 30 min and run in parallel for all analyses.

The radiolabeled [U-^{14}C]glucose and [2-^{14}C]acetate were purchased from the Amersham Pharmacia Biotech (Buckinghamshire, UK). The radiolabeled [1-^{14}C]propionate was purchased from the American Radiolabeled Chemicals Inc. (St Louis, MO, USA). The specific activity of [U-^{14}C]glucose, [1-^{14}C] propionate and [2-^{14}C]acetate was 11.7 GBq $mmol^{-1}$, 2.07 GBq $mmol^{-1}$ and 2.26 GBq $mmol^{-1}$, respectively.

Liquid scintillation counting. The uptakes of radiolabeled substrates were measured for all cultures by liquid scintillation counting before FISH and microautoradiographic procedures as described by Ariesyady *et al.* (2007b) and Ito *et al.* (2002).

Sample fixation, washing and FISH. The incubation was terminated by 4% paraformaldehyde. The fixation of the samples, washing and FISH were conducted according to Okabe *et al.* (1999) and Ito *et al.* (2002). After FISH, sample slides were stained with 4′, 6-diamidino-2-phenylindole to determine total cell numbers (Okabe *et al.*, 2007).

FISH probes. *Bacteria* and *Archaea* target oligo nucleotide probes used in this study were listed in Table 1. The probes were labeled with fluoresceinisothiocyanate or tetramethylrhodamine 5-isothiocyanate at the 5′ end.

Table 1 FISH oligonucleotide probes used in this study

Probe	*Sequence (5′–3′)*	*rRNA target site (*Escherichia coli *numbering)*	*Specificity*	*% FA*[a]	*Reference*
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)
ARC915	GTGCTCCCCCGCCAATTCCT	16S (915–934)	*Archaea*	35	Stahl and Amann (1991)
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*	20	Raskin *et al.* (1994)
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16S (19–35)	*Alphaproteobacteria* some other bacteria	20	Manz *et al.* (1992)
BET42a	GCCTTCCCACTTCGTTT	23S (1027–1043)	*Betaproteobacteria*	35	Manz *et al.* (1992)
GAM42a	GCCTTCCCACATCGTTT	23S (1027–1043)	*Gammaproteobacteria*	35	Manz *et al.* (1992)
SRB385	CGGCGTCGCTGCGTCAGG	16S (385–402)	Most *Desulfovibrionales* and other bacteria	30	Amann *et al.* (1990)
SRB385Db	CGGCGTTGCTGCGTCAGG	16S (385–402)	*Desulfovibrionaceae* and other bacteria	30	Rabus *et al.* (1996)
LGC354a	TGGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes*	35	Meier *et al.* (1999)
LGC354b	CGGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes*	35	Meier *et al.* (1999)
LGC354c	CCGAAGATTCCCTACTGC	16S (354–371)	*Firmicutes*	35	Meier *et al.* (1999)
HGC69a	TATAGTTACCACCGCCGT	23S (1901–1918)	*Actinobacteria*	20	Roller *et al.* (1994)
CF319a/b	TGGTCCGTRTCTCAGTAC	16S (319–336)	Most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*	35	Manz *et al.* (1992)
CFB563	GGACCCTTTAAACCCAAT	16S (563–580)	*Bacteroidetes*	20	Weller *et al.* (2000)
PLA46	GACTTGCATGCCTAATCC	16S (46–63)	*Planctomycetales*	30	Neef *et al.* (1998)
GNSB-941	AAACCACACGCTCCGCT	16S (941–957)	*Chloroflexi*	35	Gich *et al.* (2001)
CFX1223	CCATTGTAGCGTGTGTGTMG	16S (1223–1242)	*Chloroflexi*	35	Björnsson *et al.* (2002)
Syner195	GCAGTACTCGCGTACCTT	16S (195–212)	*Synergistes* group 4 (*Synergistetes* PD-UASB-13)	10–20	This study

Abbreviation: FISH, fluorescence *in situ* hybridization.
[a]FA, formamide concentration in the hybridization buffer.
[b]The probe can be used at any formamide concentrations.

The probe Syner195 was designed using the probe design tool of the ARB software package (Ludwig *et al.*, 2004). Probe sequences were confirmed for specificity using the probe check tool of the Ribosomal Database Project (Cole *et al.*, 2005). Specific formamide concentrations for the 9probes were experimentally determined by performing FISH at different formamide concentrations of 0%, 10%, 15%, 20% and 30%. Although no pure cultures of Syner195 target *Synergistes* group 4 bacteria have been obtained, the specific signals were identifiable in the digester sludge by the small-rod morphotype and MAR-positive signals with ^{14}C-acetate. Applying this probe to the digester sludge after incubation with ^{14}C-acetate, FISH signals of the MAR-positive cells of the characteristic morphotype became weaker above 10% formamide concentration. Thus, we selected 10% formamide as the optimal formamide concentration. The determined formamide concentration was listed in Table 1.

Autoradiographic developing procedure. Following the FISH, the autoradiographic procedure was performed directly on the cover glasses by using liquid film emulsion (LM-1, Amersham Pharmacia Biotech, Piscataway, NJ, USA) (Lee *et al.*, 1999; Kindaichi *et al.*, 2004). The optimum exposure time was determined to be 5 days for the samples incubated with [2-^{14}C]acetate and [1-^{14}C]propionate, and to be 2 days for the samples incubated with [U-^{14}C]glucose in preliminary experiments.

Microscopy and enumeration by MAR-FISH. A model LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an ultraviolet laser (351 nm and 364 nm), an Ar ion laser (450–514 nm) and two HeNe lasers (543 nm and 633 nm) were used. The formation of silver grains in the autoradiographic film was observed by using the transmission mode of the confocal laser scanning microscope system. A MAR-positive cell was defined as a cell covered with more than four

silver grains in this study (Okabe *et al.*, 2005). The numbers of MAR-positive cells and total probe-hybridized cells (or total MAR-positive cells) were determined in triplicate by directly counting at least 1000 silver grain-covered cells in randomly chosen microscopic fields of a few slides prepared for each sample.

Incubation with ^{13}C-labeled substrate ([$^{13}C_6$]glucose and [$^{13}C_3$]propionate)

In total, 25 ml slurry samples were taken from the anaerobic reactor, transferred to a 50 ml polycarbonate tube and centrifuged at 2500 *g* for 5 min. The centrifugation resulted in approximately 5 ml of sludge and 20 ml of supernatant. After replacing the gas phase of the sample tube with N_2 and CO_2 (80:20) gas, the sample tube was transferred to an anaerobic chamber containing N_2 and CO_2 (80:20). The following preparation processes and incubation were conducted in the anaerobic chamber. The supernatant was first replaced with 20 ml of the mineral solution, and then the mixture of the mineral solution and sludge was transferred to a 30-ml glass vial. For incubation with ^{13}C-labeled glucose ($^{13}C_6$, >99 at% ^{13}C; Isotec, Miamisburg, OH, USA), the vial was incubated with 2.5 mM of glucose for 48 h at 37 °C. The 2.5mM glucose was degraded to CH_4 and CO_2 via acetate and propionate as detectable intermediates during the 48-h incubation. For the incubation with ^{13}C-labeled propionate ($^{13}C_3$, >99 at% ^{13}C; Isotec), $^{13}C_3$-propionate dissolved in the mineral solution was continuously fed into the vial at a loading rate of 0.18 mmol l^{-1} h^{-1} by a microsyringe pump (IC3100; KD Scientific Inc., Holliston, MA, USA) to keep propionate concentration around 0.5 mM. The loading rate was determined at a higher propionate degradation rate of the digester sludge of the anaerobic reactor. The vial was incubated at 37 °C for 58 h under anoxic condition (in an anoxic grove box). During the 58-h incubation period, the concentration of propionate was monitored every 3–5 h and was confirmed to be in the range of 0.1–0.5 mM.

RNA extraction and fractionation

The sludge sample (25 ml) was centrifuged at 15 000 *g* for 10 min immediately after the incubation with either $^{13}C_6$-glucose or $^{13}C_3$-propionate. Total RNA was extracted from the entire harvested pellet with the FastRNA Pro Soil-Direct Kit (Qbiogene Inc., Irvine, CA, USA). The extracted RNA was purified before DNase I digestion (Lueders *et al.*, 2004a, b). The purified RNA (12 μg) was subsequently loaded with cesium trifluoroacetate equilibrium density gradient in 2.0-ml Beckman Quick-Seal polyallomer Bell Top tubes, and subjected to density gradient centrifugation with Optima TLX (Beckmann Coulter, Tokyo, Japan) at 64 000 r.p.m. and 20 °C for 36 h (Manefield *et al.*, 2002a, b). Centrifuged gradients were fractionated into 20 gradient fractions with the fraction recovery system (Bechmann Coulter) at the flow rate of 3.3 μl s^{-1} by displacement with ddH_2O using a syringe pump (Manefield *et al.*, 2002b). In fractions 1 to 20, fraction 1 was the first faction collected from the bottom of the gradient. The amount of RNA of each gradient fraction was quantified fluorometrically by RiboGreen assay (Invitrogen, Carlsbad, CA, USA) (Lueders *et al.*, 2004a). In control experiment with unlabeled RNA from the digester sludge, RiboGreen measurements showed that unlabeled RNA enriched between fractions 10 and 15 (with peak fraction 12). After the incubation with either $^{13}C_6$-glucose or $^{13}C_3$-propionate, RNA also appeared between fractions 5 and 7 (with peak fraction 6).

Reverse transcription-polymerase chain reaction, cloning, sequencing and phylogenetic analysis

For cloning and sequencing analysis, the fraction 6 that contained heavy ^{13}C-labeled RNA was amplified with the Superscript III one-step reverse transcription-polymerase chain reaction kit (Invitrogen) using bacterial primer pair 8f and 1492r (Lane, 1991). The reverse transcription-polymerase chain reaction was carried out with the following amplification program: one cycle consisting of 55 °C for 30 min (reverse transcription) and 94 °C for 2 min, and then 40 cycles consisting of 94 °C for 15 s; 54 °C for 30 s and 68 °C for 2 min, followed by final extension at 68 °C for 7 min. The reverse transcription-polymerase chain reaction product was gel-purified and cloned by using a TOPO XL PCR cloning kit (Invitrogen). Randomly selected clones were sequenced on an ABI model 3100-Avant genetic analyzer with a BigDye terminator Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared with reference 16S rRNA gene sequences available in the GenBank/EMBL/DDBJ databases using the BLAST search (Altschul *et al.*, 1997). Sequences with 97% or greater similarity were grouped into operational taxonomic units. Phylogenetic analysis was performed using the MEGA3 software package (Kumar *et al.*, 2004) after multiple alignments of data by CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic trees were constructed using neighbor-joining and maximum-parsimony methods. The confidence level for nodes was ascertained by performing a bootstrap analysis (1000 replications).

Determination of acetate degradation rates

Acetate degradation rates of the anaerobic sludge at different acetate concentrations were determined with [2-^{14}C]acetate by liquid scintillation counting. The experimental setup (the incubation condition) was the same as that for MAR-FISH (see MAR-FISH—Incubation with radiolabeled substrates). [2-^{14}C]Acetate was added with unlabeled acetate to

give final concentrations of 0.5 mM, 1.0 mM, 2.5 mM, 5 mM, 10 mM and 20 mM. Acetate is not only degraded, but also produced in the anaerobic sludge. For example, acetate could be produced by anaerobic self-degradation of the sludge and also produced by anaerobic degradation of residual substrates. Measuring the amount of [2-^{14}C]acetate makes it possible to exclude acetate production by these other processes.

Culture samples were taken at the incubation times of 0 h, 2 h, 4 h, 6 h, 8 h and 10 h. After centrifugation of the samples at 15 000 *g* for 10 min, a trace amount of sulfuric acid was added to the supernatant to de-gas ^{14}C-carbon dioxide that could be produced from the degradation of ^{14}C-acetate. Then, ^{14}C in the supernatant, that is, residual ^{14}C-acetate in the supernatant, was determined by liquid scintillation counting. The addition of sulfuric acid did not influence the measurements of acetate concentrations. Acetate degradation rates (μmol gVSS^{-1} h^{-1}) at different acetate concentrations were calculated from the slopes of liner fitting for time-dependent changes in acetate concentrations during the incubation. K_m and V_{max} were determined by the Lineweaver–Burke plot of the rates of acetate degradation versus acetate concentrations.

Acetate degradation rate of two probe-defined *Synergistes* and *Methanosaeta* groups were determined by multiplying the acetate degradation rates of the anaerobic digester sludge by the fraction of [^{14}C]acetate-utilizing MAR-positive *Synergistes* group 4 and *Methanosaeta* populations at different acetate concentrations. In this study, the acetate uptake activity (that is, the number of silver grains accumulated on the cells) of both *Synergistes* and *Methanosaeta* is more closely related to the cell number than the cell size, even though the cell size of filamentous *Methanosaeta* was bigger than one of *Synergistes*. Therefore, we used the fraction of cell numbers instead of the cell area.

Nucleotide sequence accession numbers

Sequences were deposited in the GenBank/EMBL/DDBJ database under accession numbers AB603808–AB603841.

Results

In situ *detection of acetate-utilizing archaea and bacteria*

The populations of anaerobic acetate-utilizing bacteria and archaea in anaerobic digester sludge were investigated by microautoradiography-FISH technique with ^{14}C-acetate and domain bacteria and archaea-specific oligonucleotide probes. Small-rod bacterial cells and filamentous archaeal cells were mainly detected as MAR-positive with ^{14}C-acetate (Figure 1). The MAR-positive archaeal cells were morphologically *Methanosaeta*-like cells. A significant uptake of ^{14}C-acetate was found on the small rod bacterial cells that showed bright fluorescent signals with EUB338-mixed probe, which holds the majority of MAR-positive bacterial cells. However, these EUB338-mixed probe-hybridized small rod cells could not be hybridized with general phylum and subphylum level FISH probes (Table 1). The MAR-positive cells with ^{14}C-acetate accounted for 15% of total cells.

Phylogenetic analysis of heavy ^{13}C-labeled bacterial 16S rRNA

The incubation conditions with [$^{13}C_6$]glucose and [$^{13}C_3$]propionate were carefully adjusted in order that the initial ^{13}C substrates were degraded down to methane via acetate and that RNA molecules of acetate utilizers were sufficiently labeled with produced ^{13}C-acetate. The phylogenetic affiliation of 16S rRNA sequences was analyzed for the total 48 clones of heavy ^{13}C-labeled bacterial rRNA derived from the anaerobic digester sludge incubated with 2.5 mM [$^{13}C_6$]glucose for 48 h (Figure 2). Twenty-four

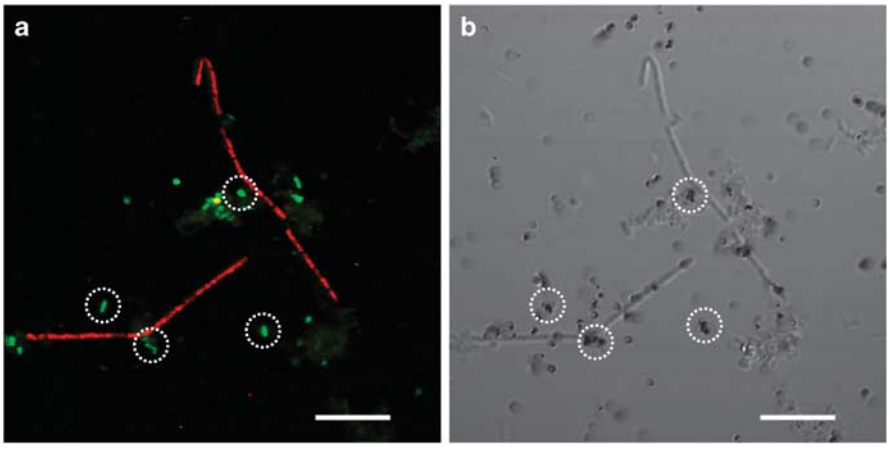

Figure 1 MAR-FISH image of acetate-utilizing bacterial and archaeal cells in the anaerobic digester sludge. (**a**) FISH image and (**b**) MAR image. *In situ* hybridization was performed with fluorescein isothiocyanate (FITC)-labeled EUB338-mixed probe and tetramethylrhodamine 5-isothiocyanate (TRITC)-labeled ARC915 probe. After incubating with 2.5 mM glucose for 1 h, 740 kBq [2-^{14}C]acetate was injected into the sample (the radiolabeled acetate concentration was 18% of total acetate concentration), and the samples were incubated for 2 h. Dotted circles indicate MAR-positive bacterial cells. Filamentous archaeal cells are also MAR-positive. Bar represents 10 μm.

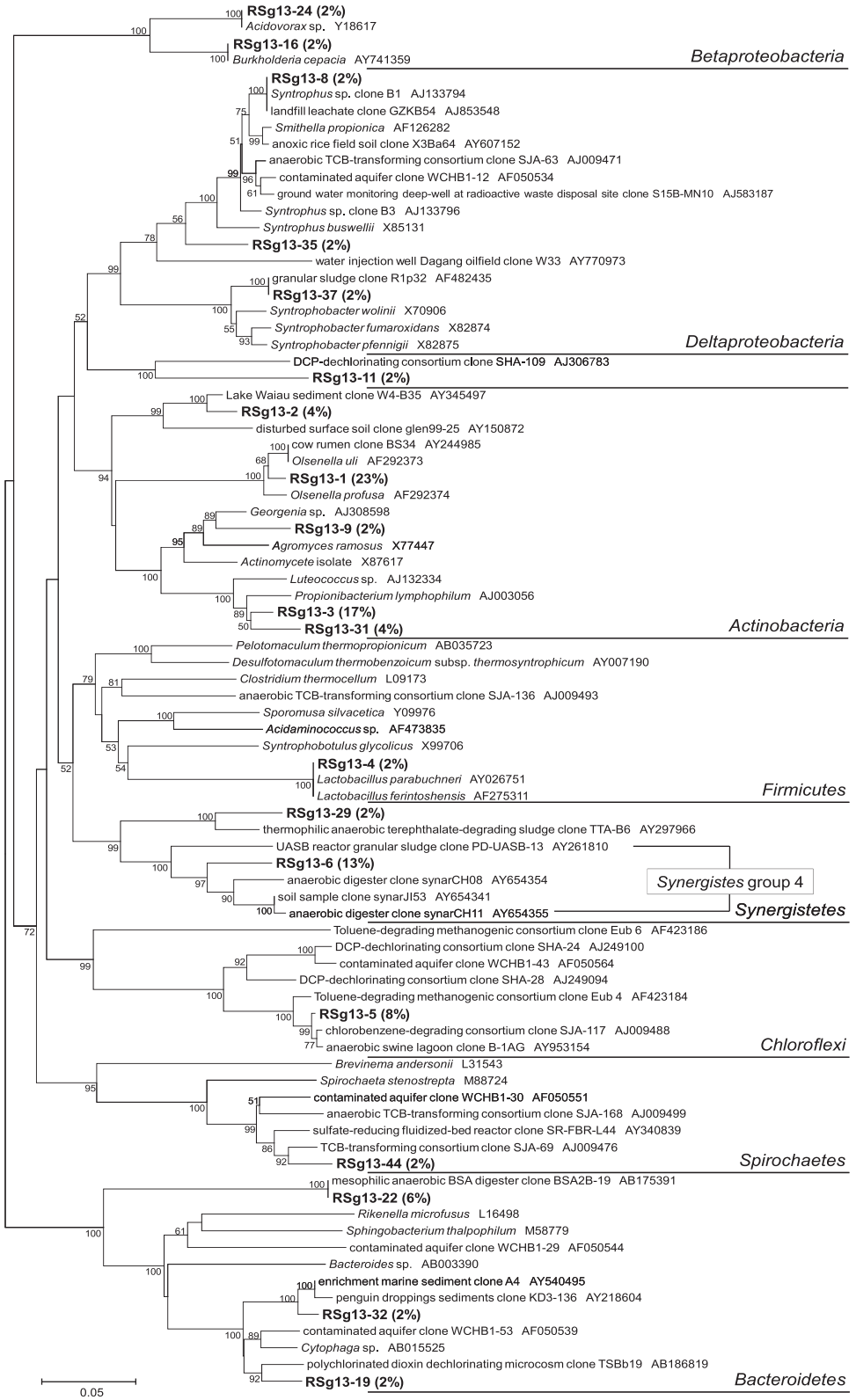

Figure 2 A phylogenetic tree showing the affiliation of 16S rRNA clones retrieved from 'heavy' fraction of the RNA, which was extracted from the anaerobic digester sludge incubated with 2.5 mM [$^{13}C_6$]glucose for 48 h (batch incubation). The numbers near branching points indicates bootstrap values. The scale bar represents 5% sequence divergence.

heavy 16S rRNA clones (50%) belonged to *Actinobacteria*, in which *Olsenella* (11 clones, 23%) and *Propionibacterium* (8 clones, 17%) were predominant. *Synergistes* (7 clones, 15%), *Bacteroides* (5 clones, 10%) and *Chloroflexi* (4 clones, 8%) followed. Six clones among the seven clones of the

Synergistes constituted a monophyletic cluster in the *Synergistes* group 4. Two clones (4%) were affiliated with propionate-degrading *Syntrophobacter* and *Smithella*, respectively.

The phylogenetic affiliation of 16S rRNA sequences was also analyzed for the total 45 clones of heavy ^{13}C-labeled bacterial rRNA derived from the anaerobic digester sludge incubated with 0.5 mM [$^{13}C_3$]propionate for 58 h (Figure 3). First, 20 heavy 16S rRNA clones (44%) belonged to the *Smithella* lineage of *Deltaproteobacteria*. Second, predominant clones belonged to *Synergistes* group 4 (9 clones, 20%). Six clones (13%) and five clones (11%) were widely distributed in *Bacteroidetes* and *Firmicutes*, respectively.

Identification of acetate-utilizing bacteria

As the results of phylogenetic analyses of heavy 16S rRNA derived from the sludge incubated with ^{13}C-glucose and ^{13}C-propionate, *Synergistes* group 4 was detected in both clone libraries, suggesting that this group could be a dominant acetate degrader. Therefore, FISH probe specific for the *Synergistes* group 4 was designed (Syner195 in Table 1) and used for MAR-FISH with [2-^{14}C]acetate to confirm that the *Synergistes* group 4 was an acetate-utilizing bacterium in the anaerobic digester sludge. FISH and MAR-FISH analyses revealed the Syner195 probe-hybridized bacteria belonging to the *Synergistes* group 4 were abundantly present in the digester sludge. The number of Syner195 probe-hybridized cells was 1.2×10^9 cells mg-VSS^{-1}, whereas the number of MX825 probe-hybridized cells was 1.4×10^9 cells mg-VSS^{-1}. The morphotype of the probe Syner195 probe-hybridized cells was a short rod, 2 μm length and 1 μm width. Dense silver grains covered on the Syner195 probe-hybridized short-rod cells (Figure 4), indicating a significant utilization of ^{14}C-acetate. This result clearly indicated that bacteria belonging to the *Synergistes* group 4 were acetate utilizers. The Syner195 probe-hybridized *Synergistes* cells were all MAR-negative when incubated with either ^{14}C-propionate or ^{14}C-glucose. Therefore, it is concluded that *Synergistes* can use neither glucose nor propionate. The *Methanosaeta*-like filamentous archaeal cells, which were hybridized with *Methanosaetaceae*-specific MX825 probe, were also MAR-positive (Figure 4). The number of silver grains accumulated on the *Methanosaeta*-like filamentous archaea was similar to that on bacteria belonging to the *Synergistes* group 4.

The involvement of *Synergistes* group 4 and *Methanosaeta*-like filamentous archaea in glucose degradation to methane and carbon dioxide was tested by MAR-FISH after incubation with [U-^{14}C]glucose for 1 h, 3 h, 12 h and 36 h. The time-course analysis by MAR-FISH with the specific probes revealed that the number of MAR-positive cells belonging to the *Synergistes* group 4 and *Methanosaeta* increased with time, indicating that glucose was degraded to methane via acetate and acetate was mainly utilized by both groups in the anaerobic digester sludge (data not shown).

Acetate utilization by Synergistes *group 4 and* Methanosaeta

Effect of acetate concentrations on the activities of the *Synergistes* group 4 and *Methanosaeta*-like filamentous archaea was investigated by MAR-FISH with the initial acetate concentrations of 0.5 mM, 1 mM, 2.5 mM, 5 mM and 10 mM, respectively (Figure 5). Incubation time was 5 h at each acetate concentration. At 0.5 mM acetate, the Syner195 probe-hybridized *Synergistes* cells accounted for approximately 35% of the total MAR-positive cells, whereas the probe MSX825-hybridized *Methanosaeta* cells accounted for 65% of the total MAR-positive cells. At 1.0 mM acetate, the percentages of the MAR-positive *Synergistes* cells and *Methanosaeta* cells were approximately 45% and 55%, respectively. However, the MAR-positive *Synergistes* cells were more abundant than the probe MSX825-hybridized *Methanosaeta* cells at 2.5 mM, 5 mM and 10 mM acetate. Fractions of total MAR-positive cells of total cells were almost constant values of 3–4% at all acetate concentrations. The result indicated that the activities of the two groups were dependent on the acetate concentrations. Other MAR-positive microorganisms were negligible and only these two groups were active acetate utilizers at all acetate concentrations.

^{14}C-acetate degradation rates of the sludge

^{14}C-acetate degradation rates of the digester sludge were determined from batch experiments for 10-h incubation at the acetate concentrations ranging from 0.5 mM to 20 mM containing 185 kBq ^{14}C-acetate (Figure 6). The acetate degradation rates enzyme kinetically increased with increasing acetate concentration. The K_m and V_{max} values of this anaerobic digester sludge were determined by the Lineweaver–Burke plot of the acetate degradation rates to be 2.8 mM and 24 μmol g-VSS^{-1} h^{-1}, respectively (Table 2). As *Synergistes* group 4 and *Methanosaeta* were dominant acetate utilizers in this anaerobic digester sludge, the overall acetate degradation rate is considered to be the sum of acetate degradation rates of *Synergistes* group 4 and *Methanosaeta*. The number of silver grain is theoretically proportional to the amount of incorporated (assimilated) ^{14}C atom into cells under the same incubation and microautoradiographic conditions. The number of silver grains accumulated on the *Methanosaeta*-like filamentous cells was similar to that on bacteria belonging to the *Synergistes* group 4 (Figure 4), indicating that the acetate degradation by MAR-positive cells of *Synergistes* group 4 is the same as that by *Methanosaeta*. It should be also assumed that the incorporation rate is proportional

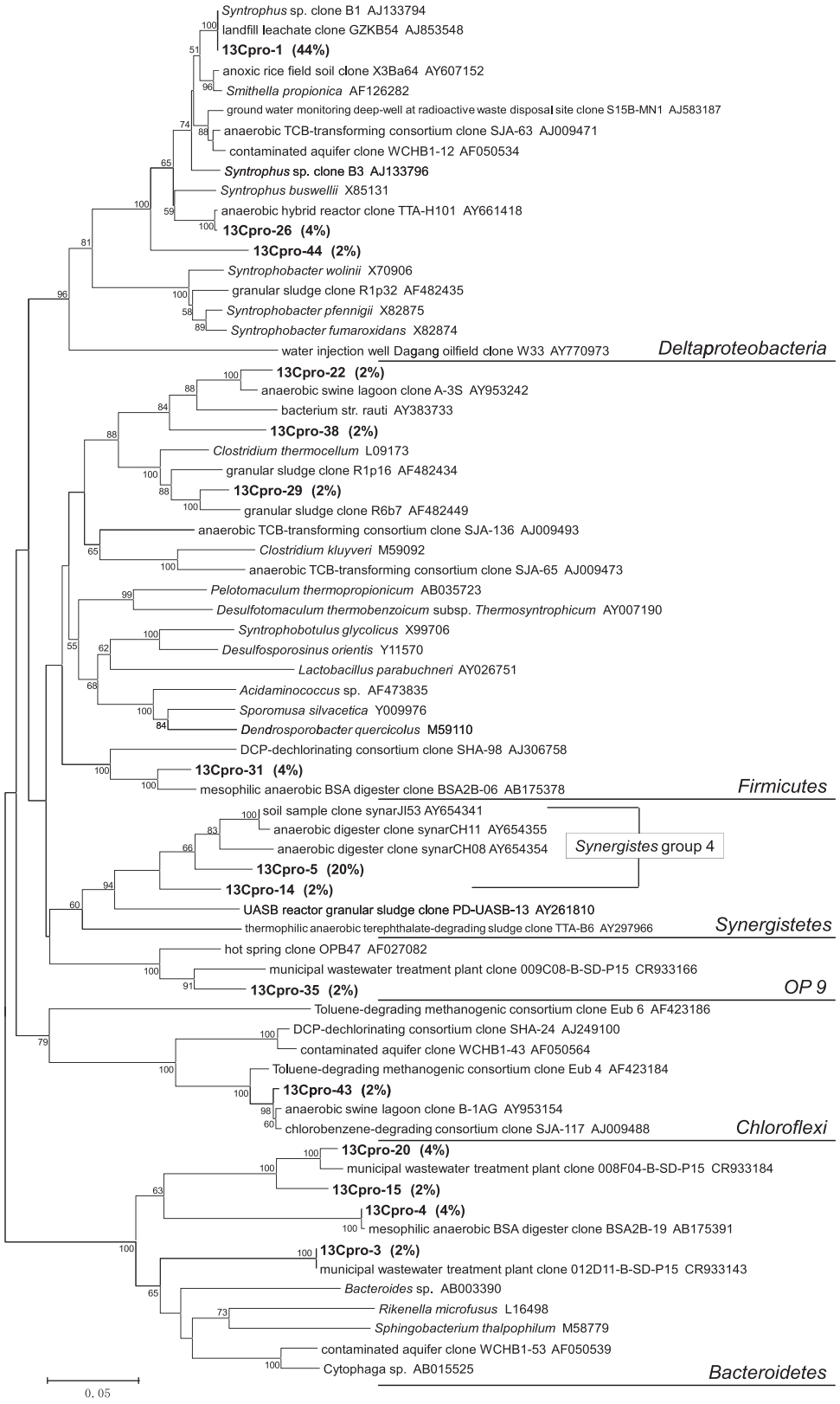

Figure 3 A phylogenetic tree showing the affiliation of 16S rRNA clones retrieved from 'heavy' fraction of the RNA, which was extracted from the anaerobic digester sludge incubated at 0.5 mM [$^{13}C_3$]propionate for 58 h (continuous-flow incubation). The numbers near branching points indicates bootstrap values. The scale bar represents 5% sequence divergence.

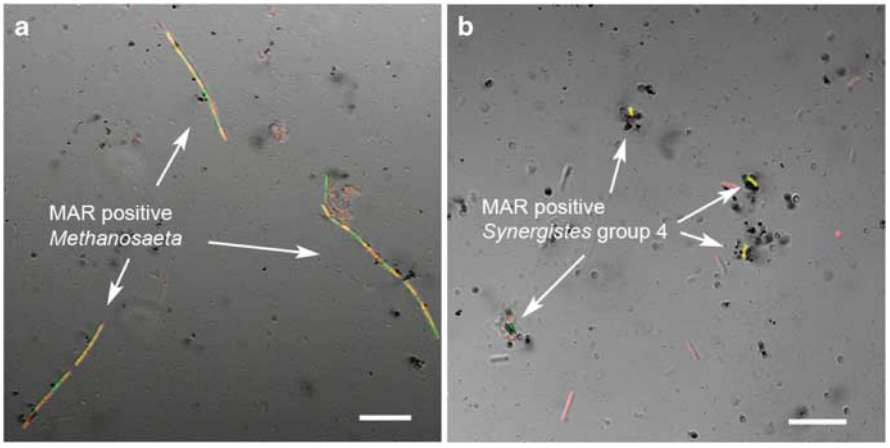

Figure 4 MAR-FISH images of acetate-utilizing archaeal and bacterial cells present in the anaerobic digester sludge incubated for 5 h at 0.5 mM acetate containing 19% [2-^{14}C]acetate. In these images, MAR-positive cells are identified with genus-specific probes, which are yellowish owing to the cross-hybridization with domain probes. (**a**) FITC-labeled MX825 probe-stained filamentous MAR-positive cells. The sample was simultaneously hybridized with TRITC-labeled ARC915 probe. (**b**) FITC-labeled Syner195 probe-stained MAR-positive cells. The sample was simultaneously hybridized with TRITC-labeled EUB338-mixed probe. Bars represent 10 μm.

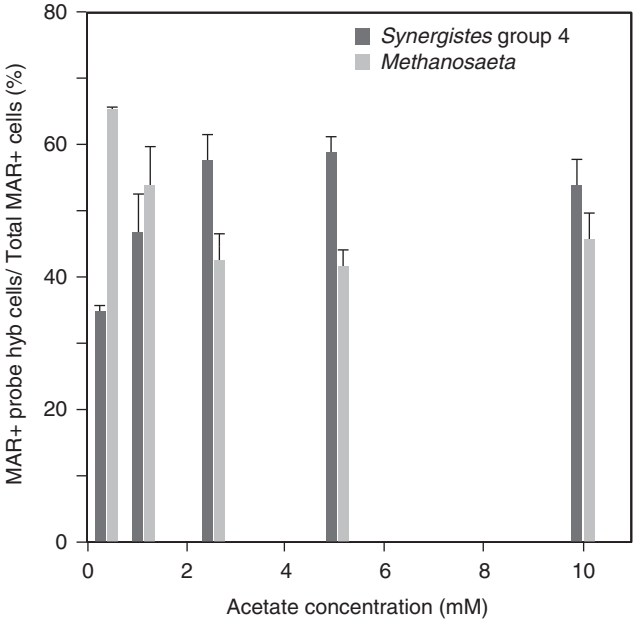

Figure 5 Relative abundance of [^{14}C]acetate-utilizing *Methanosaeta* and *Synergistes* group 4 populations at 0.5 mM, 1.0 mM, 2.5 mM, 5 mM and 10 mM acetate determined by MAR-FISH using probes of MSX825 and Syner195, respectively. The samples were incubated for 5 h at each acetate concentration containing 19% [2-^{14}C]acetate. The genus-specific probes were always combined with domain-specific probes, and the sample was counter-stained with 4′, 6-diamidino-2-phenylindole (DAPI). Error bars represent the standard errors of duplicated measurements.

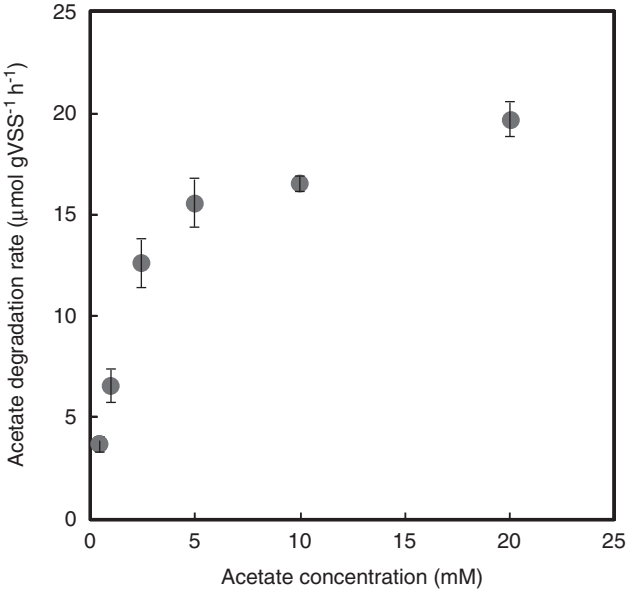

Figure 6 Acetate degradation rates of the anaerobic digester sludge incubated at 0.5 mM, 1.0 mM, 2.5 mM, 5 mM, 10 mM and 20 mM acetate containing 185 kBq [2-^{14}C]acetate for 10 h, which was determined by liquid scintillation counting. Error bars represent the standard errors of duplicated measurements.

to the degradation rate for the *Synergistes* group 4 and *Methanosaeta*, and this relationship is held at all acetate concentrations. Based on these assumptions, the individual acetate degradation rates by the *Synergistes* group 4 and *Methanosaeta* (Figure 7) could be roughly estimated from the relative abundance of [^{14}C]acetate-utilizing MAR-positive *Synergistes* group 4 and *Methanosaeta* populations (Figure 5) and specific acetate degradation rates (μmol gVSS^{-1} h^{-1}) of the anaerobic digester sludge (Figure 6). The K_m and V_{max} values for *Synergistes* group 4 were 13 mM and 36 μmol gVSS^{-1} h^{-1}, whereas those for *Methanosaeta* were 1 mM and 8 μmol gVSS^{-1} h^{-1}, respectively (Figure 7 and Table 2).

Discussion

Identification of major acetate-utilizing microorganisms in anaerobic digester sludge

Cultivation and isolation of anaerobic acetate-utilizing bacteria have been very difficult owing to their slow

Table 2 K_m and V_{max} values for acetate catabolism by the anaerobic digester sludge, *Methanosaeta* and *Synergistes* group 4

	K_m(mM)	V_{max} (μmol gVSS^{-1} h^{-1})
Anaerobic digester sludge (lab reactor)	2.8	24
Methanosaeta	1	8
Synergistes group 4	13	36

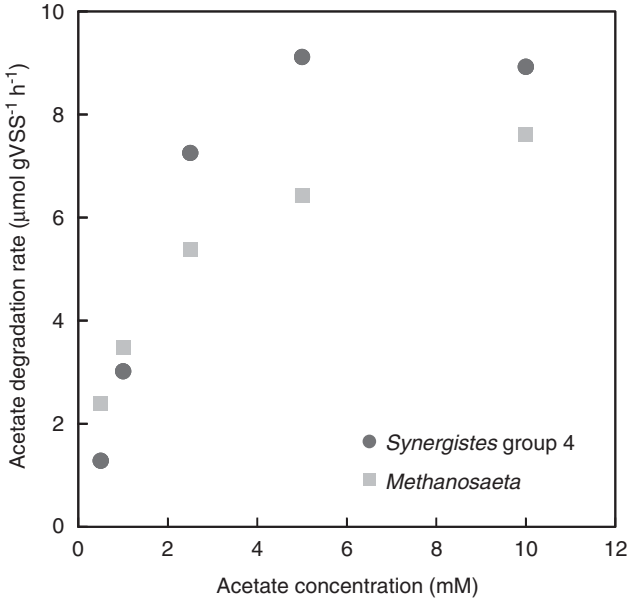

Figure 7 Acetate degradation rates of *Methanosaeta* and *Synergistes* group 4 in the anaerobic digester sludge at 0.5 mM, 1.0 mM, 2.5 mM, 5 mM and 10 mM acetate concentrations. The rates were calculated from the values of Figures 5 and 6.

and syntrophic growth with hydrogenotrophic methanogens. Therefore, abundance, diversity and phylogenetic affiliations of anaerobic acetate-utilizing bacteria in anaerobic environments including digester sludge remain largely unknown. Recent 16S rRNA gene-based phylogenetic analyses revealed the presence of a vast diversity of microorganisms in anaerobic digester sludge, the majority of which have not yet been cultivated and characterized (Chouari *et al.*, 2005; Sekiguchi, 2006; Ariesyady *et al.*, 2007a; Narihiro *et al.*, 2009).

This study was originally designed to identify bacterial groups that are responsible for degradation of glucose, propionate and acetate in anaerobic digester sludge. Therefore, we have performed RNA-SIP combined with full-cycle 16S rRNA analysis using ^{13}C-glucose, ^{13}C-propionate and ^{13}C-acetate as sole carbon source, respectively. However, the RNA-SIP with ^{13}C-acetate was failed because the incorporation rate of ^{13}C in RNA was very slow, and enough heavy RNA could not be obtained with incubation of 1–3 days. Major acetate-utilizing bacterial and archaeal populations in methanogenic anaerobic digester sludge were, therefore, identified by finding microbial groups commonly detected in heavy 16S rRNA gene clone libraries derived from ^{13}C-propionate and ^{13}C-glucose. A specific FISH probe was newly designed for the commonly detected microbial group and used for MAR-FISH to confirm acetate utilization of this bacterial group.

This study revealed for the first time that the predominant acetate-utilizing bacterium was belonging to an as-yet-unidentified *Synergistes* group 4. *Synergistetes*-affiliated microorganisms composed one core group in anaerobic digester sludge with other five groups affiliated with *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes* (Rivière *et al.*, 2009). Our *Synergistes* group 4 clones were affiliated with the phylum *Synergistetes* (formally '*Synergistes*') subdivision E, in which the genus *Aminobacterium* was classified (Jumas-Bilak *et al.*, 2009). However, *Synergistes* group 4 clones were distantly related to the amino acids-degrading *Aminobacterium*, and closely related to many anaerobic digester clones in the *Synergistetes* subdivision E. In fact, a part of the *Synergistetes* subdivision E has been described as 'Group 4 anaerobic digester' (Godon *et al.*, 2005), and more recent phylogenetic analysis reclassified the 'Group 4 anaerobic digester' into the groups PD-UASB-13 and HA73 (Hugenholtz *et al.*, 2009). Our clones were closely related to the group PD-UASB-13 (Figures 2 and 3). The bacteria belonging to the *Synergistes* have been frequently found in other full-scale anaerobic digester treating municipal wastewater sludge (Chouari *et al.*, 2005; Rivière *et al.*, 2009). However, the function of the *Synergistes* in the anaerobic digesters has not been reported yet until now, as the group PD-UASB-13 has no cultured representatives to date, and their metabolic function is presently unknown. Thus, the ability to degrade acetate by *Synergistes* group 4 would be valuable information for future cultivation and characterization of this bacterial group.

Acetate utilization by *Synergistes* group 4 is probably syntrophic acetate oxidation coupled with hydrogenotrophic methanogens. We determined $^{13}CH_4$ production from degradation of [1-^{13}C]acetate ($CH_3^{13}COOH$) by a gas chromatography–mass spectrometry. The result of gas chromatography–mass spectrometry analysis revealed about 10% of the degraded acetate was converted to $^{13}CH_4$ in anaerobic digester sludge containing 0.5 mM acetate (data not shown). $^{13}CH_4$ could be produced by syntrophic acetate oxidation coupled with hydrogenotrophic methanogens, but not by aceticlastic methanogenesis (Shigematsu *et al.*, 2004). In this study, bacteria belonging to the *Synergistes* group 4 were only predominant acetate-utilizing bacteria. Based on archaeal *16S rRNA* gene clone analysis, several clones closely related to *Methanoculleus* (2 out of total 35 clones) and *Methanosarcina* (5 out of total 35 clones) were retrieved from the anaerobic

digester sludge (data not shown). The genus *Methanoculleus* use hydrogen to reduce CO_2 to CH_4 (Boone *et al.*, 1993). *Methanosarcina* is known as acetate-utilizing methanogen, whereas many *Methanosarcina* spp. can also grow by using hydrogen to reduce CO_2 to CH_4 (Boone *et al.*, 1993). In MAR-FISH analysis with [2-^{14}C]acetate, *Methanosarcina* was not detected as MAR-positive. *Methanosarcina* might have a role as hydrogenotrophic methanogen associated with syntrophic acetate-oxidizing bacterium, *Synergistes* group 4, in the anaerobic digester sludge.

Acetoclastic methanogenesis convert methyl-based carbon (CH_3^-) of acetate into methane (Zehnder *et al.*, 1980; Zinder, 1998). Therefore, we used [2-^{14}C]acetate, but not [1-^{14}C]acetate, because acetoclastic methanogens produce $^{14}CH_4$, but not $^{14}CO_2$, from [2-^{14}C]acetate. Thus, feeding of [2-^{14}C]acetate minimizes the false MAR-positive by $^{14}CO_2$ cross-feeding to non-acetate-utilizing bacteria. On the other hand, when acetate-utilizing bacteria produced $^{14}CO_2$ from [1-^{14}C]acetate, homoacetogens can utilize the produced $^{14}CO_2$ with hydrogen and produce ^{14}C-labeled acetate, which might be another possibility that non-acetate-utilizing bacteria become MAR-positives. In fact, no other bacteria than *Synergistes* group 4 and *Methanosaeta* were found as active acetate utilizers in the MAR-FISH experiments with either [2-^{14}C]acetate or [1-^{14}C]acetate. Hence, it was negligible that $^{14}CO_2$-utilizing bacteria such as homoacetogens appeared as MAR-positives under the experimental condition applied in this study (that is, 5-h incubation with [2-^{14}C]acetate). Syner195 probe-hybridized cells were MAR-negative when cultured for 5 h with ^{14}C-bicarbonate, [1-^{14}C]propionate or [U-^{14}C]glucose (data not shown). When the incubation with ^{14}C-glucose was prolonged till 12 h, 24 h and 48 h, MAR-positive Syner195 probe-hybridized cells increased gradually. This is probably because ^{14}C-glucose was degraded to ^{14}C-acetate, and then the produced ^{14}C-acetate was utilized by *Synergistes* group 4. These results also confirm the conclusion that the *Synergistes* group 4 utilized acetate during degradation of glucose.

Acetate utilization by Synergistes *group 4 and* Methanosaeta

The K_m value (1 mM) for *Methanosaeta* (Table 2) is close to the values (0.5–0.9 mM) reported in the literature (summarized by Zinder, 1998). This result indicates that *Synergistes* group 4 is more competitive for acetate over *Methanosaeta* at high concentrations of acetate. Quantitative MAR-FISH (Nielsen *et al.*, 2003) for each microorganism should be performed to determine the more quantitative contribution of two acetate utilizers. Quantitative MAR-FISH can directly determine *in situ* acetate degradation rates in the mixed microbial populations, which would provide the more precise contribution of two acetate utilizers. Furthermore, isolation and characterization of *Synergistes* group 4 is undoubtedly necessary for understanding of their physiology.

Periodical change in acetate concentration in an anaerobic batch reactor, ranging from 0 mM to 15 mM within 2 days (Ariesyady *et al.*, 2007b), is probably the reason that both acetate utilizers, *Synergistes* group 4 and *Methanosaeta*, were present in the same culture, even though their K_m values were 10 times different. *Methanosaeta* and *Synergistes* group 4 seem to be not competitive, but cooperative for fluctuating concentration of acetate in the anaerobic batch reactor used in this study.

Four distinct bacterial species have been found as major propionate degraders in the anaerobic digester sludge (Ariesyady *et al.*, 2007b), whereas this study revealed that only two microorganisms, *Synergistes* group 4 and *Methanosaeta*, were involved in acetate degradation in the same anaerobic digester sludge. Therefore, it should be noted that acetate-degrading microbial community had less diversity than propionate-degrading community.

In conclusion, this study revealed for the first time that bacteria belonging to an as-yet-unidentified *Synergistes* group 4 were the major acetate-utilizing bacterial populations in methanogenic anaerobic digester sludge. Furthermore, this bacteria group had lower affinity to acetate and higher acetate utilization rate than *Methanosaeta*-like acetoclastic methanogen. The bacteria belonging to the *Synergistes* group 4 has been frequently found as one of core microbial groups in anaerobic digester sludge, but their function has been totally unknown. It should be noted that the combination of stable-isotope and radioisotope tracer experiments and molecular analyses (that is, RNA-SIP with full-cycle 16S rRNA analysis and MAR-FISH) is a very powerful tool to identify the *in situ* function and activity of as-yet-unidentified bacteria in mixed populations.

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