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Identification of Novel Butyrate- and Acetate-Oxidizing Bacteria in Butyrate-Fed Mesophilic Anaerobic Chemostats by DNA-Based Stable Isotope Probing

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Abstract

Butyrate is one of the most important intermediates during anaerobic digestion of protein wastewater, and its oxidization is considered as a rate-limiting step during methane production. However, information on syntrophic butyrate-oxidizing bacteria (SBOB) is limited due to the difficulty in isolation of pure cultures. In this study, two anaerobic chemostats fed with butyrate as the sole carbon source were operated at different dilution rates (0.01/day and 0.05/day). Butyrate- and acetate-oxidizing bacteria in both chemostats were investigated, combining DNA-Stable Isotope Probing (DNA-SIP) and 16S rRNA gene high-throughput sequencing. The results showed that, in addition to known SBOB, *Syntrophomonas*, other species of unclassified *Syntrophomonadaceae* were putative butyrate-oxidizing bacteria. Species of *Mesotoga*, *Aminivibrio*, *Acetivibrio*, *Desulfovibrio*, *Petrimonas*, *Sedimentibacter*, unclassified *Anaerolineae*, unclassified *Synergistaceae*, unclassified *Spirochaetaceae*, and unclassified bacteria may contribute to acetate oxidation from butyrate metabolism. Among them, the ability of butyrate oxidation was unclear for species of *Sedimentibacter*, unclassified *Synergistaceae*, unclassified *Spirochaetaceae*, and unclassified bacteria. These results suggested that more unknown species participated in the degradation of butyrate. However, the corresponding function and pathway for butyrate or acetate oxidization of these labeled species need to be further investigated.

Keywords Anaerobic digestion · DNA-stable isotope probing · Butyrate-oxidizing bacteria · Acetate-oxidizing bacteria · High-throughput sequencing

Introduction

Anaerobic digestion (AD) is an effective and environment-friendly technology for organic waste/wastewater treatment. Butyrate accounts for 17–20% of the total volatile fatty acid (VFA) intermediates during anaerobic digestion of protein wastewater, such as dairy (casein) and meat-processing (beef offal, flesh, pork flesh) wastewater [1–3]. Generally, the anaerobic oxidization of butyrate to H_2 and acetate ($\Delta G^{0'} = +48.1$ kJ/mol) is not spontaneous under standard conditions, unless it can be overcome by the collaborative interaction between syntrophic butyrate-oxidizing bacteria (SBOB) and hydrogenotrophic methanogens, which could keep a much lower H_2 partial pressure [4]. Due to the thermodynamic barrier, the accumulation of butyrate occurs easily, causes a further decrease in pH, and even leads to failure of the AD process [5]. Therefore, butyrate oxidization

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performed by SBOB is considered as a limited step for methane production.

Owing to the symbiotic relationships with hydrogenotrophic methanogens, pure cultures of only a few SBOB strains have been successfully obtained so far. These isolates include 11 mesophilic species and/or subspecies of the genus *Syntrophomonas* [6–15], two thermophilic species of the genera *Thermosyntropha* and *Syntrophothermus* [16, 17], one mesophilic species of the genus *Syntrophus* [18], and a psychrotolerant species of the genus *Algorimarina* [19]. The analysis of two SBOB genomes (*Syntrophomonas wolfei* and *Syntrophus aciditrophicus*) showed that they have multiple copies of genes related to β-oxidization for butyrate degradation [20, 21]. However, little is known about SBOB diversity in the anaerobic digester [22–24]. Therefore, the roles and contributions of SBOB during the AD process for biogas production need to be further investigated.

Considering the difficulty in isolating SBOB, it is necessary to explore the frontiers of technology for identifying novel SBOB in anaerobic digesters. The recent development of the stable isotope probing (SIP) technique, based on DNA or RNA, enables linking of metabolic function and taxonomic identity, especially for exploring the function of uncultured microorganisms [25]. Several reports have proven that SIP is an effective tool for identifying potential butyrate degraders in different methanogenic habitats [26–29]. More non-*Syntrophomonas* species, such as *Tepidanaerobacter*, *Clostridium*, *Syntrophospora*, *Syntrophomonadaceae*, *Syntrophaceae*, and *Actinobacteria*, were found to be possibly responsible for syntrophic butyrate oxidization. However, to date, only one study has investigated the diversity of butyrate-oxidizing bacteria (BOB) using SIP in methanogenic sludge [27]. Therefore, research on SBOB is of great importance for learning and regulating the AD process for protein wastewater treatment.

There are complex microbial communities and a relatively low abundance of SBOB in AD reactors for protein waste/wastewater treatment, which could reduce the chances of identifying SBOB using ^{13}C-butyrate SIP. Moreover, our previous studies demonstrated that the dilution rate (the reciprocal of hydraulic retention time, HRT) could seriously affect the community structure of VFA-degrading bacteria in a continuous stirred tank reactor (CSTR) for AD [30, 31]. Therefore, in this study, we constructed two butyrate-fed mesophilic anaerobic CSTRs and operated them at different dilution rates (0.01/day and 0.05/day) for SBOB enrichment. Then, the potential SBOB in both reactors were investigated using DNA-SIP combined 16S rRNA high-throughput sequencing. Simultaneously, acetate-oxidizing bacteria (AOB) were also analyzed, considering that acetate is an important intermediate (butyrate + $2H_2O \longrightarrow$ 2acetate + $2H_2$) during butyrate metabolism.

Materials and Methods

Construction and Operation of the Chemostats Fed with Butyrate as the Sole Carbon Source

Two anaerobic chemostats were constructed using two CSTRs, each with a working volume of 1.8 L as described previously [31]. The seed sludge was obtained from an anaerobic reactor treating kitchen waste. The chemostats, designated as BL and BH reactors, were fed with synthetic wastewater, containing butyrate as the sole carbon source (TOC 8000 mg/L) at dilution rates of 0.01/day and 0.05/day, respectively. BL and BH reactors operated at 37 °C for approximately 400 and 300 days, respectively, during which, parameters, including pH, suspended solids (SS), volatile suspended solid (VSS), total organic carbon (TOC), VFAs, were measured regularly, as described previously [32]. After the reactor reached a steady state at each dilution rate, the sludge was used for microbial community analysis and DNA-SIP experiments.

SIP Incubation with ^{13}C-Butyrate and ^{13}C-Acetate

Considering the co-existence of acetate and butyrate degraders during butyrate metabolism, the sludge was incubated separately with ^{13}C-butyrate and ^{13}C-acetate to aid SBOB identification. Incubations of ^{12}C-butyrate and ^{12}C-acetate were used as controls. Sludge was collected directly from the BL and BH reactors on days 372 and 290, respectively. Microcosms were set up individually in 50-mL serum bottles amended with 15 mL BL or BH digester sludge. After sealing with rubber stoppers and aluminum seal, and purged with nitrogen gas, cysteine-HCl (final concentration of 0.5 g/L) and resazurin (final concentration of 1 mg/L) were added as reducing agent and anaerobic condition indicator, respectively. The serum bottles were supplemented once every two days with ^{13}C or ^{12}C-substrate via a gas-tight syringe, and eight different treatments, each in duplicates, were established (Table 1). All microcosms were incubated at 37 °C and 150 rpm on a shaker. The incubation time had previously been optimized by a pretest (data not shown). Gas production was measured, with a syringe, every two days. After incubation, the remaining VFAs in each bottle were determined and sludge from each bottle was collected for DNA extraction. The [1-13C] sodium butyrate (98 atom% ^{13}C) used in this study was purchased from Shanghai Engineering Research Center of Stable Isotope in China, while [2-13C] sodium acetate (99 atom% ^{13}C) was purchased from Cambridge Isotope Laboratories, USA.

Table 1 Lists of microcosm samples, their incubation condition and DNA for sequencing

Treatment[a]	Inoculum	Substrate (concentration)[b]	Incubation time	DNA for sequencing[c]
BL-12B	BL sludge	^{12}C-sodium butyrate (6.68 mM)	30 days	Total DNA, fractions 8–10
BL-13B	BL sludge	^{13}C-sodium butyrate (6.68 mM)	30 days	Total DNA, fractions 8–10
BL-12A	BL sludge	^{12}C-sodium acetate (13.36 mM)	30 days	Total DNA, fractions 7–10
BL-13A	BL sludge	^{13}C-sodium acetate (13.36 mM)	30 days	Total DNA, fractions 7–10
BH-12B	BH sludge	^{12}C-sodium butyrate (16.7 mM)	20 days	Total DNA, fractions 8–10
BH-13B	BH sludge	^{13}C-sodium butyrate (16.7 mM)	20 days	Total DNA, fractions 8–10
BH-12A	BH sludge	^{12}C-sodium acetate (33.4 mM)	20 days	Total DNA, fractions 6–9
BH-13A	BH sludge	^{13}C-sodium acetate (33.4 mM)	20 days	Total DNA, fractions 6–9

BL BL reactor; *BH* BH reactor; *12B* ^{12}C-butytate; *13B* ^{13}C-butytate; ^{12}*A* 12C-acetate; *13A* ^{13}C-acetate.

[a] Substrate was added every two days, and the concentration was the final concentration of addition each time.

[b] After ultracentrifugation, 15 density fractions (numbered fraction 1–15 from the bottom (high density) to the top (low density), and 400 μL of each fraction) were collected from each treatment.

DNA Extraction, Density-Gradient Centrifugation, and Fractionation

Total genomic DNA of each sludge sample was extracted by CTAB method [33]. Purified DNA was prepared for density-gradient centrifugation and fractionation as described by Lueders et al. [34]. Briefly, total DNA (~ 2.5 μg) was added to Quick-Seal polyallomer tubes (6.3 mL, Beckman Coulter, Australia), along with 1.2 mL gradient buffer (GB) (containing 0.1 M Tris-HCl (pH 8.0), 0.1 M KCl, and 1 mM EDTA) and 4.8 mL CsCl solution (final buoyant density of 1.9 g/mL). Then, the tubes were sealed and centrifuged at 177,000×*g* for 40 h at 20 °C in a Beckman ultracentrifuge with Ti90 fixed angle rotor (Beckman, USA). Following centrifugation, 15 density fractions each with 400 μL, numbered fractions 1–15 from the bottom (high density) to the top (low density), were collected from each tube using a fraction recovery system (Beckman Coulter, USA). The buoyant density of each fraction was determined by a digital refractometer (AR200, Reichert, USA) [34], and DNA was recovered from each fraction by PEG6000 precipitation with glycogen [35].

In order to profile the DNA gradient distribution, bacterial and archaeal 16S rRNA genes in each fraction were quantified by qPCR, using the EcoTM real-time PCR system (Illumina, USA) with primer sets Eu27f/Eu518r [36] and Arch349f/Arch806r [37], respectively. Reaction mixtures (20 μL) were prepared with 2 μL template DNA (~ 2 g/μL and ~ 7 g/μL DNA used for amplification of bacterial and archaeal 16S rRNA gene, respectively) and 0.8 μL of each primer. qPCR conditions for bacterial 16S rRNA gene were as follows: denaturation at 95 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 40 s for a total of 40 cycles. qPCR conditions for archaeal 16S rRNA gene were as follows: denaturation at 95 °C for 40 s, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s for a total of 40 cycles. Based on qPCR results, DNA samples from several heavy density fractions and total DNA for each treatment were used for sequencing, as shown in Table 1.

High-Throughput Sequencing and Phylogenetic Analysis

The V4–V5 regions of the bacterial and archaeal 16S rRNA genes of the DNA samples were amplified using the primers, 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) [38]. Sequencing was performed on the Illumina MiSeq platform using the MiSeq v2 reagent kit (2 × 250 bp) by Chengdu Institute of Biology. Raw FASTQ files were quality-processed using the QIIME Pipeline-Version 1.7.0 (http://qiime.org/). Chimeric sequences were removed using the Uchime algorithm [39]. Operational taxonomic units (OTUs) were defined by clustering at 97% similarity. Final OTUs were taxonomically classified using Ribosomal Database Project classifier and NCBI blast [40]. Phylogenetic analysis was performed using the MEGA4 software package (http://megasoftware.net/mega4/) after multiple sequence alignment by Clustalx1.8 (http://www.clustal.org/). Phylogenetic trees were constructed using neighbor-joining method.

Nucleotide Sequence Accession Numbers

The original sequencing data is available at the National Center for Biotechnology Information database (accession no. PRJNA475621).

Results

Microbial Community of Butyrate-Fed Chemostats Operated at Different Dilution Rates

BL (0.01/day) and BH (0.05/day) reactors operated for approximately 400 and 300 days, respectively, and both retained the steady state. The performance of both reactors is displayed in Fig. S1. The biogas yield of the BL and BH reactors was stable, at approximately 80 and 900 mL/day during this period, respectively. TOC and pH in the two reactors were 40–50 mg/L and 8–8.3, respectively. No organic acid accumulation was observed, and butyrate fed into each reactor was completely mineralized. The average VSS concentration was 1.7 g/L for BL reactor and 1.5 g/L for BH reactor. The sludge from each chemostat was used for microbial community analyses.

Based on the high-throughput sequencing of 16S rRNA gene, the composition of bacterial community in both reactors was obviously different (Fig. 1). In the BL reactor, 84.22% of bacterial OTUs were assigned to four phyla: *Thermotogae* (25.65%), *Firmicutes* (22.83%), *Bacteroidetes* (18.24%), and *Proteobacteria* (17.50%) (Fig. 1a). In the BH reactor, phylum *Firmicutes* accounted for 75.33% abundance (Fig. 1b). At the genus level, *Defluviitoga*, *Mesotoga*, *Coprothermobacter*, and *Petrimonas* were the dominant genera at the dilution rate of 0.01/day (BL reactor), accounting for 13.27%, 12.37%, 10.16%, and 9.52% of the total valid reads, respectively. Several other key genera, including *Syntrophomonas* (6.09%), *Desulfovibrio* (5.71%), *Dechloromonas* (4.3%), and *Anaerobaculum* (3.35%), also were detected. Among them, *Syntrophomonas* is known as a SBOB in association with H_2-utilizing methanogens. It was observed following enrichment at the higher dilution rate (0.05/day) and became the most abundant genus (54.54%) in the BH reactor. In addition, one OTU, which could not be affiliated to any bacterial phylum, accounted for 15.19% of total bacterial reads and was the second most abundant genus in the BH reactor.

The archaeal communities of both reactors are listed in Table S1. In the BL reactor, acetoclastic *Methanosaeta* (53.62%) and hydrogenotrophic *Methanobacterium* (36.15%) and *Methanothermobacter* (7.14%) were the primary methanogens. *Methanosaeta* remained dominant in the BH reactor with an abundance of 78.60%. *Methanoculleus* (17.27%) became the most dominant hydrogenotrophic methanogen, followed by *Methanobacterium* (3.63%). *Methanothermobacter* sp. was not detected in the BH reactor.

DNA-SIP Analysis

Sludge from both reactors was incubated with ^{13}C- and ^{12}C-substrates for 30 and 20 days, respectively (Table 1). The VFA consumption and gas yield in each microcosm were measured. Two biological replicates of each treatment showed almost the same VFA consumption and biogas production. As shown in Table S2 and Fig. S2, ^{13}C- and ^{12}C-butyrate could be completely consumed by both methanogenic sludges, and the gas yields were approximate 74.18–92.40% of the theoretical value. For treatments with BL sludge, more ^{12}C-acetate (100%) than ^{13}C-acetate (about 80%) was utilized, and their gas yields were 60.15–66.9% of the theoretical value. BH sludge consumed 100% acetate, and the gas yields were around 56% of the theoretical value.

Total DNA from all microcosms was used for density-gradient centrifugation and fractionation. The DNA distribution profiles for different density fractions are illustrated by relative copies of bacterial and archaeal 16S rRNA genes (Fig. 2; Fig. S3). As shown in Fig. 2a, heavy density fractions, 1.71 to 1.72 g/mL (fraction 8), contained more bacterial 16S rRNA genes (29.11% and 17.85%) in the ^{13}C-butyrate treatments with BL sludge than in the ^{12}C-controls (8.91% and 7.48%). For ^{13}C-acetate treatments with BL sludge (Fig. 2b), 13.7% and 10.5% of bacterial 16S rRNA genes were enriched at a density of 1.73 g/mL (fraction 7). Figure S3a shows that the abundance of archaeal 16S rRNA genes increased from 14.8% and 8.05% (^{12}C-controls) to 52.52% and 36.33% (^{13}C-butyrate treatments) in the heavy fractions. More archaeal 16S rRNA genes (37.4% and 20.5%) were obtained in the heavy density DNA fractions from ^{13}C-acetate treatments with BL sludge (Fig. S3b). Similarly, about 18.66% and 16.91% bacterial 16S rRNA genes were enriched at density fractions ranging from 1.72 to 1.73 g/mL (fraction 8) in the ^{13}C-butyrate treatments with BH sludge (Fig. 2c). At the same density fractions, archaeal 16S rRNA genes presented with 3.77% and 6.38% abundance (Fig. S3c). For the ^{13}C-acetate treatments with BH sludge, 13.99% and 14.59% of 16S rRNA genes (Fig. 2d) were found at a density of 1.73 g/mL (fraction 7), and more archaeal 16S rRNA genes (14.23% and 13.19%) were obtained in the heavy density fractions (Fig. S3d). These results suggested that bacterial and archaeal species were labeled successfully by ^{13}C-substrates. In order to be closer to the potential BOB and AOB, the 16S rRNA genes in the heavy density fractions (fraction 8–10 for butyrate of BL and BH, fraction 7–10 for acetate of BL and fraction 6–9 for acetate of BH) were used for sequencing analyses. Whole DNA for 16 samples from eight different treatments (Table S2) were sequenced.

Phylogenetic Identification of the Labeled Bacterial and Archaeal Species

Bacterial Community at the Phylum Level

Microbial community data obtained from total DNA from ^{12}C- and ^{13}C-treatments were compared at the phylum level,

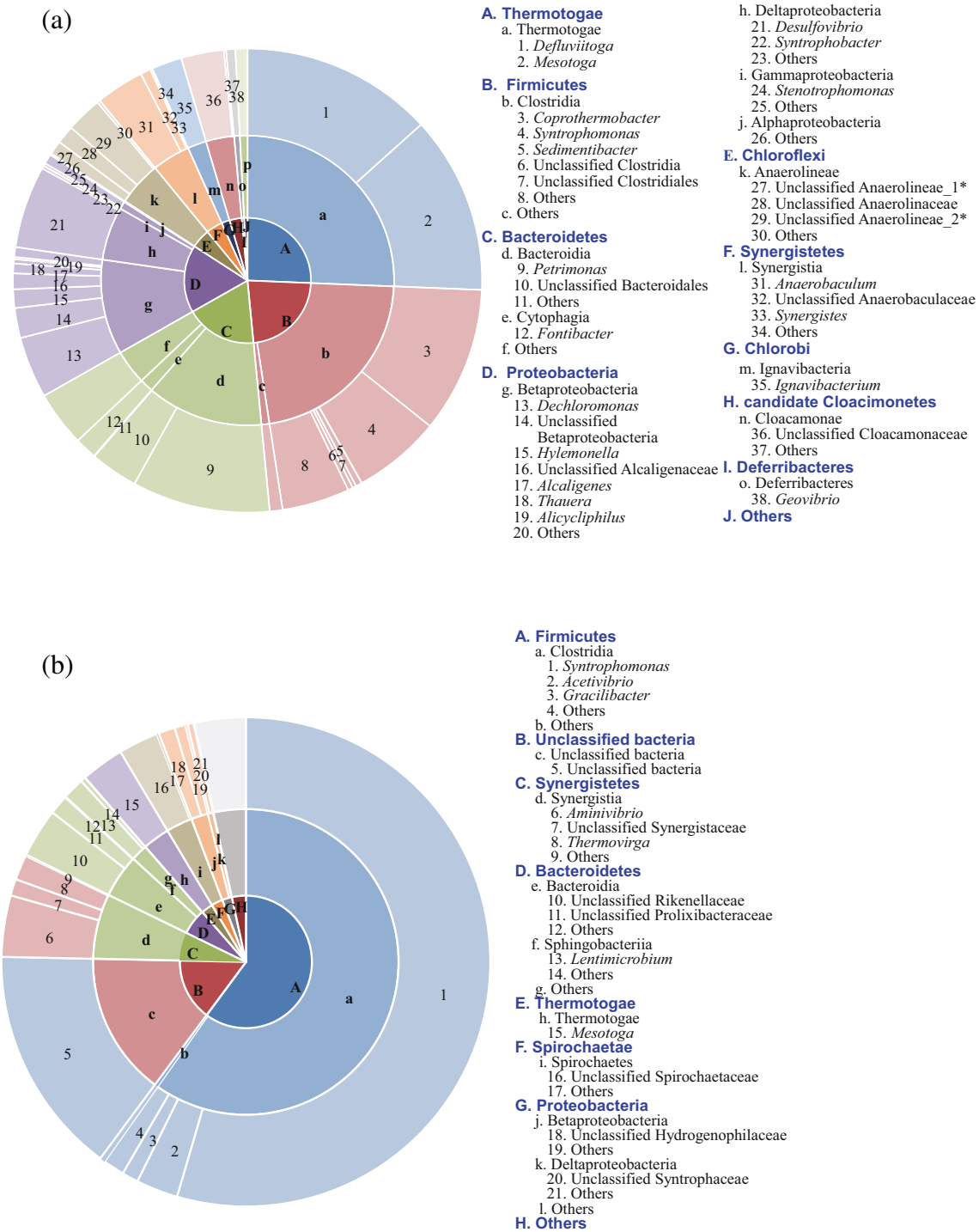

Fig. 1 Composition of bacterial community in two mesophilic butyrate-fed chemostats. **a** BL reactor: operated at a dilution of 0.01/day. **b** BH reactor: operated at a dilution of 0.05/day. *Unclassified Anaerolineae_1 and Unclassified Anaerolineae_2 were the two different OTUs affiliated to class *Anaerolineae*

using 16S rRNA gene sequences. The results showed that the composition and abundances of phyla were similar in the total DNA from BL or BH sludge treated with ^{12}C- and ^{13}C-substrates (data not shown). Phyla *Proteobacteria*, *Thermotogae*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, and *Synergistetes* were predominant in BL sludge treatments, while *Firmicutes*, *Synergistetes*, *Spirochaetae*, *Thermotogae*, *Bacteroidetes*, and unclassified bacteria presented in BH sludge treatments.

For ^{13}C-butyrate-treated BL sludge (Fig. 3a), *Firmicutes* and *Synergistetes* (two dominant phyla), accounted for

11.17% and 6.47% of the total reads in fraction 8 (1.718 g/mL), respectively, while there were only 1% of *Firmicutes* and *Synergistetes* in the corresponding ^{12}C-control. In addition, the abundance of *Bacteroidetes* increased nearly three times, both in fraction 8 (from 0.14% in ^{12}C-butyrate treatment to 0.44% in ^{13}C-butyrate treatment) and fraction 9 (from 2.83% in ^{12}C-butyrate treatment to 7.52% in ^{13}C-butyrate treatment). More phyla related to acetate oxidation were observed in BL sludge, especially in fraction 7 (1.727 g/mL) and fraction 8 (1.714 g/mL) (Fig. 3a). *Firmicutes* was enriched in fraction 7 and 8 with an abundance of 5.44% and 9.48%, respectively, while it was only 0.21% and 1.16% in the corresponding fraction of ^{12}C-control. *Synergistetes* was enriched in fraction 7 (from 0.09% in ^{12}C-acetate treatment to 3.48% in ^{13}C-acetate treatment) and in fraction 8 (from 1.23% to 3.02%). Moreover, phyla *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Thermotogae* slightly increased in abundance in fraction 8 of DNA treated with ^{13}C-acetate, compared with the ^{12}C-control.

For ^{13}C-butyrate-treated BH sludge (Fig. 3b), four key phyla were significantly enriched, largely in the DNA of fraction 8 (1.727 g/mL), including *Firmicutes* (5.21%), *Synergistetes* (6.81%), *Spirochaetae* (1.88%), and unclassified bacteria (2.42%), while phylum *Thermotogae* (0.27%) slightly increased. These phyla accounted for 0.44%, 0.21%, 0.15%, 0.39%, and 0.04% in fraction 8 of DNA from the ^{12}C-butyrate treatment, respectively. For acetate-treated BH sludge, *Synergistetes* was enriched, largely in fractions 6–8 (1.749 to 1.722 g/mL) of DNA from the ^{13}C-acetate treatment, with an abundance of 5.75%, 10.91%, and 10.01%, respectively, compared with the abundances from the ^{12}C-acetate treatment (0.17%, 0.31%, and 2.08%, respectively). The abundance of two other phyla, *Firmicutes* and unclassified bacteria, was 2–3 times higher in fractions 7 and fraction 8 of DNA from ^{13}C-acetate treatment than those in the ^{12}C-control. In addition, small quantities of *Spirochaetae*, *Thermotogae*, and *Bacteroidetes* were enriched in the heavy density DNA fraction from ^{13}C-acetate treatments. The results above suggested that much more phyla involved in the butyrate and acetate oxidation.

Bacterial Community at the Genus Level

There were more apparent differences between the bacterial communities in heavy density fractions of ^{13}C- and ^{12}C-treatments at the genus level (Figs. 4, 5, and 6). The abundance of several represented bacteria increased in the heavy density ^{13}C-treated DNA fractions, especially focusing on fraction 8 for both butyrate and acetate.

Compared with ^{12}C-butyrate-treated BL sludge, *Syntrophomonas* (BL-OTU281, 7.61%), unclassified *Synergistaceae* (BL-OTU29, 4.43%), *Sedimentibacter* (BL-OTU152, 0.59%), and unclassified *Syntrophomonadaceae* (BL-OTU7514, 0.63%) populations increased separately, ten-fold in fraction 8 of DNA from the ^{13}C-butyrate treatments (Figs. 4a, b, e and 6). In acetate-treated BL sludge, *Sedimentibacter* (BL-OTU152) and unclassified *Synergistaceae* (BL-OTU29) were greatly enriched. *Sedimentibacter* populations increased from 0.05% in ^{12}C-acetate treatments to 5.25% in ^{13}C-acetate treatments (105-fold increase) in fraction 8, while unclassified *Synergistaceae* increased from 0.02 to 1.77% (88.5-fold increase) in fraction 7 (Figs. 4c–e and 6). In addition, the abundance of *Mesotoga* (BL-OTU23, 1.92%), unclassified *Anaerolineae*_1 (BL-OTU45, 1.79%), *Desulfovibrio* (BL-OTU146, 1.25%), and *Petrimonas* (BL-OTU95, 0.9%) in the eighth density ^{13}C-acetate-treated DNA fractions were higher than those from ^{12}C-controls, which suggested that these bacteria were enriched by ^{13}C-acetate.

Compared with ^{12}C-butyrate-treated BH sludge, unclassified *Synergistaceae* (BH-OTU209, 6.10%) and *Syntrophomonas* (BH-OTU324, 2.75%) populations increased by 47- and 18-fold, respectively, in the 8th density DNA fractions from the ^{13}C-butyrate treatments (Figs. 5a, b, e and 6). *Syntrophomonas* (BH-OTU80, 2.01%), unclassified *Spirochaetaceae* (BH-OTU142, 1.80%), unclassified bacteria (BH-OTU344, 2.42%), and *Mesotoga* (BH-OTU48, 0.27%) were enriched 5–9-fold in the same DNA fraction. Compared with ^{12}C-acetate-treated BH sludge, unclassified *Synergistaceae* (BH-OTU209, 10.61% in fraction 7), *Acetivibrio* (BH-OTU287, 1.23% in fraction 8), and unclassified *Spirochaetaceae* (BH-OTU142, 3.02% in fraction 8) were enriched 40-, 17-, and 5-fold, respectively, in DNA from ^{13}C-acetate treatments (Figs. 5c–e and 6). The abundance of three other genera increased slightly in heavy density DNA fractions from ^{13}C-acetate treatments compared with the ^{12}C-control, including unclassified bacteria (BH-OTU344, 1.79%), *Mesotoga* (BH-OTU48, 0.6%), and *Aminivibrio* (BH-OTU82, 0.71%).

Archaeal Community at the Genus Level

Archaeal community studies at the genus level showed that both acetotrophic and hydrogenotrophic methanogens were enriched by the ^{13}C-substrate (Figs. S4 and S5). Compared with the ^{12}C-butyrate-treated BL sludge microcosm, *Methanosaeta* (21.17%), *Methanoculleus* (14.63%), *Methanobacterium* (4.70%), and *Methanothermobacter* (3.62%) were enriched 3.3–6.7-fold in fraction 8 of ^{13}C-butyrate treatments (Fig. S5). Four genera, including *Methanosaeta, Methanothermobacter*, *Methanobacterium*, and unclassified *Methanomassiliicoccaceae*, were largely enriched following ^{13}C-acetate treatments. Unclassified *Methanomassiliicoccaceae* and *Methanosaeta* particularly presented with an abundance of 4.97% and 20.13% in the 7th density DNA fractions from ^{13}C-acetate treatments, while

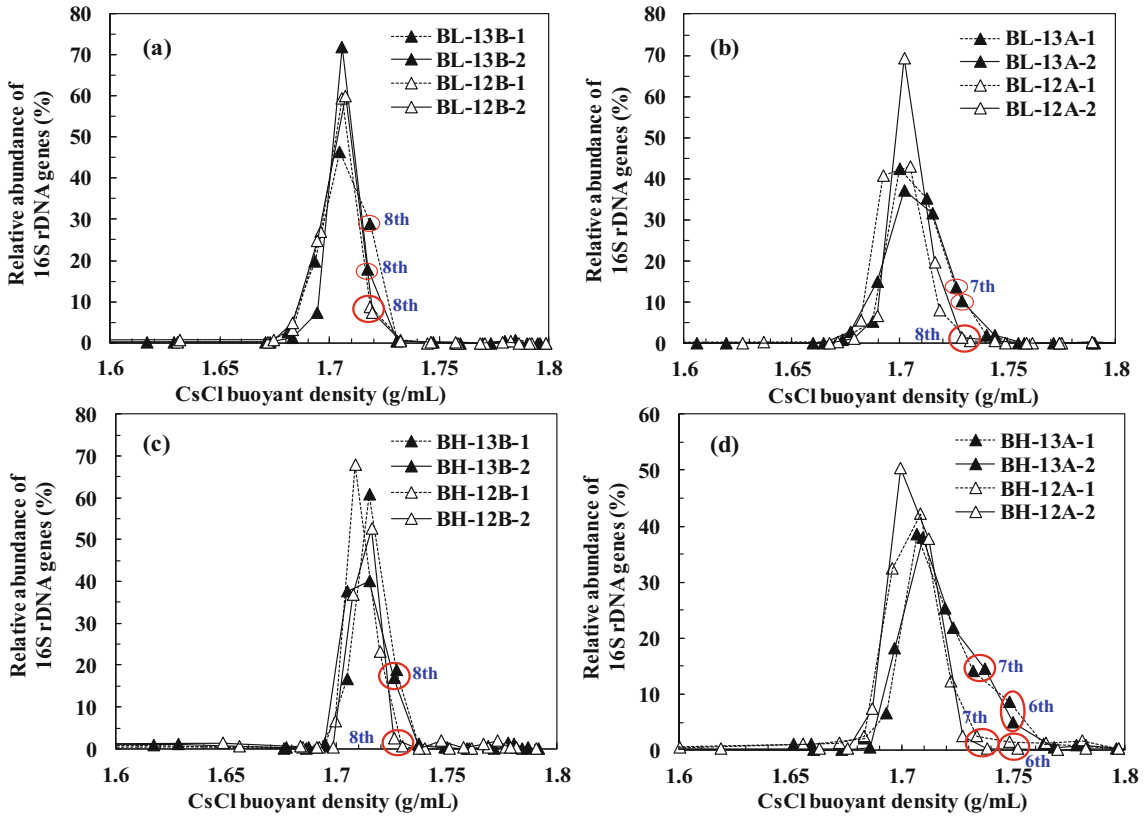

Fig. 2 Relative abundance of bacterial 16S rRNA genes in the gradient fractions. **a** BL sludge (0.01/day), butyrate; **b** BL sludge, acetate; **c** BH sludge (0.05/day), butyrate; (b) BH sludge, acetate. 6–8th, the number of fractions after gradient fractionation

there were only 0.01% and 0.35% in the corresponding ^{12}C-controls, respectively. In the BH sludge microcosm (Fig. S5), *Methanosaeta*, *Methanoculleus*, and *Methanobacterium* increased in the heavy fractions of ^{13}C-butyrate treatments compared with ^{12}C-control treatments, while the enrichment of *Methanosaeta* and *Methanoculleus* was observed in the heavy fractions of ^{13}C-acetate treatments.

Discussion

In this study, two mesophilic chemostats fed with butyrate as the sole carbon source were constructed and operated at different dilution rates. The potential butyrate-oxidizing bacteria (BOB), acetate-oxidizing bacteria (AOB), and methanogens in both chemostats were investigated by DNA-SIP and 16S rRNA high-throughput sequencing. The results showed that different species were responsible for butyrate and acetate oxidation in both chemostats.

Among the species enriched in the heavy density DNA fractions from ^{13}C-butyrate and ^{13}C-acetate-treated BL sludge, only two *Syntrophomonadaceae* OTUs (BL-OTU281 and BL-OTU7514) were enriched in the heavy density DNA fractions from ^{13}C-butyrate treatment. *Syntrophomonas* (BL-OTU281) and unclassified *Syntrophomonadaceae* (BL-OTU7514) shared 98% and 93% sequence identity with *Syntrophomonas wolfei*, respectively. The 10-fold enrichment observed for both OTUs suggests that they played key roles in butyrate oxidation. Four genera were enriched in the heavy density DNA fractions from ^{13}C-acetate treatment, including *Mesotoga* (BL-OTU23), unclassified *Anaerolineae* (BL-OTU45), *Desulfovibrio* (BL-OTU146), and *Petrimonas* (BL-OTU95). This suggested that these OTUs may be related to acetate oxidation. BL-OTU23 was affiliated to *Mesotoga infera* VNs100 (97% similarity), a members of the phylum *Thermotogae*. According to metagenomic analysis results, Nobu et al. found that *Mesotoga* may syntrophically oxidize acetate through a previously uncharacterized pathway [41]. BL-OTU45 showed 98% similarity with function-unknown uncultured clone 22 (MH040197) and clone B146 (HQ640609), belonging to class *Anaerolineae* of phylum *Chloroflexi*. Several reports have suggested that syntrophic metabolism of butyrate and propionate could occur by filamentous *Anaerolineaceae* and *Methanosaeta* via direct interspecies electron transfer (DIET) during the AD process [42, 43]. Therefore, the syntrophic oxidation of acetate via DIET may have occurred between *Anaerolineae* and *Methanosaeta* in this study, considering the high abundance of *Methanosaeta* in BL sludge; however, this requires further investigation. BL-

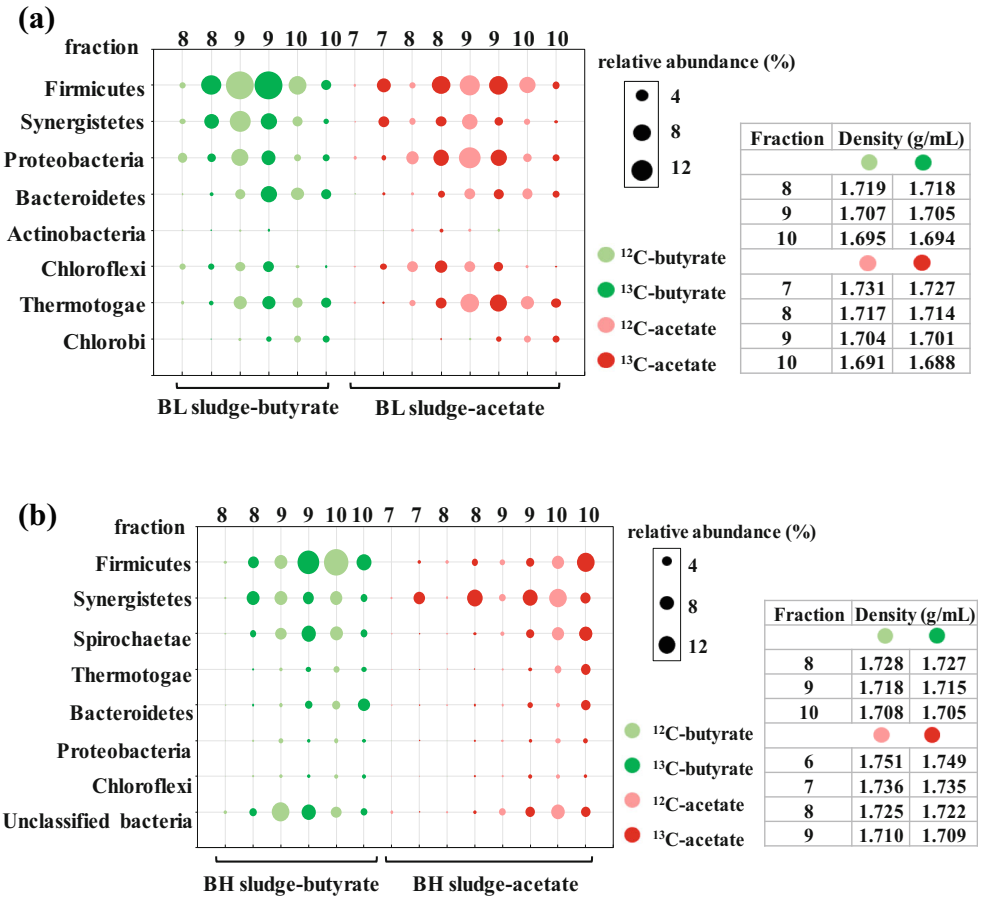

Fig. 3 The representative bacterial communities of DNA-SIP samples at the phylum level. (**a**) BL sludge. (**b**) BH sludge; 6–10th fraction: the number of fractions after gradient fractionation

OTU146 and BL-OTU95 showed highest similarity with *Desulfovibrio oryzae* PETROMIC B02 (AY664600) (96%) and *Petrimonas sulfuriphila* BN3 (97%), respectively. *Desulfovibrio* spp. are known as sulfate-reducing bacteria [44], and to date, only the butyrate oxidation ability of *Desulfovibrio butyratiphilus* sp. nov. BSY^{T} has been described [45]. Recently, several bacteria in the sulfate-reducing sediment were labeled by ^{13}C-acetate, including unclassified *Desulfobacteraceae* and *Desulfovibrio* [46]. Here, we inferred that these sulfate reducers may utilize acetate for energy production or growth, as a building block of biosynthesis. *Petrimonas sulfuriphila* is a strictly anaerobic bacteria, which is able to utilize sugars as carbon and energy sources, and reduce sulfur to sulfide with hydrogen [47]. Therefore, the roles of *Petrimonas* in BL sludge remain unclear; however, the labeling of this bacterium suggests that it may be related to acetate oxidation. BL-OTU152 and BL-OTU29 were largely enriched in the heavy density DNA fractions from both ^{13}C-butyrate and ^{13}C-acetate treatments, suggesting that both OTUs may be mostly involved in acetate oxidation. BL-OTU152 was closely related to *Sedimentibacter* (96%) of the family *Synergistaceae*, and species in this genus are often identified as amino acid-utilizing bacteria [48, 49]. Recently, several fermentative microorganisms including *Sedimentibacter* were found in acetate-fed microbial fuel cells, although acetate was a non-fermentative substrate [50, 51]. Regueiro et al. believed that *Sedimentibacter* played an important role in the degradation of accumulated VFAs, considering that *Sedimentibacter* appeared or increased in population and remained until VFA levels decreased in a temperature-changed AD system [52]. Thus, *Sedimentibacter* labeled by ^{13}C-acetate (105-fold increase) in our study suggests that it is an acetate-oxidizing bacterium. BL-OTU29 also showed high similarity (98%) with another amino acid-fermenting bacterium, *Synergistaceae* DZ-S4 (MF185666), isolated from a municipal anaerobic sewage sludge digester. Although most cultured microbes belonging to family *Synergistaceae* have the ability to degrade amino acids into VFAs [53, 54], some species from *Synergistaceae* may also ferment VFAs via syntrophic relationships with methanogens during anaerobic digestion [55]. Hence, the roles of this OTU in BL sludge may be related to acetate oxidation. The simultaneous labeling of BL-OTU152 and BL-OTU29 by ^{13}C-butyrate may be due to cross-feeding.

Among the species enriched in the heavy density DNA fractions from ^{13}C-butyrate and ^{13}C-acetate-treated BH

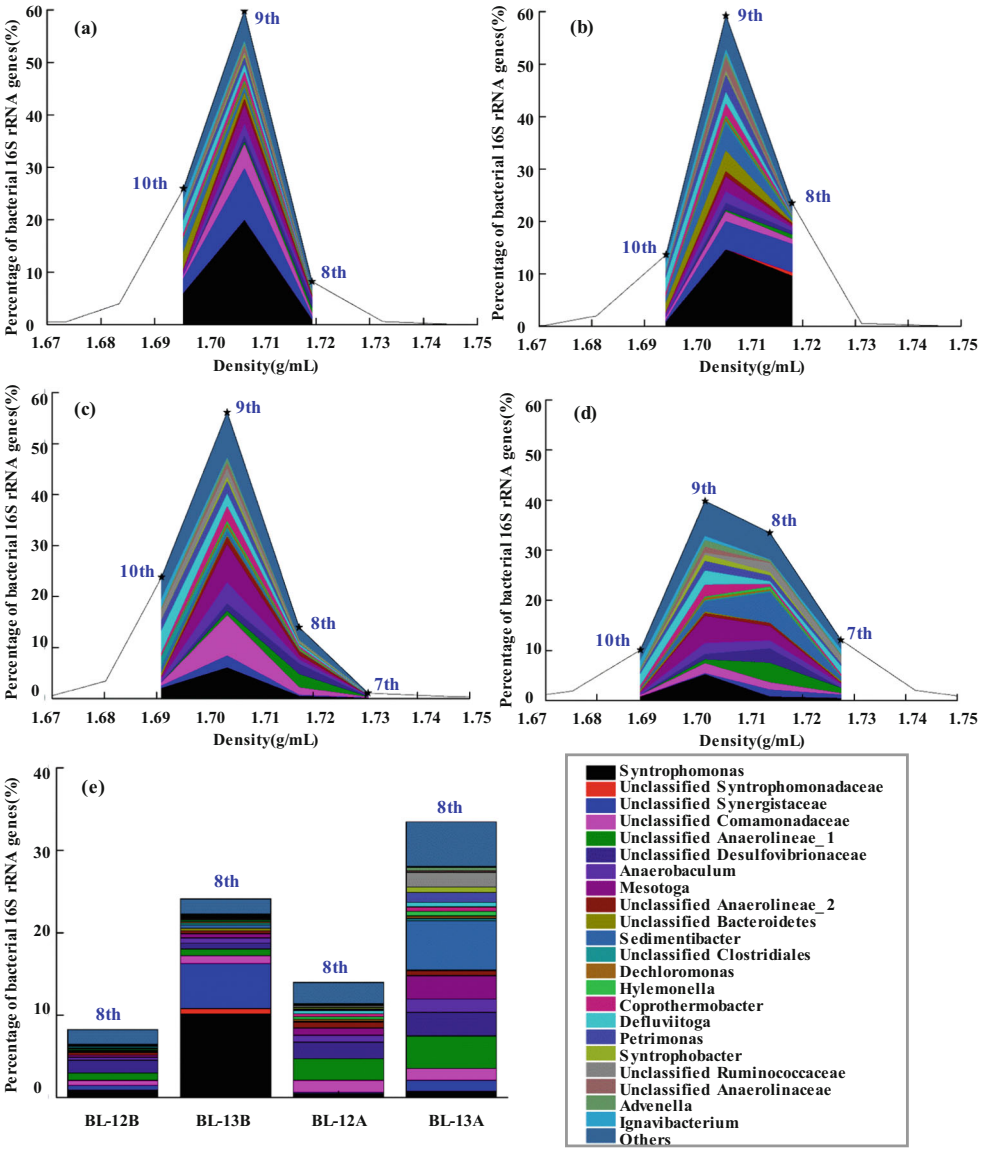

Fig. 4 The relative bacterial abundances of DNA-SIP samples from BL sludge at the genus level. (**a**) BL-12B. (**b**) BL-13B. (**c**) BL-12A. (**d**) BL-13B. (**e**) Sample in the 8th fraction

sludge, two syntrophic BOB, *Syntrophomonas* BH-OTU80 and BH-OTU324, were only enriched from ^{13}C-butyrate treatment. They showed 98% and 96% similarity with *Syntrophomonas wolfei* Goettingen G311 and *Syntrophomonas zehnderi* OL-4, respectively. *Aminivibrio* BH-OTU82 and *Acetivibrio* BH-OTU287 were only enriched in the heavy density DNA fractions from ^{13}C-acetate treatment, suggesting that they play key roles in acetate oxidation. BH-OTU82 was most closely related to *Aminivibrio pyruvatiphilus* 4F6E (99% similarity), an amino acid-degrading bacterium when in co-culture with the hydrogen-utilizing methanogen *Methanobacterium formicicum* JCM 10132(T) [54]. According to the phylogenetic tree, BH-OTU82 belonged to Synergistes group 4 (clone RSg13-6 and clone 13Cpro-5 in the Fig. 6), which was recently identified by RNA-SIP and MAR-FISH as the only predominant acetate-utilizing bacteria in anaerobic digester sludge [56]. In addition, Synergistes group 4 was found to have maximum utilization rate and high K_m for acetate, and they are more competitive than acetoclastic *Methanosaeta* at high acetate concentrations. Taken together, BH-OTU82 may play a role in syntrophic oxidation of acetate in BH sludge. BH-OTU287 had 96% similarity with *Acetivibrio cellulolyticus* HL-2, a well-known anaerobic cellulolytic microorganism [57]. However, no report referred to its function in acetate oxidation, and this needs to be further confirmed. Four common OTUs were enriched in the heavy density DNA fractions from both the ^{13}C-butyrate and ^{13}C-acetate treatments, including BH-OTU209, BH-OTU142, BH-OTU48, and BH-OTU 344. These were most likely involved in acetate oxidation. BH-OTU209 was most closely related to *Synergistaceae* clone VHW_D_R9 (JQ085712, 99% similarity) from a two-stage digester treating solid wastes. And, it had 95% similarity with *Thermovirga lienii* DSM 17291, an amino-acid-

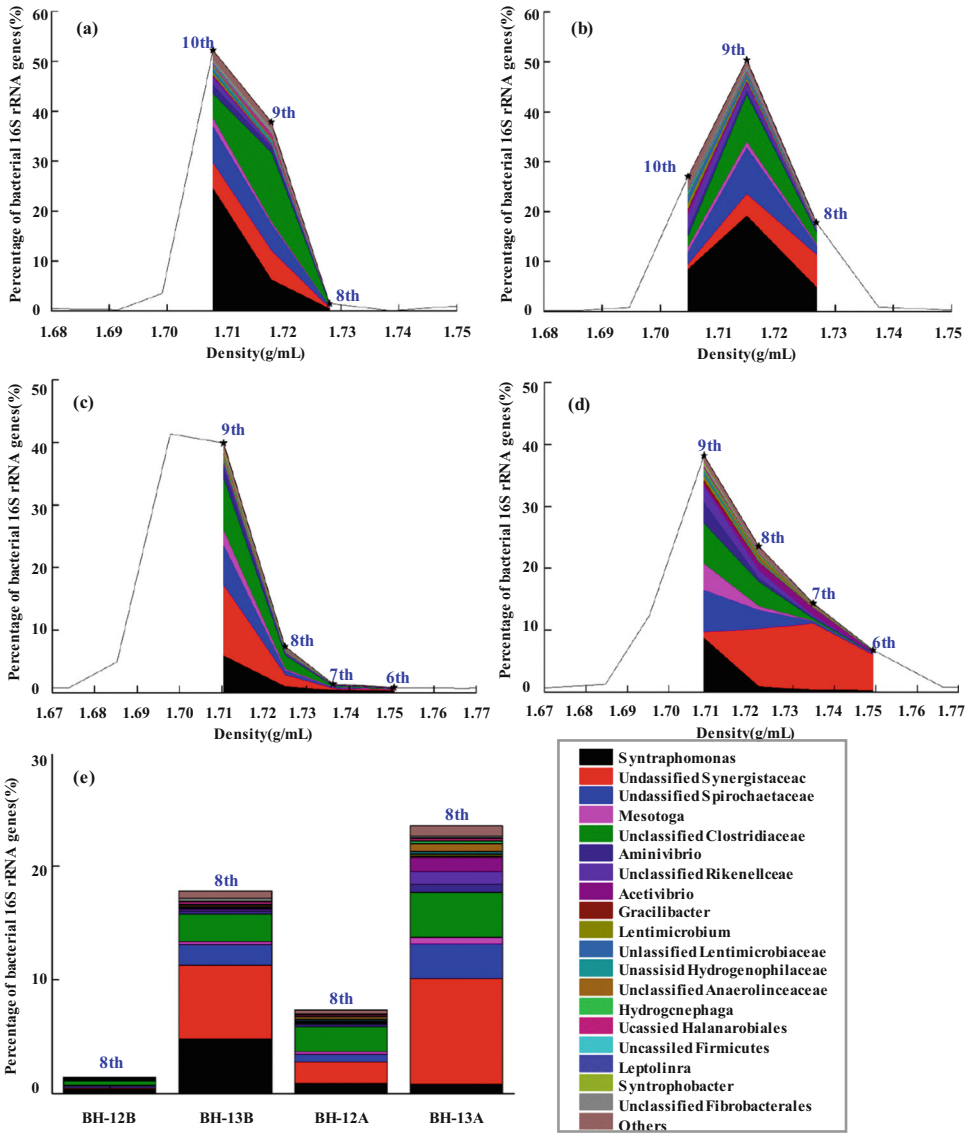

Fig. 5 The relative bacterial abundances of DNA-SIP samples from BH sludge at the genus level. (**a**) BH-12B. (**b**) BH-13B. (**c**) BH-12A. (**d**) BH-13B. (**e**) Sample in the 8th fraction

degrading bacterium [53]. In other studies in our lab, the same OTU was also largely enriched in different mesophilic digester sludges when incubated with ^{13}C-acetate or ^{13}C-propionate (data not shown). Xu et al. [58] reported that one OTU (99% similarity with BH-OTU209) accounted for nearly half of the total reads in the anaerobic reactor fed with acetate. Therefore, BH-OTU209 may have the ability for syntrophic acetate oxidation. BH-OTU142 only had 94% similarity with the pure-culture bacterium, *Rectinema cohabitans* HM (NR_156915). It was affiliated to uncultured *Spirochaetaceae* clone F3 (MG674678, 99% similarity), which was obtained from an anaerobic butyrate oxidation system. Selective enrichment of *Spirochaetes* was observed during the AD process, accepting VFAs, especially acetate, as substrate, and suggesting the possible role of *Spirochaetes* in syntrophic acetate oxidation [59]. BH-OTU48 was affiliated to *Mesotoga infera* VNs100 (99% similarity). BH-OTU344 was highly abundant (15.19%) in the original BH reactor sludge (Fig. 1b). It showed 99% similarity with uncultured clone QEDR1AF11 obtained from an anaerobic digestion system treating sludge [60], but could not be affiliated to any bacterial phylum (Fig. 5). Taken together, the four OTUs may be putative AOB, and cross-feeding may lead to the labeling of them in the ^{13}C-butyrate treatments.

The community analysis above showed that different species were retrieved from different sludges. From the DNA-SIP results, species from *Syntrophomonadaceae* may be putative BOB in both the BL and BH sludges. Liu et al. determined, using DNA-SIP, that *Syntrophomonadaceae*, together with the methanogens, *Methanosarcinaceae* and *Methanocellales*, were responsible for syntrophic oxidation of butyrate in paddy soil [29]. But, in other environments, more non-*Syntrophomonadaceae* bacteria were labeled by ^{13}C-butyrate [26–28]. This may be because *Syntrophomonadaceae*-affiliated species were more easily enriched in chemostats during

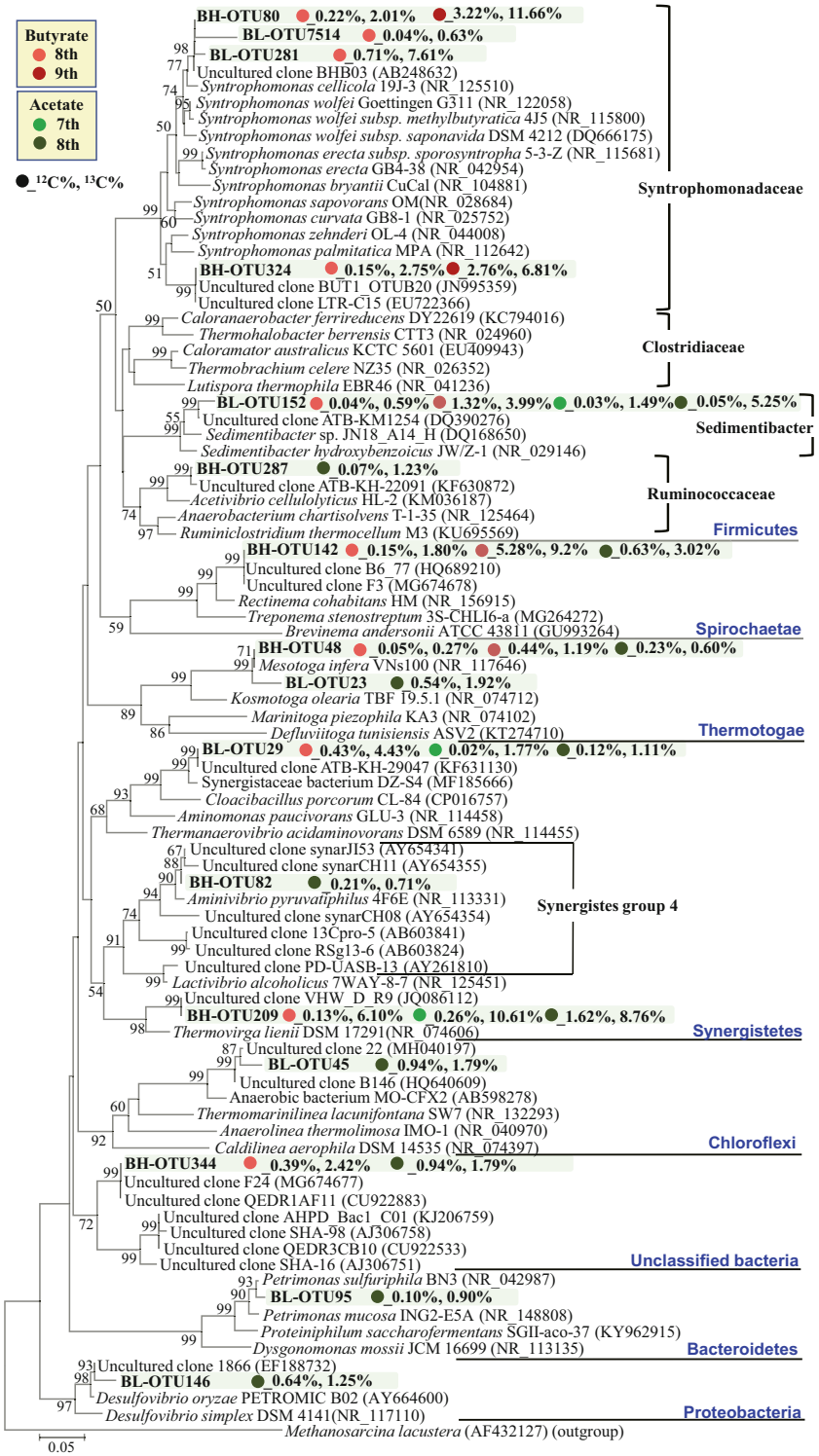

Fig. 6 Neighbor-joining phylogenetic tree of ^{13}C-substrate labeled representative bacteria

long-term incubation using butyrate as the sole carbon source. The diversity of bacteria labeled by ^{13}C-acetate was higher than expected in this study. Species from *Mesotoga*, unclassified *Anaerolineae*, *Desulfovibrio*, *Petrimonas*, *Sedimentibacter*, and unclassified *Synergistaceae* may be the AOB in the BL sludge, while species from *Aminivibrio*, *Acetivibrio*, *Mesotoga*, unclassified *Synergistaceae*, unclassified *Spirochaetaceae*, and unclassified bacteria may be related to acetate oxidation in the BH sludge. Similar to our results, several reports, using DNA- or RNA-SIP identified some species belonging to *Desulfovibrionaceae*, *Synergistaceae*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* as acetate

utilizers [46, 56, 61]. Hao et al. also observed that species from the classes *Clostridia*, *Thermotogae*, and *Spirochaetes* were labeled by ^{13}C-acetate in thermophilic methanogenic reactors with high ammonia levels [62].

Regarding archaea in ^{13}C-butyrate-treated BL or BH sludge, nearly all methanogens, except *Methanomassiliicoccus* in BL sludge, existing in the original chemostats were labeled, which suggested that they were all involved in methane production from butyrate. Acetotrophic *Methanosaeta* and hydrogenotrophic *Methanoculleus* were particularly largely concentrated in the heavy fractions. Tang et al. also found that these two genera dominated in a mesophilic butyrate-degrading methanogenic reactor, at low dilution rates [31]. Lower H_2 partial pressures of *Methanoculleus* than other hydrogenotrophic methanogens may have permitted its dominant position in both chemostats [63]. In fact, hydrogenotrophic methanogens, including *Methanoculleus*, *Methanobacterium*, and *Methanothermobacter*, should compete for hydrogen with each other due to their different hydrogen affinities [64]. This may allow the enrichment and labelling of only *Methanothermobacter* in the BL sludge. Similarly, different hydrogenotrophic methanogens were enriched in ^{13}C-acetate treatments with BL (*Methanobacterium* and *Methanothermobacter*) and BH (*Methanoculleus*) sludges. Detection of hydrogenotrophic methanogens in our study suggests that syntrophic acetate oxidation could occur [65].

In conclusion, microbial community analyses showed that DNA-SIP successfully identified butyrate and acetate oxidizers. Different species were retrieved from two chemostats operated at different dilution rates, suggesting that more uncultured bacteria played roles in butyrate degradation during AD. However, some functional bacteria, which have slower growth rates and lower abundance in the sludge, may be difficult to identify using DNA-SIP. In addition, the presence of these species identified by DNA-SIP does not mean activity, which should be further confirmed by culture-dependent technology and RNA approach (such as RNA-SIP and metatranscriptomics).

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