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Patterns of Syntrophic Interactions in Methanogenic Conversion of Propionate

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Abstract

Methanogenesis is central to anaerobic digestion processes. The conversion of propionate as a key intermediate for methanogenesis requires syntrophic interactions between bacterial and archaeal partners. In this study, a series of methanogenic enrichments with propionate as the sole substrate were developed to identify microbial populations specifically involved in syntrophic propionate conversion. These rigorously-controlled propionate enrichments exhibited functional stability with consistent propionate conversion and methane production; yet, the methanogenic microbial communities experienced substantial temporal dynamics, which has important implications on the understanding of mechanisms involved in microbial community assembly in anaerobic digestion. *Syntrophobacter* was identified as the most abundant and consistent bacterial partner in syntrophic propionate conversion regardless of the origin of the source culture, the concentration of propionate or the temporal dynamics of the culture. In contrast, the methanogen partners involved in syntrophic propionate conversion lacked consistency, as the dominant methanogens varied as a function of process condition and temporal dynamics. *Methanoculleus* populations were specifically enriched as the syntrophic partner at inhibitory levels of propionate, likely due to the ability to function under unfavorable environmental conditions. Syntrophic propionate conversion was carried out exclusively via transformation of propionate into acetate and hydrogen in enrichments established in this study. Microbial populations highly tolerant of elevated propionate, represented by *Syntrophobacter* and *Methanoculleus*, are of great significance in

understanding methanogenic activities during process perturbations when propionate accumulation is frequently encountered.

Key Points

- *Syntrophobacter* was the most consistent bacterial partner in propionate metabolism
- Diverse hydrogenotrophic methanogen populations could serve as syntrophic partners
- *Methanoculleus* emerged as a methanogen partner tolerant of elevated propionate

Keywords Methanogenesis, Syntrophy, *Syntrophobacter*, *Methanoculleus*, Anaerobic digestion

Introduction

Anaerobic digestion of organic substrates, as a methanogenic conversion process, has attracted much attention for the recovery of methane as a renewable energy source and the potential for reducing carbon emission. Propionate is an important intermediate in methanogenic processes, accounting for approximately one third of the biogenic methane production in nature (McCarty and Smith 1986). During methanogenic conversion, propionate is typically transformed into acetate, carbon dioxide and hydrogen, which becomes exergonic only when a sufficiently low hydrogen partial pressure is maintained by hydrogen-consuming methanogens (Schink 1997). As a result, anaerobic propionate conversion requires syntrophic interactions between fermentative populations that convert propionate into acetate/hydrogen and hydrogenotrophic methanogen populations

capable of consuming hydrogen to very low levels of partial pressure (Enzmann et al. 2018).

The significance of anaerobic propionate conversion in methanogenic processes has been extensively documented. Perturbations in methanogenic processes are frequently characterized by the accumulation of propionate, an indication of the inhibition of syntrophic propionate conversion (Capson-Tojo et al. 2017; Pullammanppallil et al. 2001; Liu et al 2010). Furthermore, accumulation of propionate is often accompanied by spikes in hydrogen partial pressure, suggesting the inhibition of hydrogenotrophic methanogen activities by the accumulation of organic acids (Rajendran et al. 2020; Ye et al. 2018). Indeed, anaerobic propionate conversion, monitored as propionate accumulation, has been considered as a performance indicator for anaerobic digestion processes (Jannat et al. 2021; Li et al. 2017b; Tale et al. 2011).

Given the significance of propionate in methanogenic processes, efforts have been made to study the syntrophic partners involved in propionate conversion. In anaerobic digestion processes, syntrophic bacteria considered to be involved in propionate degradation are associated with genera including *Syntrophobacter, Pelotomaculum*, and *Smithella* (Liu et al. 1999; de Bok et al. 2001; Imachi et al. 2007). One study developed long-term methanogenic microbial communities enriched by propionate and identified *Syntrophobacter* as the primary syntrophic partner in propionate conversion; however, the dominance of *Syntrophobacter* was not consistently observed (Narihiro et al., 2015). In another study where propionate-enriched methanogenic microbial communities were established at acidic pH, *Smithella* was identified as the most abundant syntrophic

propionate oxidizer, followed by *Syntrophobacter* and *Pelotomaculum* (Li et al. 2018). Other studies show the involvement of diverse microbial populations in methanogenic propionate conversion, likely due to variations in process conditions (Capson-Tojo et al. 2017; Han et al. 2020; Puengrang et al. 2020; Venkisteshwaran et al 2016). The presence of diverse hydrogenotrophic methanogens identified in these studies has raised further questions on the specificity of certain methanogens as syntrophic partners. Given the inconsistencies in process conditions encountered in previous studies, it remains ambiguous what factors influence the selection of syntrophic partners in anaerobic propionate conversion.

Therefore, with the objective to characterize microbial populations specifically involved in anaerobic propionate conversion, propionate was used as the sole substrate to establish a series of long-term methanogenic communities driven by syntrophic propionate conversion followed by microbial community analysis with high-throughput sequencing of 16S rRNA gene-based amplicon libraries.

Materials and methods

Medium composition and enrichment conditions

Enrichment cultures were developed using sodium propionate as the sole substrate with a 10% (v/v) transfer of various source cultures into sterile anaerobic medium according to previously established protocols (Chen and He 2016). The anaerobic medium was prepared with the following recipe (per liter): 1.0 g NaCl, 0.5 g KCl, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.3 g NH_4Cl, 1.7 g KH_2PO_4, 3.0 g K_2HPO_4, 0.0015 g $CaCl_2 \cdot 2H_2O$, 1.5 mg $FeCl_2 \cdot 4H_2O$,

0.19 mg $CoCl_2 \cdot 6H_2O$, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.07 mg $ZnCl_2$, 0.036 mg $Na_2MoO_4 \cdot 2H_2O$, 0.024 mg $NiCl_2 \cdot 6H_2O$, 0.008 mg $Na_2WO_4 \cdot 2H_2O$, 0.006 mg H_3BO_3, 0.006 mg $Na_2SeO_3 \cdot 5H_2O$, 0.002 mg $CuCl_2 \cdot 2H_2O$, 0.5 mg NaOH, 0.25 mL 0.1% Resazurin, 0.048g $Na_2S \cdot 9H_2O$, 0.031 g L-cysteine, and 1 mL of the Wolin vitamin solution (which contained 0.02 mg/L biotin, 0.02 mg/L folic acid, 0.1 mg/L pyridoxine hydrochloride, 0.05 mg/L riboflavin, 0.05 mg/L thiamine, 0.05 mg/L nicotinic acid, 0.05 mg/L pantothenic acid, 0.001 mg/L vitamin B_{12}, 0.05 mg/L *p*-aminobenzoic acid, and 0.05 mg/L thioctic acid). The pH of this anaerobic medium was adjusted to 7.2 with 1.0 M K_2HPO_4 or KH_2PO_4. The medium recipe was intended to minimize the presence of carbonate and bicarbonate to facilitate carbon balance calculations. The medium was heated to boil and purged with N_2 to eliminate oxygen before the addition of resazurin and $Na_2S \cdot 9H_2O$. The medium (100 mL) was then dispensed into 160-mL serum bottles flushed with pure N_2 and sealed with butyl rubber stoppers and aluminum caps. Subsequently, the medium was autoclaved and cooled before inoculation with the source culture.

Strict anaerobic techniques were used throughout in experimental manipulations. Growth substrates were added from sterile anaerobic stock solutions after autoclaving. Sterile syringes and needles, used for substrate addition and sampling, were flushed with N_2 prior to use. Each feeding of propionate resulted in a final concentration of 15 mM in the enrichment cultures. Ten feedings of propionate were added to each enrichment culture. A feeding was initiated when methane production from the previous feeding ceased. All enrichment cultures were kept at 37±1 ºC in a shaking incubator (80 rpm).

Long-term propionate enrichments

In order to obtain cultures highly enriched in syntrophic populations involved in anaerobic propionate conversion, duplicate long-term propionate enrichment cultures were developed with the digestate from an established bench-scale continuous anaerobic digester (fed with sucrose as the sole substrate) as the source culture. The source anaerobic digester was maintained with a solids retention time of 33 days and an organic loading rate of 0.21 g sucrose/L/day. Utilization of sucrose stabilized at 90% when the inocula were taken from the source anaerobic digester. Five generations of enrichment cultures were established with each generation of enrichments receiving repeated feedings of 15 mM of propionate as the sole substrate. The 1^{st}-generation propionate enrichments were established by a 10% inoculum of the source culture into fresh medium. The next-generation enrichments were subsequently developed by a 10% inoculum from the previous-generation enrichments following the completion of 10 feedings of 15 mM propionate. In total, 50 feedings of 15 mM propionate were administered to the enrichment cultures during a course of 420 days.

Utilization of acetate/butyrate by long-term propionate enrichments

To test the ability of the long-term propionate enrichments to utilize other important intermediates of the anaerobic food web, 6^{th}-generation propionate enrichments were developed by a 10% inoculum from the 5^{th}-generation enrichments following the completion of 10 feedings of 15 mM propionate. After the 6^{th}-generation enrichments

received 5 feedings of 15 mM propionate, one set of duplicate 6th-generation enrichments were fed with sodium acetate (20 mM) and the other set of duplicate 6th-generation enrichments were fed with sodium butyrate (10 mM).

Enrichments with 150 mM propionate

The concentration of propionate was evaluated as a key process parameter that could influence the microbial community involved in anaerobic propionate conversion. To compare with long-term propionate enrichments established with 15 mM of propionate, additional enrichments with 150 mM of propionate were developed with a 10% inoculum of the same source culture. The 1st-generation 150 mM propionate enrichments received one feeding of 150 mM propionate. When methane production ceased, 2nd-generation 150 mM propionate enrichment were established by a 10% inoculum from the 1st-generation 150 mM enrichments. Biomass samples were taken for analysis when methane production ceased following one feeding of 150 mM propionate in the 2nd-generation enrichments.

Propionate enrichment with various source cultures

In order to evaluate the potential diversity of microbial populations involved in syntrophic propionate degradation, enrichment cultures with propionate as the sole substrate were set up using previously described protocols (Chen and He, 2016) using a series of source cultures as the inoculum. The enrichments were established by a 10% (v/v) transfer of various source cultures into sterile anaerobic medium without

replication. The source cultures included dilute diary manure (D) (Zhang et al., 2011), beef cattle manure (C), excess sludge from a secondary municipal wastewater treatment facility (S), digestate from a bench-scale anaerobic bioreactor developed with sucrose as the sole substrate (W), and digestate from a bench-scale anaerobic bioreactor developed with dilute diary manure as the sole substrate (L) (Chen et al., 2012). Characteristics of these source cultures are provided in supplementary material (Table S1).

Analytical methods

Production of CH_4 and CO_2 in the enrichments was measured using a water displacement method (Demirer and Speece 1998). Gas composition was determined using a gas chromatograph (HP5890, Agilent, Santa Clara, California, USA) equipped with a thermal conductivity detector and Carbonxen®-1000 column (Supelco, Bellefonte, Pennsylvania, USA) using argon carrier gas at a flow rate of 5 mL/min. The temperatures for injection, oven (column), and detection were set at 150 ºC, 125 ºC, and 170 ºC, respectively. Chemical oxygen demand (COD) was measured following Standard Method 5220D (APHA 2005) with CHEMetrics™ COD Vials Kits (Midland, Virginia, USA). Volatile fatty acids, including acetate, propionate, and butyrate, were quantified by a gas chromatograph equipped with a flame ionization detector and a Stabilwax®-DA column (Restek, Bellefonte, Pennsylvania, USA), using helium as the carrier gas at a flow rate of 20 mL/min with 1:20 split ratio. The injection and detection temperatures were set at 250 ºC and 300 ºC, respectively. The temperature gradient for the oven (column) began at 80

ºC for 1 minute, followed by an increase of 10 ºC/min to 220 ºC, and then held steady at 220 ºC for 5 minutes. Samples were acidified to pH < 2 with 0.5 mL of 85% H_3PO_4.

Microbial community analysis

Biomass samples from enrichments were prepared individually without pooling by centrifugation at 14,000× g and the pellets were preserved at -80ºC for microbial community analysis by sequencing. Community DNA was extracted using FastDNA™ Spin Kit for Soil (MP Biomedicals, Irvine, California, USA) according to the manufacturer's instructions (Protocol Revision #116560200-201608) and purified with the Genomic DNA Clean & Concentrator™-10 kit (Zymo Research, Irvine, California, USA). The quality and quantity of the DNA extracts were determined using the NanoDrop ND-3300 fluorospectrometer (Thermo Scientific, Waltham, Massachusetts, USA).

Polymerase chain reactions (PCR) were performed by targeting the V4 region of the 16S rRNA gene with a cocktail mix (25 µL) containing 12 µL Phusion flash Master Mix (Thermo Scientific, Waltham, Massachusetts, USA), 1 µL forward primer (515F: GTGCCAGCMGCCGCGGTAA), 1 µL reverse primer (806R: GGACTACHVGGGTWTCTAAT with a unique 12-base specific barcode for each sequence), 2 µL (100 to 150 ng) DNA template, and 9 µL ultra-pure water. The PCR program was set as follows: one cycle of 94 ºC for 3 minutes, 35 cycles at 94 ºC for 45 seconds (denaturation), 55 ºC for 1 minute (annealing), and 72ºC for 1 minute and a half (elongation), and a final extension at 72 ºC for 10 minutes. Post PCR, the quality and

concentrations of amplicons were measured on an Agilent 2100 Bioanalyzer Instruments with Agilent DNA 7500 chips (Santa Clara, California, USA). The DNA concentration of libraries was determined using the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, Massachusetts, USA) and were diluted with 10 mM Tris/0.05% tween buffer (pH = 8.5). The libraries were amended with 20% PhiX control kit (Illumina, San Diego, California, USA) to increase library diversity. The library (10 pM) was subsequently loaded to an Illumina MiSeq System with a MiSeq Reagent Kit v2 (300-cycles) (Illumina, San Diego, California, USA) and the Metagenomic workflow was selected to execute the 16S protocol using the MiSeq Reporter software (MSR). DNA extraction, PCR amplification, and sequencing were completed separately for each sample.

Data Processing

The acquired amplicon sequences were processed in QIIME 2 using the DADA2 pipeline, followed by Scikit-learn classifier with the SILVA v.138 reference database (Yilmaz et al. 2014). Raw sequence reads were deposited at the Sequence Read Archive (SRA) of GenBank with accession numbers SAMN12719674 - SAMN12719684 under Project PRJNA564675.

Results

Performance of long-term propionate enrichment cultures

In order to obtain cultures highly enriched in syntrophic populations involved in anaerobic propionate conversion, five generations of enrichment cultures were established in this study with each generation of enrichments receiving repeated feedings of 15 mM of propionate as the sole substrate. The 1st-generation propionate enrichments were established by a 10% inoculum from an existing continuous anaerobic digester into fresh medium. The next-generation enrichments were subsequently developed by a 10% inoculum from the previous-generation enrichments following the completion of 10 feedings of 15 mM propionate. In total, 50 feedings of 15 mM propionate were administered to the enrichment cultures during a course of 420 days (Fig. 1).

Performance of the enrichment cultures was monitored as methane production. Immediately following the inoculation of the 1st-generation enrichments, the lag time between the first feeding of 15 mM propionate and generation of detectable methane was 11 days (Fig. 1A). In comparison, this lag time was reduced significantly to 4 days in the 2nd-generation enrichment cultures, suggesting the enrichment of microbial communities adapted to propionate utilization (Fig. 1B). In subsequent generations of enrichment cultures, i.e. the 3rd-, 4th-, and 5th-generation enrichments, the lag time remained identical at 3 days (Fig. 1C-E), indicative of functional stability of the microbial communities enriched by propionate. Cumulative methane production was similar in enrichment cultures across all five generations, averaging 805 ± 38 mL/L following 10 feedings of propionate, which corresponds to a propionate : methane molar ratio of 4 : 7 and is

consistent with the stoichiometry of syntrophic propionate oxidation (Schink 1997). These results demonstrated that the long-term enrichment cultures, particularly those of the 3rd, 4th, and 5th generations, were likely comprised of functionally stable syntrophic microbial communities for the conversion of propionate to methane.

Impact of long-term propionate enrichment on methanogenic microbial community

In order to identify microbial populations enriched specifically for syntrophic propionate conversion, microbial community composition was compared between the original source culture of propionate enrichments, which was taken from an existing anaerobic digester fed with sucrose as the substrate, and functionally stable propionate enrichment cultures, i.e. the 3rd- and 5th-generation propionate enrichments.

It is evident that methanogen populations were numerically more important for the utilization of propionate than sucrose in methane production, as the relative abundance of archaeal populations increased by more than three times from 16% in the original source culture to more than 60% in the propionate enrichment cultures (Fig. 2A). Accordingly, the relative abundance of bacterial populations decreased from 84% in the original source culture to less than 40% in the propionate enrichments (Fig. 2A). These results are consistent with the positioning of propionate in the anaerobic food web that does not require extensive hydrolytic and fermentative processing, typically carried out by bacterial populations, prior to methanogenesis (Li et al. 2012; Muller 2010).

Syntrophic microbial populations in long-term propionate enrichment

Since anaerobic propionate conversion to methane requires syntrophic interactions (Schink 1997), the microbial populations present in the long-term propionate enrichments were expected to be involved in syntrophic propionate conversion. A closer examination of the microbial communities at finer taxonomic resolution revealed that the archaeal populations in the original source culture were dominated by acetoclastic methanogens represented exclusively by *Methanosaeta*, with a relative abundance of more than 60% of all archaeal sequences. The remainder of the archaeal populations in the original source culture were comprised of hydrogenotrophic methanogens, represented primarily by the genus *Methanobacterium* (Fig. 2B). Following propionate enrichment, the dominant methanogen populations shifted to hydrogenotrophic methanogens including *Methanobacterium*, *Methanospirillum*, *Methanoculleus*, and a population associated with the family *Methanomassiliicoccaceae* (Fig. 2B), which were expected to be the hydrogen-consuming partners involved in syntrophic propionate conversion. It should be noted that the syntrophic methanogen partners differed considerably between the 3rd- and 5th-generation propionate enrichments (Fig. 2B). In the 3rd-generation propionate enrichments, *Methanospirillum* and *Methanobacterium* were the two most abundant syntrophic methanogen partners, comprising 47±1% and 26±5% of the archaeal community, respectively. In contrast, the methanogen populations in the 5th-generation propionate enrichments were dominated by *Methanobacterium*, contributing to 67±7% of the archaeal sequences, whereas *Methanospirillum* only accounted for 12±7% of the archaeal sequences (Fig. 2B). Given that the long-term propionate enrichments were stable in process performance (Fig. 1), the sizable temporal shifts observed in

hydrogenotrophic methanogen populations in these functionally-stable enrichment cultures pointed to the potential lack of specificity of methanogen partners in syntrophic propionate conversion.

The most abundant bacterial populations detected in the propionate enrichments included those closely related to *Syntrophobacter*, *Mesotoga*, W5 (family *Cloacimonadaceae*), and *Thermovirga* (Fig. 2C). Similar to the methanogens, substantial shifts in bacterial populations were observed from the 3rd-generation to the 5th-generation propionate enrichments. However, *Syntrophobacter* persisted as the most abundant bacterial population in the propionate enrichments, providing evidence that *Syntrophobacter* could be a consistent partner in syntrophic propionate conversion.

Anaerobic propionate metabolism in long-term propionate enrichments

Two metabolic pathways have been proposed for syntrophic conversion of propionate to acetate and hydrogen before methanogenesis (Wang and He 2019): 1) oxidation of propionate to acetate and hydrogen; and 2) dismutation of propionate to acetate and butyrate followed by β-oxidation. It could be deduced that the latter pathway would require syntrophic populations to be capable of butyrate utilization.

Thus, to ascertain the pathway responsible for syntrophic propionate conversion in the long-term propionate enrichments, acetate (20 mM) or butyrate (10 mM) was added as the sole substrate into long-term propionate enrichment cultures, i.e. 6th-generation enrichments, and methane production was monitored (Fig. 3). Expectedly, acetate was immediately utilized by the long-term propionate enrichments for methane production

(Fig. 3A), consistent with acetate being a common intermediate in both pathways for syntrophic conversion of propionate. In comparison, no methane production was detected from butyrate addition to the long-term propionate enrichments (Fig. 3B), indicative of the lack of capacity for butyrate utilization. These results show that syntrophic propionate conversion in the long-term propionate enrichments of this study likely proceeded via transformation of propionate into acetate and hydrogen.

Microbial community analysis found that the addition of acetate into long-term propionate enrichments led to the greater involvement of acetoclastic methanogenesis with a marked increase in the relative abundance of *Methanosaeta* from 9±0% to 25±1% (Fig. 4), which was expected given that acetate is an exclusive substrate for acetoclastic methanogenesis. Another significant change in response to acetate addition was the decrease in the relative abundance of *Syntrophobacter* from 16±1% to 10±0% (Fig. 4). With the metabolic shift to acetoclastic methanogenesis in response to acetate addition, it was predictable that microbial populations specifically associated with syntrophic conversion of propionate, such as *Syntrophobacter*, would experience a decline. Nevertheless, this observation further confirmed the importance and specificity of *Syntrophobacter* in propionate conversion. It should be noted that *Smithella* populations were not detected in the long-term propionate enrichments (Fig. 2).

Impact of propionate concentration on syntrophic populations

The association of syntrophic partners in anaerobic propionate conversion could be shaped by process conditions. In this study, the concentration of propionate was

evaluated as a key process parameter potentially impacting syntrophic population composition. To compare with long-term propionate enrichments established with 15 mM of propionate (Fig. 1), additional enrichments with 150 mM of propionate were developed with the same source culture.

One distinction between the two sets of enrichments was the difference in COD removal, which is a measure of the utilization of organic carbonaceous substrates (Fig. 5A). The enrichment cultures developed with 15 mM of propionate achieved nearly complete COD removal of 97.2±0.2% (Fig. 5A). However, the use of a higher concentration of propionate (i.e. 150 mM) led to incomplete substrate utilization with a substantial decline in COD removal to 66.4±0.8%. Further analysis revealed that the incomplete substrate utilization associated with the higher propionate concentration could be attributed to the presence of residual propionate at the concentration of 12.6±0.4 mM (Fig. 5B), suggesting the partial inhibition of syntrophic propionate conversion activity. More importantly, the use of 150 mM propionate as the substrate resulted in the accumulation of significant levels of acetate, averaging 65.0 mM (Fig. 5B), evidence of markedly reduced activity for acetoclastic utilization of acetate.

Indeed, microbial community analysis indicated diminished presence of acetoclastic methanogens, e.g. *Methanosaeta*, in the enrichment cultures established with 150 mM propionate as compared with those grown with 15 mM propionate (Fig. 6). These results support the postulation that incomplete propionate utilization was due to the suppression of acetoclastic methanogens and the ability to consume acetate as an intermediate from syntrophic propionate conversion.

The use of higher concentrations of propionate (150 mM) gave rise to the dominance of *Methanoculleus*, accounting for more than 88% of the methanogen populations in the enrichments developed with 150 mM propionate (Fig. 6). Given that *Methanoculleus* was a minor methanogen population in enrichments derived from lower concentrations of propionate (15 mM), it is likely that *Methanoculleus* was more tolerant of elevated propionate and acetate, which is a product of anaerobic propionate conversion, than other hydrogenotrophic methanogens as syntrophic partners for anaerobic propionate conversion. In contrary to the dominance of *Methanoculleus* in syntrophic populations developed from higher concentrations (150 mM) of propionate, the hydrogenotrophic methanogen populations were more diverse in enrichments established with lower levels of propionate (15 mM), including *Methanospirillum* and *Methanobacterium* (Fig. 6), suggesting the possibility that multiple hydrogenotrophic methanogen partners were involved in syntrophic propionate conversion.

In contrast to the considerable shifts in methanogen populations in response to propionate concentration, *Syntrophobacter* remained as the most abundant bacterial population notwithstanding the concentration of propionate tested in this study (Fig. 6), further supporting the importance of *Syntrophobacter* as a syntrophic partner in propionate conversion. In addition to *Syntrophobacter*, *Mesotoga*, W5 (family *Cloacimonadaceae*), and *Thermovirga* also represented bacterial populations present at both high and low propionate concentrations (Fig. 6), suggesting the potential involvement of these populations as syntrophic partners.

Impact of source culture on syntrophic populations

It could be argued that populations found in syntrophic propionate conversion would be dependent on the composition of the source culture. To test this hypothesis, propionate enrichments were established with a diverse series of source cultures as the inoculum, including dilute diary manure (D), beef cattle manure (C), excess sludge from a secondary municipal wastewater treatment facility (S), digestate from a bench-scale anaerobic bioreactor developed with sucrose as the sole substrate (W), and digestate from a bench-scale anaerobic bioreactor developed with dilute diary manure as the sole substrate (L).

As revealed by microbial community analysis, the source culture did have an impact on the syntrophic communities involved in anaerobic propionate conversion. For example, the composition of hydrogenotrophic methanogen populations was distinct in all five enrichments (Fig. 7). *Methanofollis* was the most abundant hydrogenotrophic methanogen population in S enrichments, representing 19.5% of the entire syntrophic community; however, *Methanofollis* was not detected in any of the enrichment cultures inoculated with other source cultures. *Methanospirillum* was the most numerically important hydrogenotrophic methanogen in W enrichments, contributing to 30.9% of the entire syntrophic community in W enrichments; however, *Methanospirillum* represented a minor methanogen population in all other enrichments with relative abundance less than 5% (Fig. 7). These results again demonstrated the lack of consistent specificity of methanogen partners in syntrophic propionate conversion, which needs to be validated in

further studies using more source cultures of diverse characteristics with higher levels of replication.

Despite considerable variations in the bacterial community composition between propionate enrichments developed with different source cultures, one common feature was the presence of *Syntrophobacter* as the most abundant bacterial population (Fig. 7), again pointing to the significance of *Syntrophobacter* as a consistent syntrophic partner in anaerobic propionate conversion. Other bacterial populations identified consistently in all enrichments included sequences associated with *Mesotoga*, *Aminobacterium*, and *Spirochaetaceae*, suggesting the potential involvement of these populations in syntrophic propionate conversion.

Discussion

Consistent involvement of *Syntrophobacter* in syntrophic propionate conversion

In this study, concentration of propionate and origin of source culture were tested as two important variables that could potentially influence the composition of bacterial populations in syntrophic propionate conversion. Indeed, bacterial populations exhibited substantial divergence as a function of propionate concentration (Fig. 6) and source culture (Fig. 7). More unexpectedly, functionally-stable long-term propionate enrichments established in this study were found to experience major temporal shifts in syntrophic bacterial populations (Fig. 2), instead of continued increase in specific populations over time as expected for enrichment cultures.

Despite considerable population variations in the propionate enrichments developed with different substrate concentrations, source cultures, and substantial temporal dynamics in microbial community composition, *Syntrophobacter* emerged as the most abundant and consistent syntrophic partner in propionate conversion (Fig. 2, 6, & 7). This is consistent with the identification of *Syntrophobacter* as a key bacterial partner for syntrophic propionate oxidation in previous studies (Boone and Bryant 1980; Chen et al. 2005; De Bok et al. 2003; Houwen et al. 1990). Other bacterial populations frequently identified in the propionate enrichments of this study included *Mesotoga*, *Thermovirga*, and *Aminobacterium* (Fig. 2, 6, & 7). The roles of these bacterial populations have not been well defined. While these populations could be directly involved in propionate conversion as syntrophic partners, it is possible that certain populations could sustain as scavengers of cell debris in the enrichments or constitute the so-called dark matter as part of an intricate food web involved in syntrophy (Nobu et al. 2015). Evidently, further efforts are need to elucidate the metabolic functions of these bacterial populations in syntrophic conversion of propionate.

Diversity of hydrogenotrophic methanogens in syntrophic propionate conversion

While *Syntrophobacter* was identified as a consistent partner in syntrophic propionate conversion, a consistent methanogen partner was not found. A diversity of hydrogenotrophic methanogens were involved in propionate conversion, including *Methanobacterium*, *Methanoculleus*, *Methanofollis*, *Methanospirillum*, and *Methanolinea* (Fig. 7), each establishing dominance with different substrate concentrations or source

cultures. It should be noted that some of these methanogens have been previously reported to interact with *Syntrophobacter* in syntrophic propionate oxidation (Botsch and Conrad 2011; Dong et al. 1994; Gallert and Winter 2008; Li et al. 2017a). Significant temporal shifts in hydrogenotrophic methanogen populations were observed in functionally-stable long-term propionate enrichments when operational conditions remained unchanged (Fig. 2B). These results indicate the lack of specificity in hydrogenotrophic methanogens to syntrophic propionate conversion.

The dominance of *Methanoculleus* is remarkable in the conversion of inhibitory levels (i.e. 150 mM) of propionate (Fig. 6). *Methanoculleus* populations have been reported for the ability to function under unfavorable environmental conditions such as elevated ammonia levels (Angenent et al. 2002; Barret et al. 2013; Barret et al. 2012), high organic loadings (Acharya et al. 2011; Botello Suárez et al. 2018), and thermophilic conditions (Fontana et al. 2016; Lavania et al. 2014). Thus, it is unsurprising that *Methanoculleus* was also able to function at high levels of propionate, suggesting the potential of *Methanoculleus* in sustaining methanogenic activities during process perturbations when propionate inhibition is more likely to occur (Chen et al. 2020).

Pathway for syntrophic propionate conversion

Two metabolic pathways with identical overall stoichiometry have been proposed for syntrophic conversion of propionate to acetate and hydrogen before methanogenesis: 1) oxidation of propionate to acetate and hydrogen; and 2) dismutation of propionate to acetate and butyrate followed by β-oxidation (Wang and He 2019). In propionate

enrichments developed in this study, microbial community analysis did not detect the presence of *Smithella* (Fig. 2 & 7), which is the only known genus involved in the dismutation of propionate to acetate and butyrate (Dolfing 2013; Liu et al. 1999). Furthermore, the long-term propionate enrichments were unable to utilize butyrate (Fig. 3), a key immediate from propionate dismutation. These results show that syntrophic propionate conversion was carried out exclusively via transformation of propionate into acetate and hydrogen in this study.

The absence of *Smithella* and the propionate dismutation pathway, however, could be attributed to the cultivation conditions used in the propionate enrichments. High propionate concentrations were found to be linked to the absence of the propionate dismutation pathway in anaerobic digestion processes (Li et al. 2015; McMahon et al. 2004; Wang et al. 2019). It has been suggested that *Syntrophobacter* was more tolerant of propionate accumulation than *Smithella* in anaerobic digestion (McMahon et al. 2004; Wang et al. 2019). Given that the concentrations of propionate used in this study were considerable higher than those typically encountered in methanogenic treatment processes, it is likely that the configurations of propionate enrichments in this study were unfavorable for *Smithella* and the propionate dismutation pathway, or the source cultures used in this study did not contain *Smithella*. In addition, these factors might have contributed to the absence of *Pelotomaculum* as a syntrophic partner in these enrichments. Future studies using lower concentrations of propionate and source cultures of more diverse origins are needed to investigate process conditions that would facilitate the propionate dismutation pathway.

Authors' contributions LC and QH conceived and designed research. LC conducted experiments. LC analyzed data. LC, QH, and CDC wrote the manuscript. All authors read and approved the manuscript.

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Data availability Sequence data is available at the Sequence Read Archive (SRA) of GenBank (https://www.ncbi.nlm.nih.gov/sra) with accession numbers SAMN12719674 - SAMN12719684. Other data and material for this article are available upon request.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Fig. 1 Methane production in long-term enrichment cultures with repeated feedings of 15 mM propionate. The 1st-generation enrichments (a) were established by a 10% inoculum from an existing continuous anaerobic digester into fresh medium. The 2nd-generation (b), 3rd-generation (c), 4th-generation (d), and 5th-generation (e) enrichments were developed by a 10% inoculum from the 2nd-generation, 3rd-generation, and 4th-generation enrichments, respectively, subsequent to the completion of 10 feedings of 15 mM propionate. Each generation of enrichments received 10 feedings with each feeding designated by a red arrow: from left to right—1st to 10th feeding. Each colored line indicates methane production following a feeding of 15 mM propionate. Data points were means of duplicate enrichments with the error bars showing standard deviations

Fig. 2 Relative abundance of microbial populations in long-term propionate enrichments and the source culture in replicates. a): community composition at the domain level; b) bacterial populations; and c) archaeal populations. Microbial populations in B and C are shown at the genus level. Only populations with an average relative abundance greater than 1% are shown

Fig. 3 Methane production in long-term propionate enrichments following the addition of 20 mM acetate (a) or 10 mM butyrate (b). Addition of acetate or butyrate to the long-term propionate enrichments, which were 6th-generation propionate enrichments, occurred

following 5 feedings of 15 mM propionate. Each color line indicates methane production following a feeding of 15 mM propionate, 20 mM of acetate, or 10 mM butyrate. Data points were means of duplicate enrichments with the error bars showing standard deviations

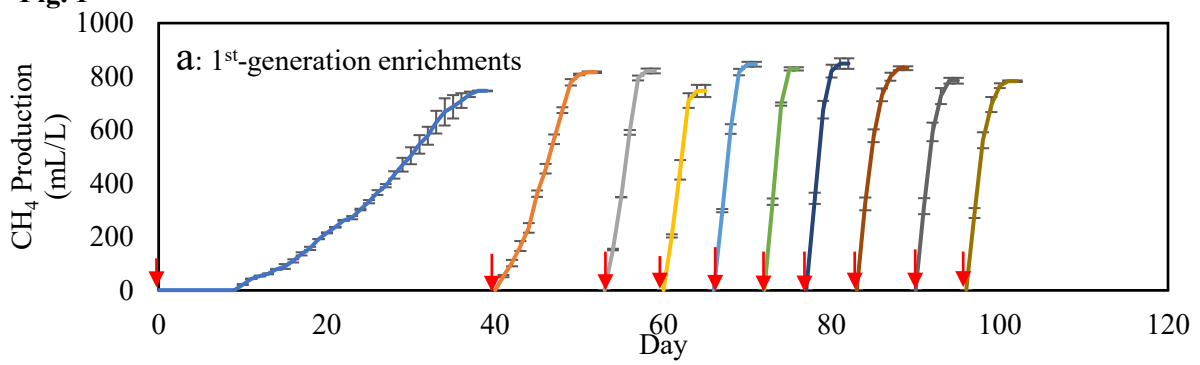
Fig. 4 Changes in microbial community composition at the genus level before and after the utilization of 20 mM acetate in long-term propionate enrichments. Addition of 20 mM acetate to the long-term propionate enrichments, which were 6th-generation propionate enrichments, occurred following 5 feedings of 15 mM propionate. Red: relative abundance of microbial populations in the long-term propionate enrichments before the addition of 20 mM acetate as the substrate; green: relative abundance of microbial populations in the enrichments following the utilization of 20 mM acetate as the substrate. Data shown were means of duplicate enrichments with the error bars indicating standard deviations

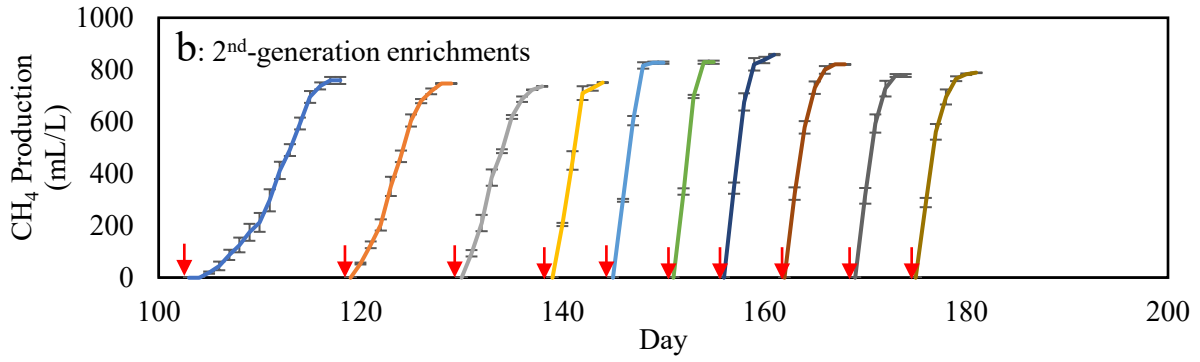
Fig. 5 Performance of anaerobic propionate conversion in enrichment cultures fed with 15 mM or 150 mM of propionate as the sole substrate. a) substrate utilization measured as COD removal; and b) concentration of residual propionate and acetate. Data for enrichment cultures fed with 15 mM propionate were shown as the means of duplicate 3rd- and 5th-generation enrichments as illustrated in Fig. 1, with the error bars indicating standard deviations

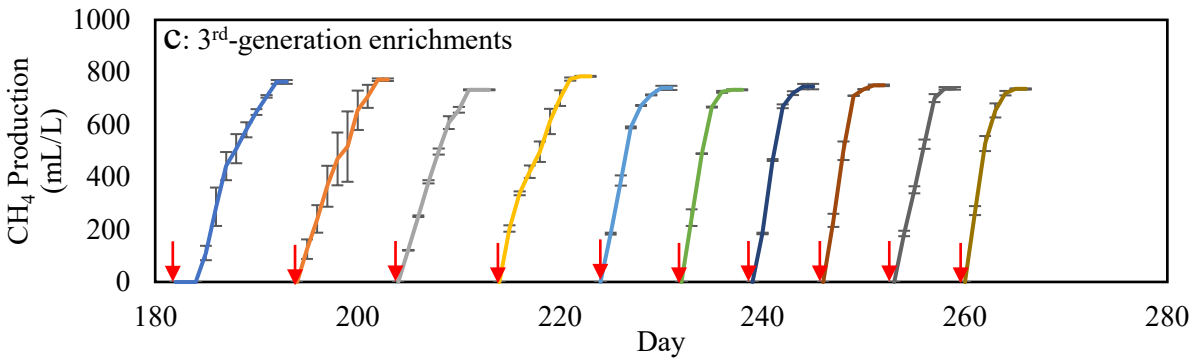
Fig. 6 Comparisons of microbial populations in enrichments developed with 15 mM or 150 mM of propionate in replicates.

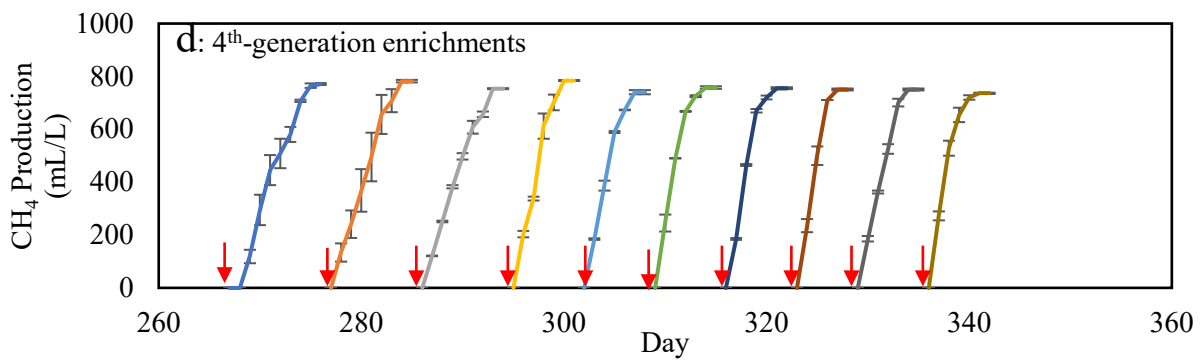
Fig. 7 Microbial community composition in propionate enrichments developed with a series of source cultures including dilute diary manure (D), beef cattle manure (C), excess sludge from a secondary municipal wastewater treatment facility (S), digestate from a bench-scale anaerobic bioreactor developed with sucrose as the sole substrate (W), and digestate from a bench-scale anaerobic digester developed with dilute diary manure as the sole substrate (L). Shown are genera with average relative abundance > 1%

Fig. 1


a: 1st-generation enrichments
CH4 Production (mL/L)
1000
800
600
400
200
0
0
20
40
60
80
100
120
Day


b: 2nd-generation enrichments
CH4 Production (mL/L)
1000
800
600
400
200
0
100
120
140
160
180
200
Day


c: 3rd-generation enrichments
CH4 Production (mL/L)
1000
800
600
400
200
0
180
200
220
240
260
280
Day


d: 4th-generation enrichments
CH4 Production (mL/L)
1000
800
600
400
200
0
260
280
300
320
340
360
Day

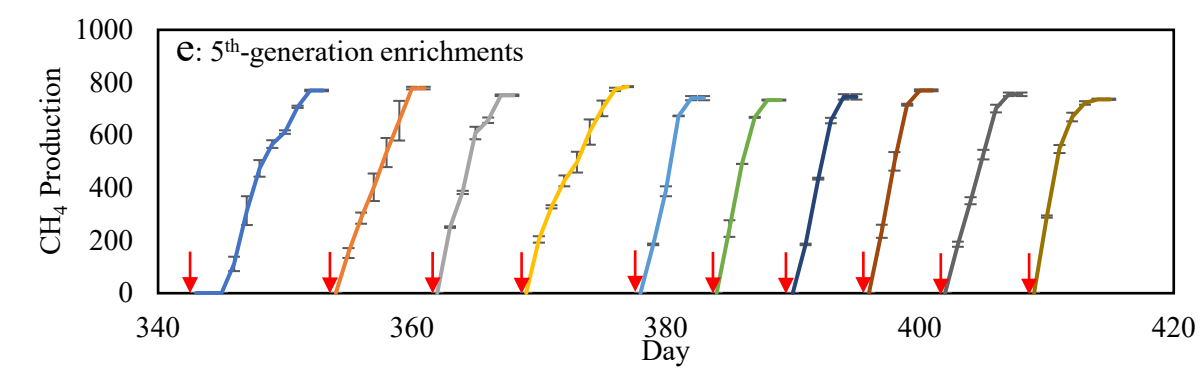
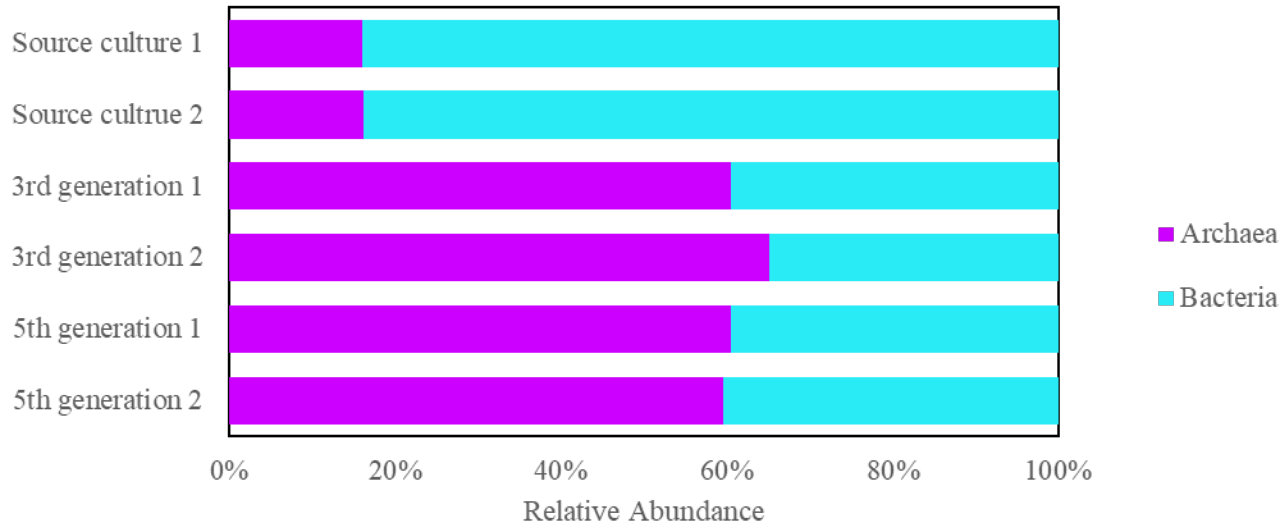
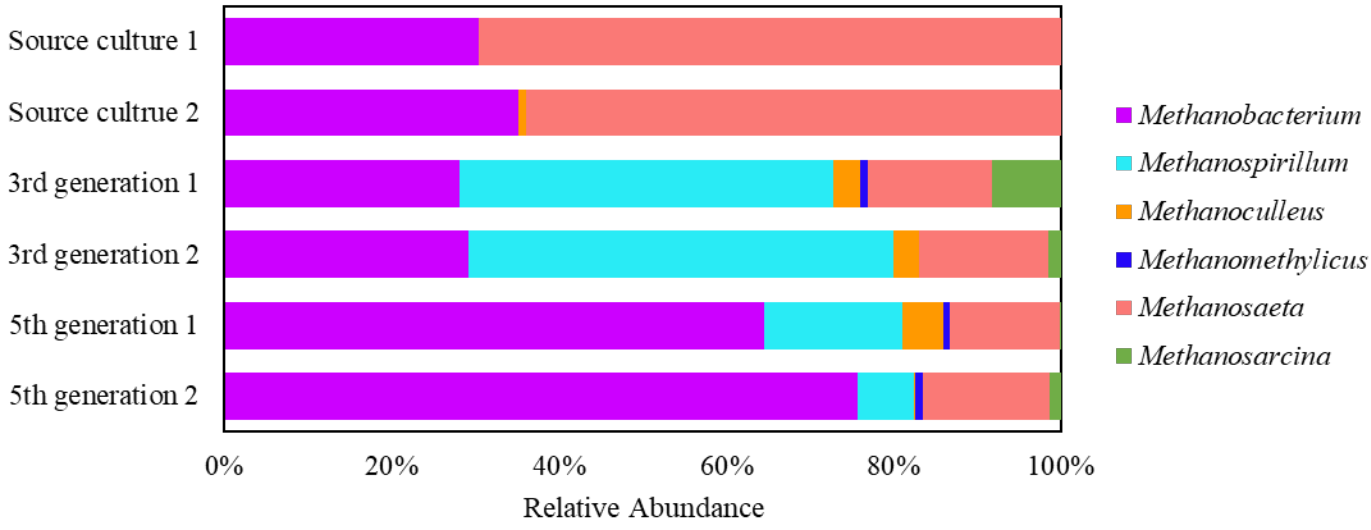

e: 5th-generation enrichments
CH4 Production
1000
800
600
400
200
0
340
360
380
400
420
Day

Fig. 2


Source culture 1
Source cultrue 2
3rd generation 1
3rd generation 2
5th generation 1
5th generation 2
0%
20%
40%
60%
80%
100%
Relative Abundance
Archaea
Bacteria


Source culture 1
Source cultrue 2
3rd generation 1
3rd generation 2
5th generation 1
5th generation 2
0%
20%
40%
60%
80%
100%
Relative Abundance
Methanobacterium
Methanospirillum
Methanoculleus
Methanomethylicus
Methanosaeta
Methanosarcina

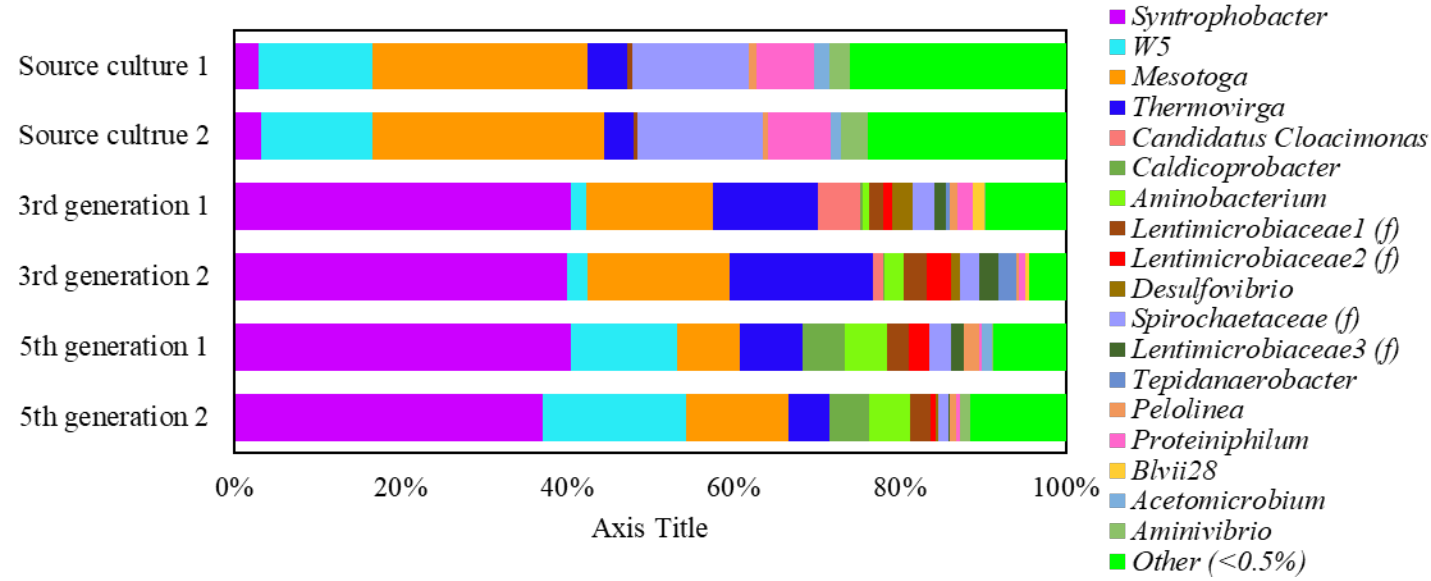
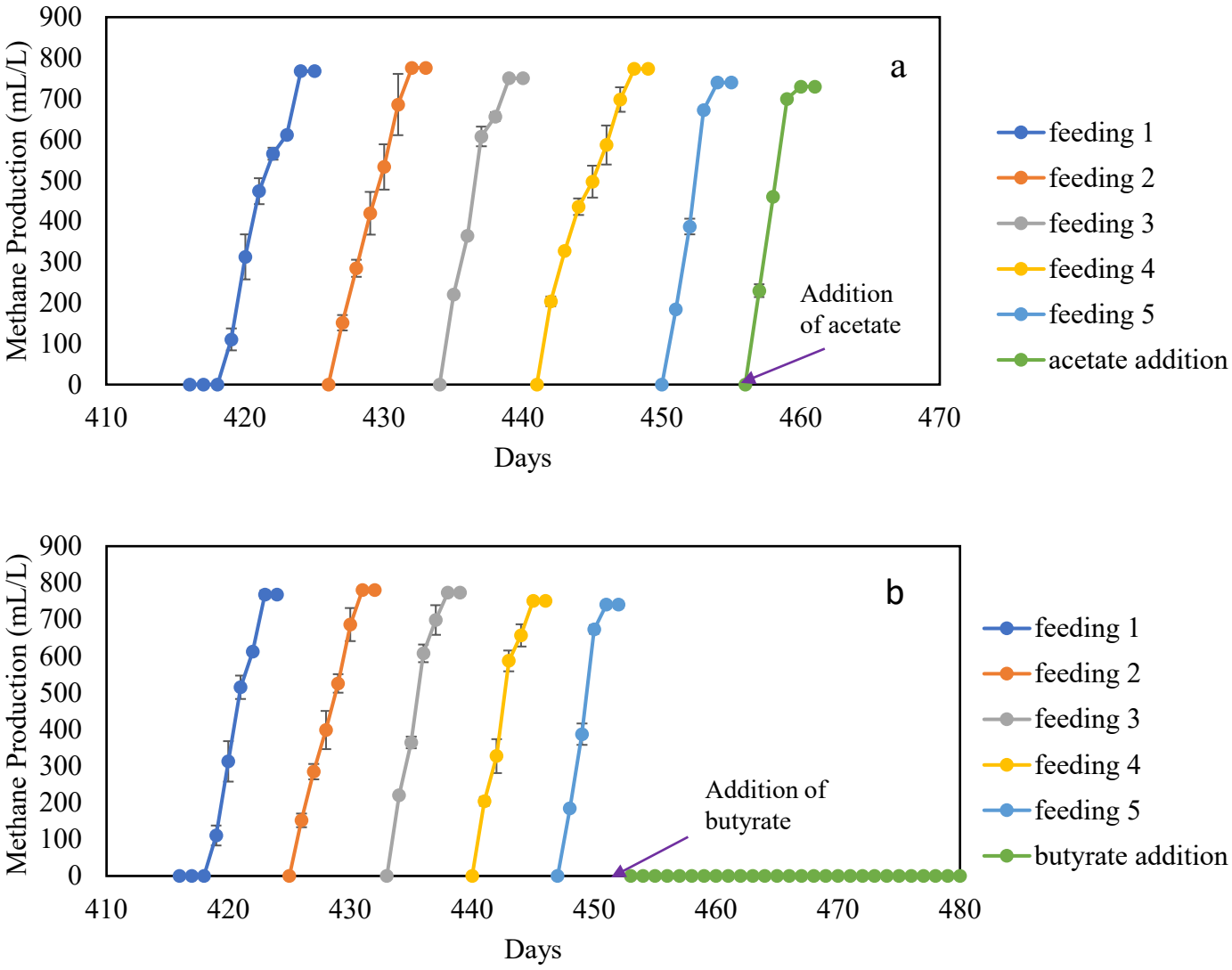

Source culture 1
Source cultrue 2
3rd generation 1
3rd generation 2
5th generation 1
5th generation 2
0%
20%
40%
60%
80%
100%
Axis Title
Syntrophobacter
W5
Mesotoga
Thermovirga
Candidatus Cloacimonas
Caldicoprobacter
Aminobacterium
Lentimicrobiaceae1 (f)
Lentimicrobiaceae2 (f)
Desulfovibrio
Spirochaetaceae (f)
Lentimicrobiaceae3 (f)
Tepidanaerobacter
Pelolinea
Proteiniphilum
Blvii28
Acetomicrobium
Aminivibrio
Other (<0.5%)

Fig. 3


a
Methane Production (mL/L)
900
800
700
600
500
400
300
200
100
0
410
420
430
440
450
460
470
Days
Addition of acetate
feeding 1
feeding 2
feeding 3
feeding 4
feeding 5
acetate addition

b
Methane Production (mL/L)
900
800
700
600
500
400
300
200
100
0
410
420
430
440
450
460
470
480
Days
Addition of butyrate
feeding 1
feeding 2
feeding 3
feeding 4
feeding 5
butyrate addition

Fig. 4

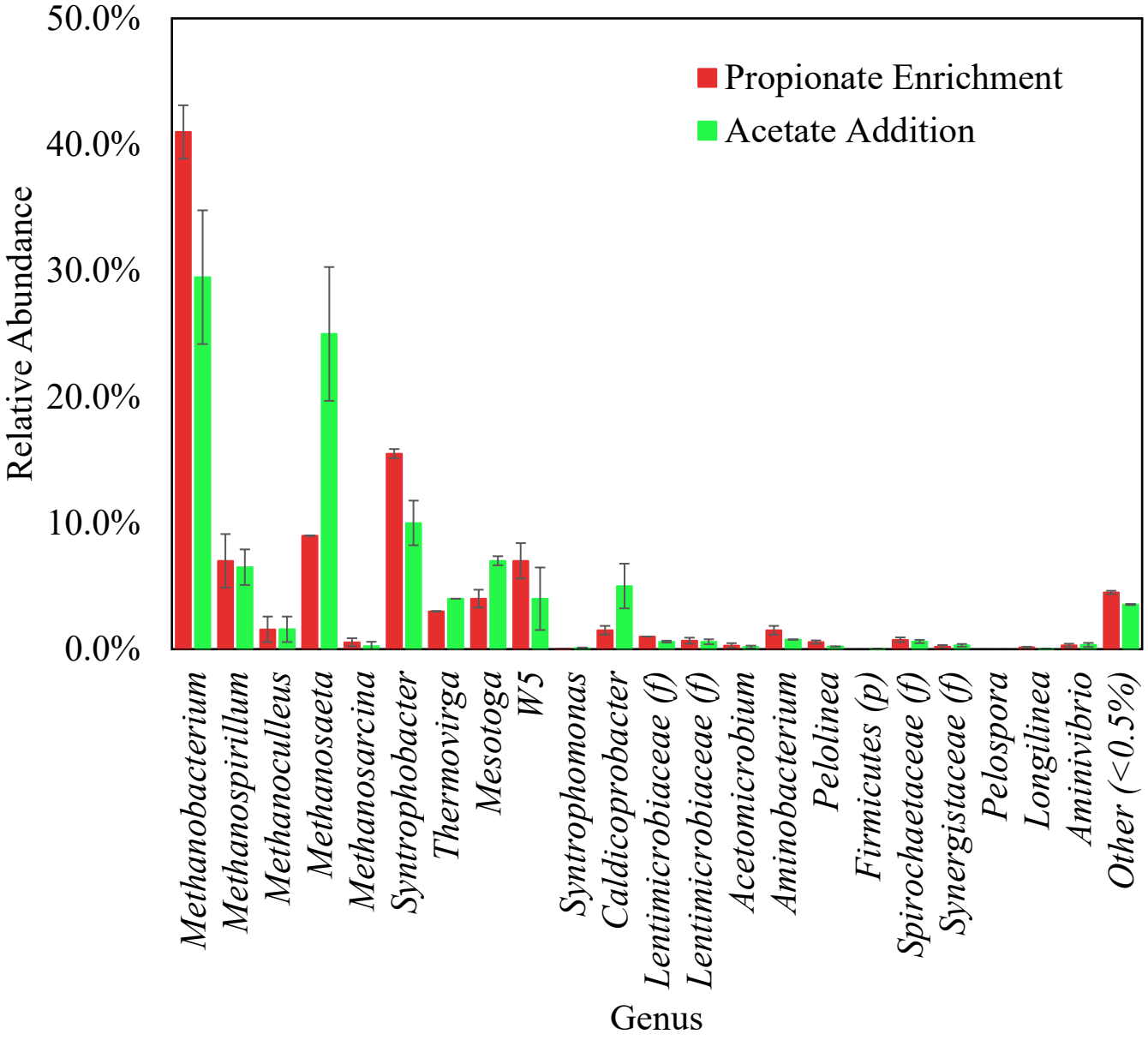

50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
Relative Abundance
Propionate Enrichment
Acetate Addition
Methanobacterium
Methanospirillum
Methanoculleus
Methanosaeta
Methanosarcina
Syntrophobacter
Thermovirga
Mesotoga
W5
Syntrophomonas
Caldicoprobacter
Lentimicrobiaceae (f)
Lentimicrobiaceae (f)
Acetomicrobium
Aminobacterium
Pelolinea
Firmicutes (p)
Spirochaetaceae (f)
Synergistaceae (f)
Pelospora
Longilinea
Aminivibrio
Other (<0.5%)
Genus

Fig. 5

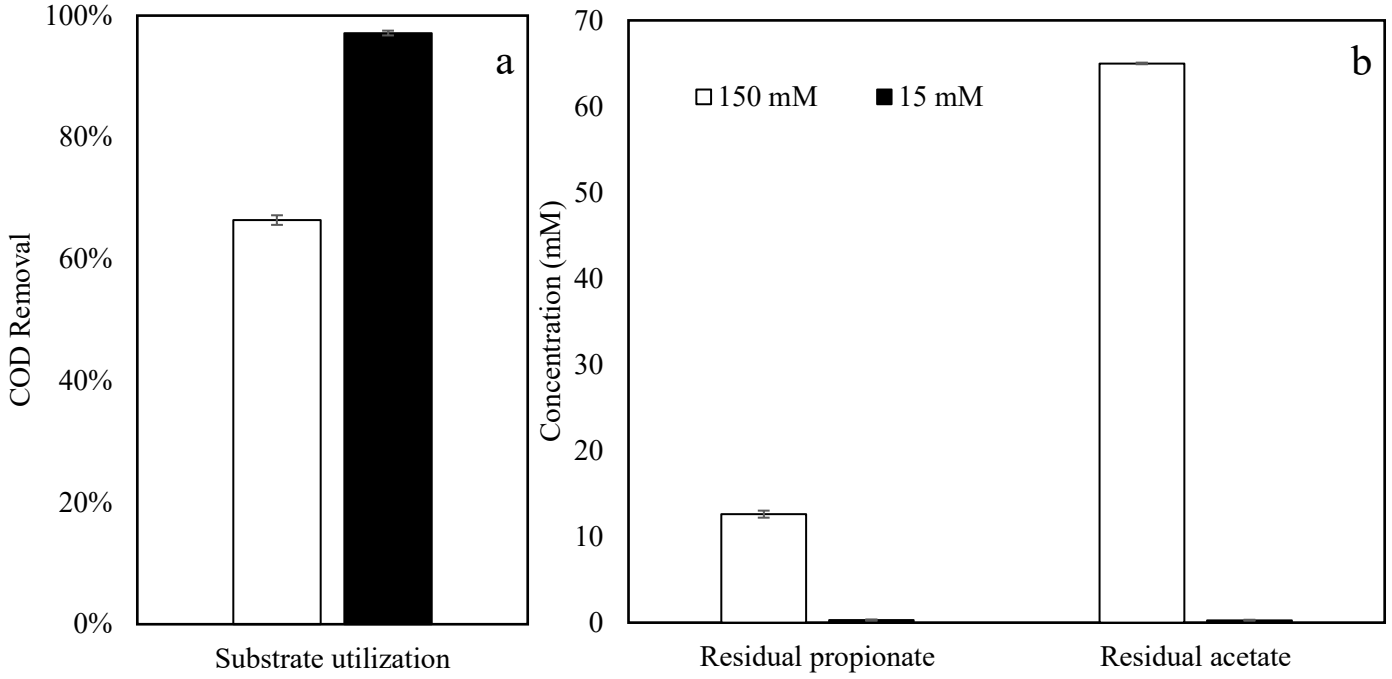

a
100%
80%
60%
40%
20%
0%
COD Removal
Substrate utilization
b
70
60
50
40
30
20
10
0
Concentration (mM)
150 mM
15 mM
Residual propionate
Residual acetate

Fig. 6

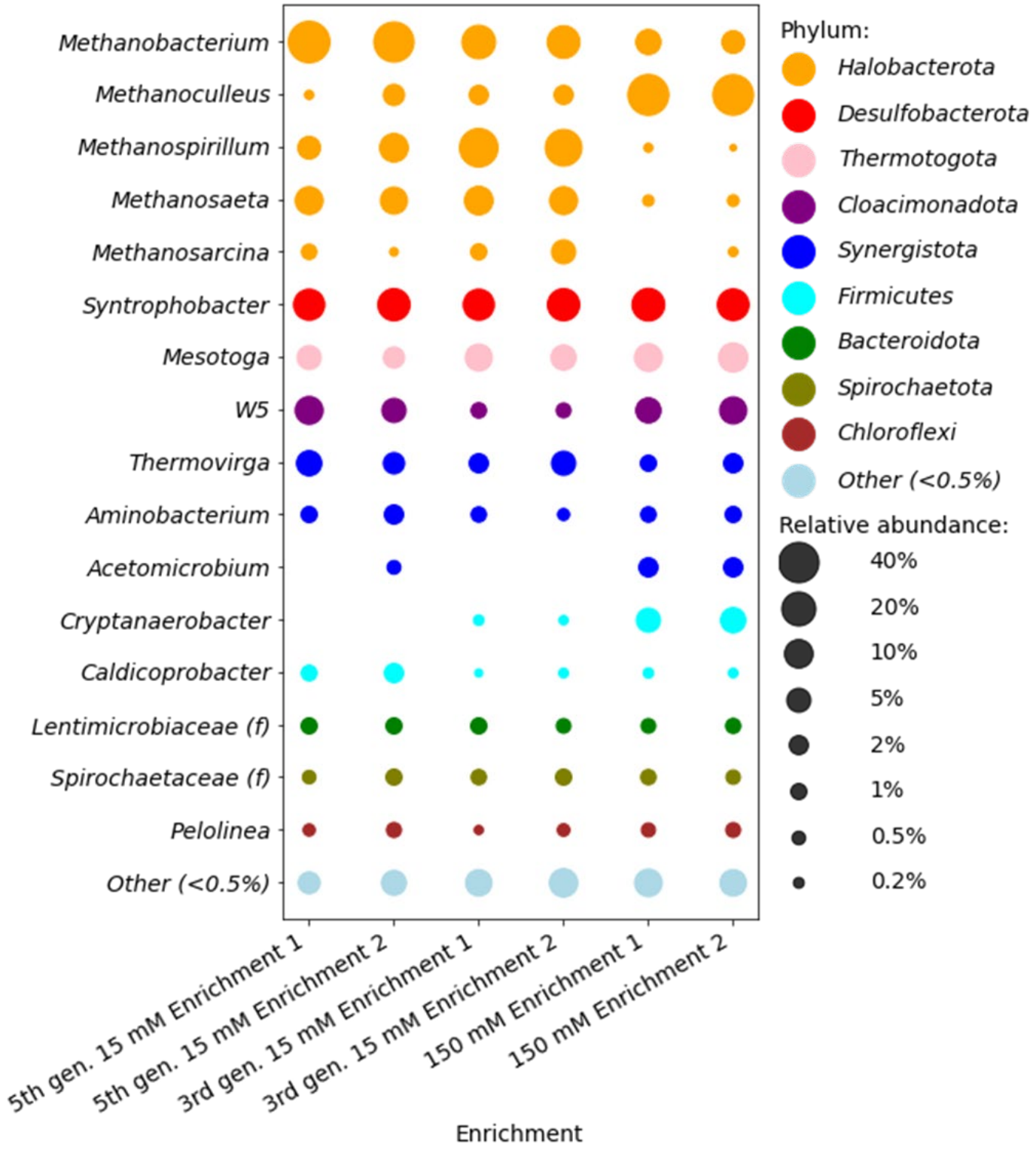
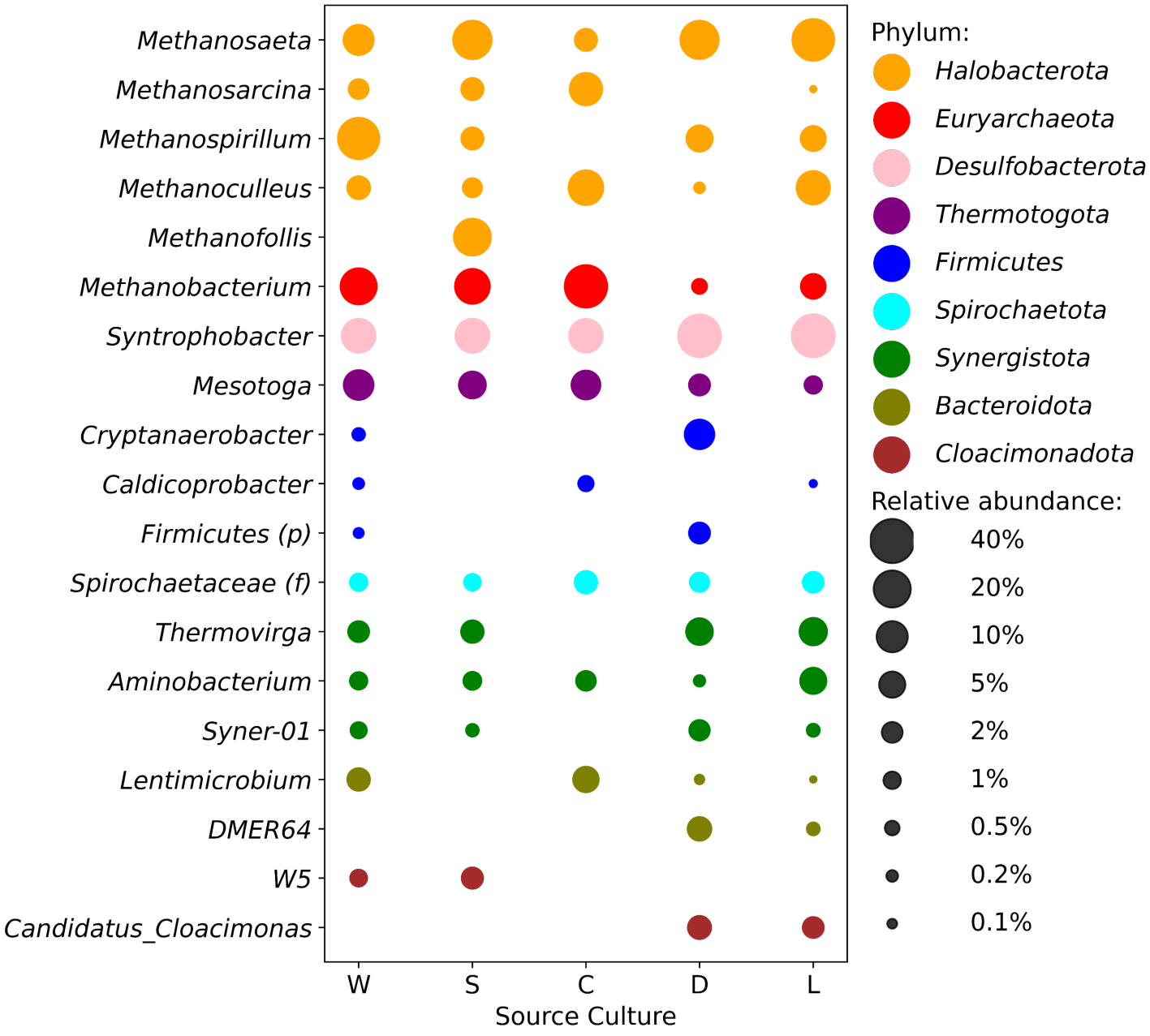

Methanobacterium
Methanoculleus
Methanospirillum
Methanosaeta
Methanosarcina
Syntrophobacter
Mesotoga
W5
Thermovirga
Aminobacterium
Acetomicrobium
Cryptanaerobacter
Caldicoprobacter
Lentimicrobiaceae (f)
Spirochaetaceae (f)
Pelolinea
Other (<0.5%)
5th gen. 15 mM Enrichment 1
5th gen. 15 mM Enrichment 2
3rd gen. 15 mM Enrichment 1
3rd gen. 15 mM Enrichment 2
150 mM Enrichment 1
150 mM Enrichment 2
Enrichment
Phylum:
Halobacterota
Desulfobacterota
Thermotogota
Cloacimonadota
Synergistota
Firmicutes
Bacteroidota
Spirochaetota
Chloroflexi
Other (<0.5%)
Relative abundance:
40%
20%
10%
5%
2%
1%
0.5%
0.2%

Fig. 7


Methanosaeta
Methanosarcina
Methanospirillum
Methanoculleus
Methanofollis
Methanobacterium
Syntrophobacter
Mesotoga
Cryptanaerobacter
Caldicoprobacter
Firmicutes (p)
Spirochaetaceae (f)
Thermovirga
Aminobacterium
Syner-01
Lentimicrobium
DMER64
W5
Candidatus_Cloacimonas
W
S
C
D
L
Source Culture
Phylum:
Halobacterota
Euryarchaeota
Desulfobacterota
Thermotogota
Firmicutes
Spirochaetota
Synergistota
Bacteroidota
Cloacimonadota
Relative abundance:
40%
20%
10%
5%
2%
1%
0.5%
0.2%
0.1%