environmental microbiology reports

Environmental Microbiology Reports (2019) **11**(4), 558–570 doi:10.1111/1758-2229.12759

Brief Report

Candidatus Syntrophosphaera thermopropionivorans: a novel player in syntrophic propionate oxidation during anaerobic digestion

Stefan Dyksma* **and Claudia Gallert**
Faculty of Technology, Microbiology – Biotechnology, University of Applied Sciences Emden/Leer, Emden, Germany.

Summary

Propionate is an important intermediate in the anaerobic mineralization of organic matter. In methanogenic environments, its degradation relies on syntrophic associations between syntrophic propionate-oxidizing bacteria (SPOB) and *Archaea.* However, only 10 isolated species have been identified as SPOB so far. We report syntrophic propionate oxidation in thermophilic enrichments of *Candidatus* Syntrophosphaera thermopropionivorans, a novel representative of the candidate phylum *Cloacimonetes.* In enrichment culture, methane was produced from propionate, while *Ca.* S. thermopropionivorans contributed 63% to total bacterial cells. The draft genome of *Ca.* S. thermopropionivorans encodes genes for propionate oxidation via methymalonyl-CoA. Phylogenetically, *Ca.* S. thermopropionivorans affiliates with the uncultured *Cloacimonadaceae* W5 and is more distantly related (86.4% 16S rRNA gene identity) to *Ca.* Cloacimonas acidaminovorans. Although *Ca.* S. thermopropionivorans was enriched from a thermophilic biogas reactor, *Ca.* Syntrophosphaera was in particular associated with mesophilic anaerobic digestion systems. 16S rRNA gene amplicon sequencng and a novel genus-specific quantitative PCR assay consistently identified *Ca.* Syntrophosphaera/ *Cloacimonadaceae* W5 in 9 of 12 tested full-scale biogas reactors thereby outnumbering other SPOB such as *Pelotomaculum*, *Smithella* and *Syntrophobacter.* Taken together the ubiquity and abundance of *Ca.* Syntrophosphaera, those SPOB might be key players for syntrophic propionate metabolism that have been overlooked before.

Introduction

In anoxic environments such as sediments, water-logged soils and intestines of animals, syntrophic microbial communities are important for organic carbon mineralization to methane and carbon dioxide. Moreover, those methanogenic consortia are key to treat municipal and industrial organic waste as well as sewage and wastewater sludge for biogas production in a process called anaerobic digestion (AD). A constraint of anaerobic syntrophy is the oxidation of short-chain fatty acids (SCFA), for example, propionate and butyrate by acetogenic bacteria to produce molecular hydrogen, carbon dioxide and acetate, the precursors for methanogenesis. Those bacterially mediated oxidation reactions are thermodynamically unfavourable under standard conditions (reaction *A* and *B*) and only possible if the concentrations of acetate, formate and in particular hydrogen are kept low (Thauer *et al.,* 1977; Schink, 1992).

$$\mathrm{A}: CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2 \left(\Delta G^{0'} = 72\,\mathrm{kJ/reaction}\right)$$

$$\mathrm{B}: CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \left(\Delta G^{0'} = 48\,\mathrm{kJ/reaction}\right)$$

Thus, the fermentation of propionate and butyrate depends on syntrophic interaction between SCFA-oxidizing bacteria that transfer reducing equivalents to hydrogenotrophic *Archaea.* Propionate degradation has been considered as a rate-limiting step in AD (Amani *et al.,* 2011), and propionate accumulation was frequently reported when the process became unstable (de Bok *et al.,* 2004; Wang *et al.,* 2006; Gallert and Winter, 2008). However, propionate was among the main intermediates in the carbon flow to methane accounting for up to 35% of the total methanogenesis (Glissmann and Conrad, 2000).

Received 2 November, 2018; revised Month, 2019; accepted 12 April, 2019. *For correspondence. E-mail stefan.dyksma@hs-emden-leer.de; Tel. +49 4921 807 1483; Fax. +49 4921 807 1000.

Syntrophic propionate oxidation seems to be a niche process carried out by rather specialized microorganisms (Müller *et al.,* 2010; Li *et al.,* 2012). To date, only seven mesophilic syntrophic propionate-oxidizing bacteria (SPOB) have been isolated: *Syntrophobacter wolinii*, *S. pfennigii*, *S. fumaroxidans*, *S. sulfatireducens*, *Smithella propionica*, *Pelotomaculum schinkii* and *P. propionicum* (Boone and Bryant, 1980; Wallrabenstein *et al.,* 1995; Harmsen *et al.,* 1998; Liu *et al.,* 1999; Chen *et al.,* 2005; de Bok *et al.,* 2005; Imachi *et al.,* 2007). In addition, three thermophilic SPOB have been isolated and described: *P. thermopropionicum*, *Desulfotomaculum thermocisternum* and *D. thermobenzoicum* subsp. *thermosyntrophicum* (Nilsen *et al.,* 1996; Imachi *et al.,* 2002; Plugge *et al.,* 2002). *Smithella propionica* is the only known species so far that degrades propionate through the C6 dismutation pathway to acetate and butyrate, whereas butyrate is eventually oxidized to molecular hydrogen (H_2) and acetate (de Bok *et al.,* 2001). All other SPOB use the methylmalonyl-CoA pathway for propionate oxidation to acetate, H_2 and CO_2 (Plugge *et al.,* 1993). Although the pathways for syntrophic propionate metabolism are mostly understood, the extent of the phylogenetic diversity of SPOB is still unclear.

Metagenomic sequencing has been used to reconstruct the genome of the bacterium *Candidatus* Cloacimonas acidaminovorans from a digester treating wastewater sludge (Pelletier *et al.,* 2008). Its genome properties suggested the capability of a syntrophic lifestyle and propionate oxidation (Pelletier *et al.,* 2008). Recently, syntrophic propionate metabolism among members of the candidate phylum *Cloacimonetes* was further supported by single-cell-, metagenomics and transcriptomics (Rinke *et al.,* 2013; Nobu *et al.,* 2015). Uncultured *Cloacimonetes* closely related to *Ca.* C. acidaminovorans expressed methymalonyl-CoA pathway genes with high homology to those found in *Pelotomaculum* (Nobu *et al.,* 2015).

Most studies concerning SPOB are only focused on the well characterized genera *Smithella*, *Syntrophobacter* and *Pelotomaculum.* However, beyond the usual suspects, there are likely other bacteria that take part in a key process in anoxic environmental and engineered ecosystems, the syntrophic oxidation of propionate. Using cultivation-based techniques a novel SPOB from a thermophilic biogas reactor was enriched and physiologically characterized. Based on physiological and genomic characteristics, it was provisionally classified as *Candidatus* Syntrophosphaera thermopropionivorans (gen. nov., sp. nov.) affiliating with the candidate phylum *Cloacimonetes.* Using newly designed genus-specific primer sets and quantitative PCR, *Ca.* Syntrophosphaera was identified in various biogas reactors at similar or higher cell numbers than *Smithella*, *Syntrophobacter* and *Pelotomaculum*, suggesting a pivotal role of this newly described SPOB in a "bottleneck" of AD.

Results and discussion

Thermophilic propionate-degrading enrichment culture

Bicarbonate-buffered mineral medium without additional electron acceptors was inoculated with <1% digestate from a thermophilic biogas reactor (PFR1)-treating biowaste (Table 1). Cultures were repeatedly fed with propionate to a maximum concentration of 10 mM. After 4 month of incubation at 55 °C and continuous dilution by successive transfers (n = 4) of 1% culture into fresh medium, a total of approximately 100 mM propionate was consumed. Microscopic examination revealed an enrichment of small spherical cells. For functional characterization of the microbial community in the enrichment culture (ENR1), a metagenome was sequenced totaling to 2.37 Gb. Phylogenetic analysis of all 16S rRNA gene fragments extracted from the metagenome has shown that the uncultured *Cloacimonadaceae* W5 were highly enriched in comparison to the initial microbial community composition accounting for an increase in sequence abundance up to 44%, whereas other known SPOB were hardly found (Supplementary Information Fig. S1). A metagenomic bin with high coverage was recovered from the enrichment culture representing 95.6% of a complete genome (Table 2). Based on phylogenetic and genomic data, it was proposed that the enriched SPOB represents a novel genus and species, hereinafter referred to as *Candidatus* Syntrophosphaera thermopropionivorans.

Phylogeny of *Ca*. Syntrophosphaera thermopropionivorans

The 16S rRNA gene extracted from the genome bin was used to resolve the phylogenetic placement of *Ca.* S. thermopropionivorans by three different treeing methods. As there were only few largely complete meta- and single-cell genomes available for the candidate phylum *Cloacimonetes*, the 16S rRNA gene rather than concatenated marker genes was used for the phylogenetic reconstruction. In a consensus tree of 276 nearly full-length 16S rRNA sequences, *Ca.* S. thermopropionivorans formed a monophyletic cluster with uncultured bacteria of the *Cloacimonadaceae* W5 (Fig. 1). Although few sequences of the *Cloacimonadaceae* W5-group were recovered from oxygen minimum zones (OMZ) and spring sediments (Elshahed *et al.,* 2007; Walsh *et al.,* 2009), the vast majority of sequences were obtained from mesophilic biogas reactors (Chouari *et al.,* 2005; Tang *et al.,* 2007; Rivière *et al.,* 2009; Krakat *et al.,* 2011; Ito *et al.,* 2012) underscoring their allegiance with such methanogenic environments. *Ca.* S. thermopropionivorans displayed 86.4% 16S rRNA gene identity to *Candidatus* Cloacimonas acidaminovorans, an uncultivated bacterium that has been described by metagenomic analysis of an anaerobic

Table 1. Basic characteristics of the sampled biogas reactors and performed experiments.

Sample name (sampling date)	Reactor type	Temperature (°C)	Substrate	Wet weight (g/ml)	Percent dry matter (w:w)	Performed experiments
PFR1 (2017-10-05)	Plug flow reactor (full-scale)	54	Biowaste	1.21 ± 0.07	23.19 ± 1.27	a, b, c
CSTR1 (2017-06-08)	Continuous stirred tank reactor (full-scale)	37	Wastewater sludge	1.02 ± 0.04	1.28 ± 0.13	b, c
CSTR2 (2017-10-26)	Continuous stirred tank reactor (full-scale)	37	Manure	1.05 ± 0.03	4.38 ± 0.04	a, b, c
CSTR3 (2018-05-08)	Continuous stirred tank reactor (full-scale)	47	Maize silage	0.93 ± 0.05	6.68 ± 0.34	b, c
CSTR4 (2018-05-08)	Continuous stirred tank reactor (full-scale)	45	Maize silage	1.01 ± 0.03	6.21 ± 0.68	b, c
CSTR5 (2018-05-07)	Continuous stirred tank reactor (full-scale)	45	Maize silage + sugar beet (90:10)	1.04 ± 0.08	5.77 ± 0.41	b, c
CSTR6 (2018-05-07)	Continuous stirred tank reactor (full-scale)	45	Maize silage + sugar beet (90:10)	0.97 ± 0.07	5.87 ± 0.13	b, c
CSTR7 (2018-05-07)	Continuous stirred tank reactor (full-scale)	43	Maize silage	1.03 ± 0.13	6.58 ± 0.56	b, c
CSTR8 (2018-05-07)	Continuous stirred tank reactor (full-scale)	44	Maize silage + cattle manure + grass silage (70:20:10)	1.00 ± 0.06	7.90 ± 0.39	b, c
CSTR9 (2018-04-03)	Continuous stirred tank reactor (full-scale)	43	Maize silage + sugar beet (90:10)	0.97 ± 0.02	7.03 ± 0.12	b, c
CSTR10 (2018-05-08)	Continuous stirred tank reactor (full-scale)	43	Maize silage + grass silage (90:10)	0.99 ± 0.14	8.74 ± 0.46	b, c
CSTR11 (2018-05-08)	Continuous stirred tank reactor (full-scale)	43	Maize silage + grass silage (85:15)	1.01 ± 0.02	8.50 ± 0.51	b, c
CSTR12 (2018-03-08)	Continuous stirred tank reactor (lab-scale)	43	Maize silage	1.03 ± 0.02	4.49 ± 0.07	b, c
CSTR13 (2017-06-08)	Continuous stirred tank reactor (lab-scale)	39	Biowaste	1.04 ± 0.03	2.06 ± 0.06	b, c
ENR1 (2018-02-13)	Enrichment culture (inoculated from PFR1)	55	Propionate	n.d.	n.d.	b, c
ENR2 (2018-02-13)	Enrichment culture (inoculated from CSTR2)	37	Propionate	n.d.	n.d.	b, c

[a]enrichment culture.
[b]16S rRNA gene sequencing.
[c]qPCR; n.d. not determined.

digester of a wastewater treatment plant (Pelletier *et al.,* 2008). Based on the taxonomic thresholds presented by Yarza *et al*. (2014), the 16S rRNA sequence identity of 86.4% is strong evidence for a distinct genus or even a distinct family (genus threshold 94.5%, family threshold 86.5%). Based on the taxonomy depicted in the phylogenetic tree (SILVA database SSU Ref NR release 132, Quast *et al.,* 2013), all treeing methods support a second sequence cluster classified as *Cloacimonadaceae* W5 that is only distantly related to *Ca.* S. thermopropionivorans and likely represent an independent linage (Fig. 1). To analyse the phylogenetic affiliation of *Ca.* S. thermopropionivorans in more detail using total genomic information, average nucleotide identities (ANI) were calculated to the closest related completely sequenced bacterium, *Ca.* Cloacimonas acidaminovorans. Two-way ANI and orthoANI (Richter and Rosselló-Móra, 2009; Yoon *et al.,* 2017) revealed identities of 78.92% and 68.38% respectively. This further supports that *Ca.* S. thermopropionivorans represent a novel genus.

Table 2. General genome statistics and selected metabolic features encoded in the metagenome bin of *Ca.* S. thermopropionivorans and in *Ca.* C. acidaminovorans' genome.

Name	*Candidatus* Syntrophosphaera thermopropionivorans	*Candidatus* Cloacimonas acidaminovorans
Accession	SMOG01000000	NC_020449.1
Sample origin	Thermophilic plug flow reactor (55 °C) digesting biowaste (PFL1)	Mesophilic digester (33 °C), Evry wastewater treatment plant
Genome statistics		
Assembly size (bp)	1,753,479	2,246,820
GC (%)	36.6	37.9
Predicted CDS	1,463	1,897
Scaffolds	58	1
N50	63,948	–
Marker gene completeness (%)[b]	95.6	100
Number of RNAs/tRNAs	46/42	52/46
rRNAs	5S, 16S, 23S	5S, 16S, 23S
Metabolism		
Respiration	RnfABCDEG, confurcating hydrogenase (NADH dehydrogenase/FeFe hydrogenase group A3), formate dehydrogenase-O, V-type ATPase	RnfABCDEG, confurcating hydrogenase (NADH dehydrogenase/FeFe hydrogenase group A3), formate dehydrogenase-O, V-type ATPase
Inorganic phosphate uptake	Pit and Pst system	Pit system
Amino acid degradation	+	+
Methylmalonyl-CoA pathway	+[a]	+
Other		
Oxidative stress response	Superoxide reductase, ruberythrin, rubredoxin, rubredoxin-oxygen reductase, *Bacteroides* aerotolerance operon	Superoxide reductase, ruberythrin, rubredoxin, peroxiredoxin, rubredoxin-oxygen reductase, hydroxyacylglutathione hydrolase, *Bacteroides* aerotolerance operon
FeFe hydrogenase group C1 (unknown function)	+	+
TypeIV pili	+	+

[a]Not all methylmalonyl-CoA pathway genes were identified.
[b]CheckM (Parks *et al.,* 2015).

Syntrophic propionate oxidation by *Ca*. S. thermopropionivorans

To the best of our knowledge, this study represents the most progressed cultivation success of a member from the candidate phylum *Cloacimonetes.* However, unexpected instability and complete stagnation of growth, as also reported for SPOB cultures of *Pelotomaculum* (Stams *et al.,* 1992; Imachi *et al.,* 2000), hampered the purification of defined co-cultures of *Ca.* S. thermopropionivorans so far. Therefore, syntrophic propionate oxidation was studied in enrichment cultures derived from ENR1 after a total of six successive transfers. Replicated cultures were inoculated (5% inoculum) and fed with 5 mM propionate. After the first 5 mM propionate were depleted, the cultures were flushed with N_2:CO_2 gas (80:20) to remove the produced methane from headspace and controls were amended with 20 mM 2-bromoethanesulfonate (BES) to inhibit methanogenesis. All cultures were fed again with approximately 7.5 mM propionate. Acetate did not accumulate during the incubation to concentrations above 0.4 mM (not shown), indicating acetate consumption by syntrophic acetate oxidizing bacteria (SAOB) or acetoclastic methanogens. 16S rRNA gene amplicons from the enrichment culture indicate a low abundance of acetoclastic *Methanosarcina*, whereas known SAOB have not been identified (Supplementary Information Figs S1 and S3). Over the period of 24 days, propionate was completely consumed and methane was produced. BES-amended controls did not show methane production or propionate oxidation (Fig. 2). Although propionate was the exclusive source for H_2 amended to the medium, the stoichiometry for the conversion of propionate to methane without acetate accumulation was not as expected. A significant fraction of proteolytic *Coprothermobacter* in the enrichments (Supplementary Information Fig. S3) possibly growing on proteinaceous material of cell debris may also contributed to overall methane production (Sasaki *et al.,* 2011), which ultimately led to more methane formed as it was possible solely from propionate oxidation. CARD-FISH performed after the incubation period revealed that *Ca.* Syntrophosphaera and *Bacteria* contributed 43.9 $\pm$ 13% and 69.6 $\pm$ 9.8% to total DAPI-stained cells respectively (Fig. 2), thus identifying *Ca.* Syntrophosphaera as the dominant organism. A yield of 0.52 $\pm$ 0.26 g protein per mole propionate was determined for the enrichment culture.

Genome properties and mechanisms for syntrophy

The relatively small but largely complete draft genome recovered from the enrichment culture consists of

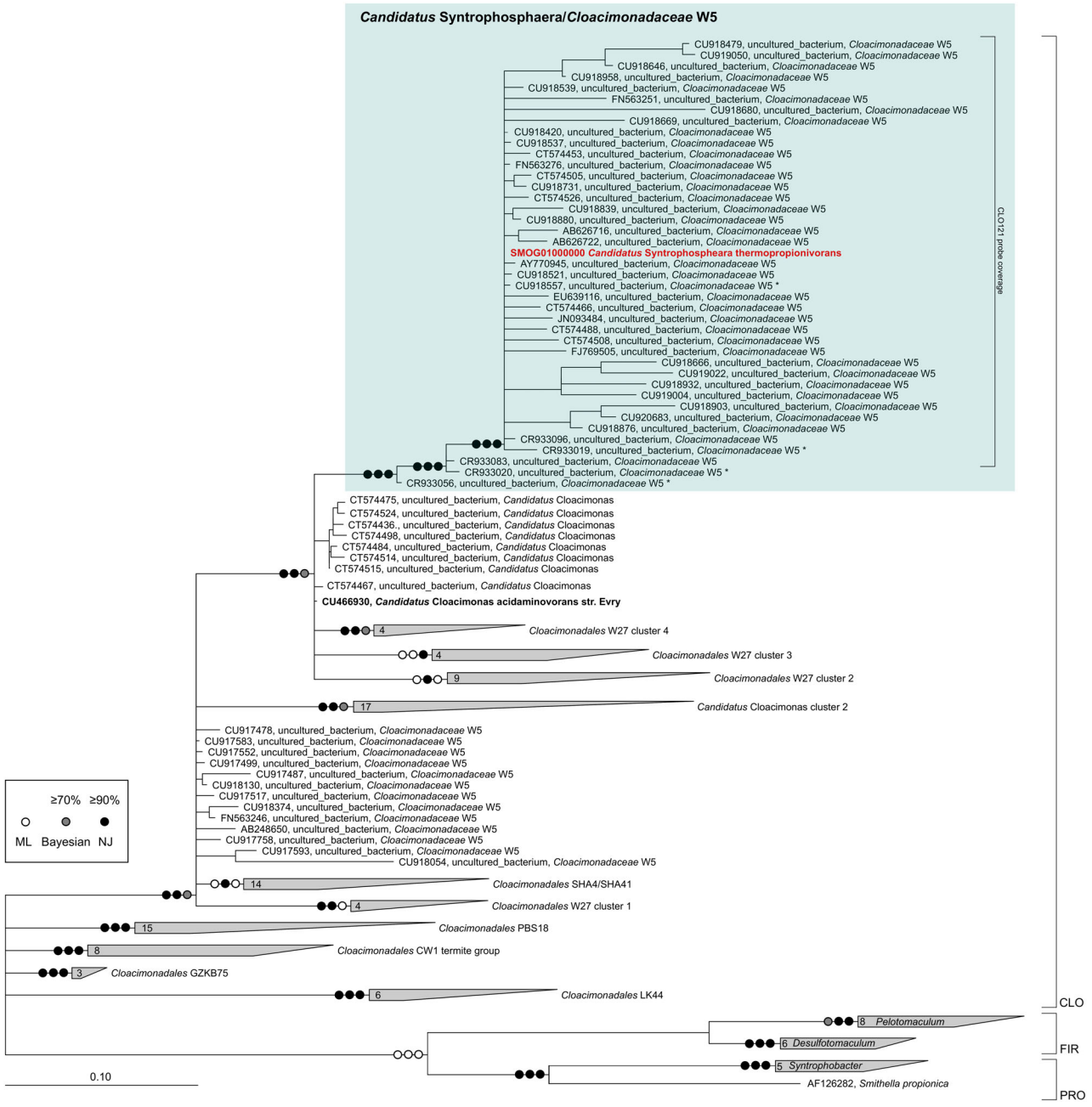

Fig. 1. 16S rRNA phylogeny of the *Cloacimonetes* and *Candidatus* Syntrophosphaera/*Cloacimonadaceae* W5. Consensus tree of nearly full-length 16S rRNA sequences using Maximum Likelihood (RAxML), Bayesian inference (MrBayes) and Neighbour-Joining (NJ) algorithm. Circles indicate bootstrap and consensus support. Diverging phylogenies are displayed as multifurcations. *Ca.* S. thermopropionivorans affiliate with the *Cloacimonadaceae* W5. For clarity only, a subset of the tree is depicted. The taxonomy refers to the SILVA database release 132. Coverage of probe CLO121 is indicated. Asterisks indicate sequences that were not targeted by probe CLO121.

1,753,479 nucleotides with a GC content of 36.6%. Analysis of the sequence bin using checkM software (Parks *et al.,* 2015) indicated low level of contamination (2.2%) that is likely from a very similar strain (strain heterogeneity of 100%). One complete rRNA operon (5S, 16S and 23S) and a second copy of the 23S rRNA gene have been identified. A total of 1,463 predicted genes were identified of which 1,082 could be assigned to a biological function. Despite the phylogenetic distance between *Ca.* S. thermopropionivorans and *Ca.* C. acidaminovorans, their potential metabolic functions encoded in the genomes are similar (Table 2). Genomic determinants for a syntrophic lifestyle and propionate oxidation are shared by those microorganisms (Table 2; Pelletier *et al.,* 2008). Like in many other syntrophic bacteria (Sieber *et al.,* 2012), their genomes encode an electron confurcating

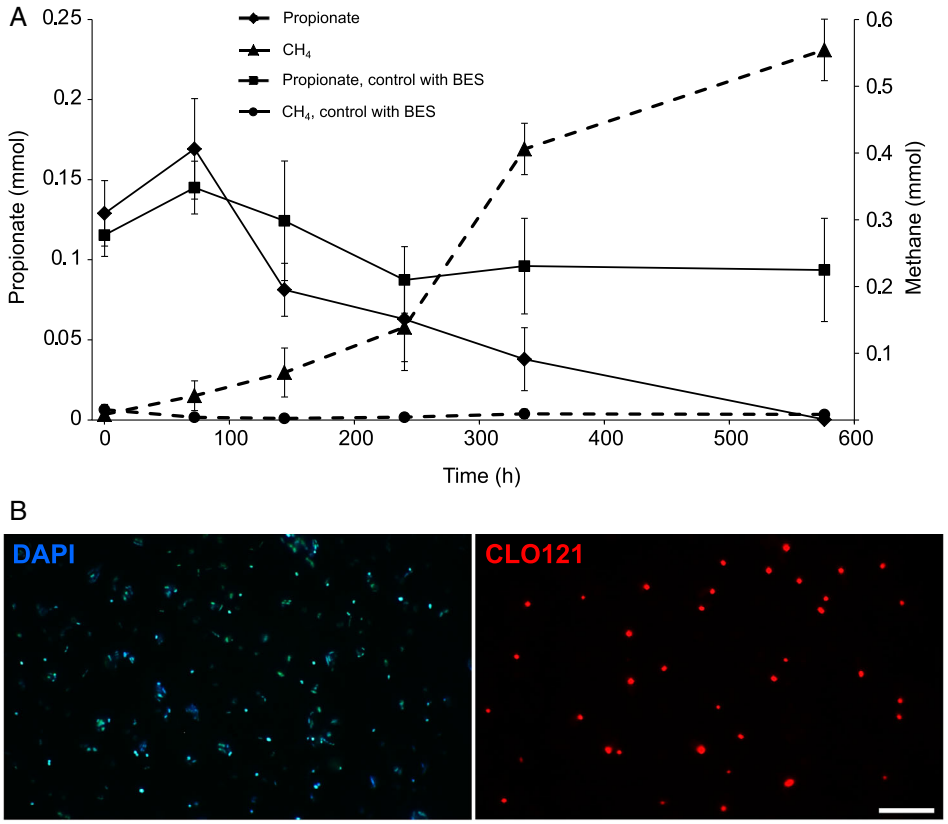

Fig. 2. Syntrophic enrichment culture growing on propionate (A) and visualization of *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 cells (B).
A. Methane production from propionate degradation. Control cultures amended with BES did not show methane production.
B. Identification of *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 cells using specific CARD-FISH probe (CLO121; in red). Scale bar refers to 10 μm. In blue, DAPI-stain.

hydrogenase (ECHyd) that couples the production of hydrogen from NADH with the oxidation of reduced ferredoxin. Reverse electron transport is driven in combination with a Na^+/H^+ translocating ferredoxin:NAD^+ oxidoreductase (Rnf complex) that generates reduced ferredoxin. Alternatively, formate production from NADH may be also involved in reverse electron transport through NADH-dependent formate dehydrogenase (Table 2; Sieber *et al.,* 2010). *Ca.* S. thermopropionivorans likely facilitates propionate oxidation via methylmalonyl-CoA (Table 2); however, not all methylmalonyl-CoA pathway genes have been identified in the draft genome assembly (Supplementary Information Fig. S2). The genes-encoding propionate metabolism showed the highest sequence homology to those of *Ca.* C. acidaminovorans (56%–87% sequence identity). Moreover, the genetic potential for propionate oxidation using the methylmalonyl-CoA pathway was consistently found among members of the *Cloacimonetes* (Pelletier *et al.,* 2008; Nobu *et al.,* 2015). The draft genome of *Ca.* S. thermopropionivorans includes all genes for histidine and aspartate degradation as well as the complete lysine fermentation pathway (Kreimeyer *et al.,* 2007) except one gene that is missing; therefore, the capability to ferment amino acids seems likely. However, lysine (10 mM) fed to an enrichment culture derived from ENR1 did not support growth and methane formation was not observed. The absence of electron transport chains for utilization of terminal electron acceptors, for example, nitrate, nitrite, sulphate and other sulphur compounds further support the fermentative lifestyle. Whereas *Ca.* C. acidaminovorans is only capable of inorganic phosphate uptake through the constitutively expressed low-affinity Pit system (Rosenberg *et al.,* 1977; van Veen, 1997), *Ca.* S. thermopropionivorans also harbour a high-affinity phosphate-specific ABC transporter (Pst system) indicating a physiological advantage at limiting phosphate concentrations (Vershinina and Znamenskaya, 2002).

Furthermore, a set of genes required for assembly of type IV pili is encoded in the genome. Type IV pili carry out various functions in bacteria such as motility, adherence, micro-colony formation, DNA uptake (Melville and Craig, 2013) as well as carrying electric current during direct interspecies electron transfer (DIET) (Reguera *et al.,* 2005; Summers *et al.,* 2010). Syntrophic microorganisms transfer reducing equivalents either through soluble carriers like H_2 and formate (interspecies hydrogen

or formate transfer) or using DIET. In the latter, electrically conductive pili (e-pili) can mediate extracellular electron exchange. A high density of aromatic amino acids without large gaps of aromatic amino acid free regions in the pilus assembly protein are key to yield electrical conductance (Vargas *et al.,* 2013; Walker *et al.,* 2018). The density of aromatic amino acids in the pilus assembly protein of *Ca.* S. thermopropionivorans (10.8%) and *Ca.* C. acidaminovorans (11%) exceeds those of *Geobacter sulfurreducens* (8.5%) (Supplementary Information Fig. S3), which is among the best studied model organisms capable of DIET (e.g., Vargas *et al.,* 2013; Adhikari *et al.,* 2016). Despite the strong indication to possess e-pili, the capability of DIET for *Ca.* Syntrophosphaera and *Ca.* Cloacimonas would be speculative since their genomes encode no c-type cytochromes that were proposed to be an important component for electron transfer. Liu *et al*. (2015) demonstrated that the conductive iron mineral magnetite can compensate for the lack of e-pili associated cytochromes thereby facilitating DIET. Interestingly, in AD of sewage sludge, the most pronounced increase in abundance was shown for *Ca.* Cloacimonas after addition of magnetite (Wang *et al.,* 2018). The majority of studies to date concerning DIET are focused on very few model organisms although other important but understudied microorganisms most likely bear the potential to circumvent H_2 production by developing electrical connections. If *Ca.* Syntrophosphaera and related *Cloacimonetes* participate in DIET, for example, in natural environments that can be rich in conductive minerals such as sediments or in engineered ecosystems still awaits elucidation by additional research.

Ca. Syntrophosphaera/*Cloacimonadaceae* W5 are common members of the prokaryotic community in mesophilic biogas reactors

To identify SPOB and to examine if the *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 were a regular component of the microbial community in anaerobic digestion we used 16S rRNA gene amplicon sequencing (iTags) of the V3-V4 region (>400 bp). Nearly 1 mio quality trimmed reads from 12 full-scale and 2 laboratory-scale digesters as well as 2 propionate-degrading enrichment cultures were kept for phylogenetic analysis (Supplementary Information Table S1). *Smithella* and *Syntrophobacter* were only detected in anaerobic digestion of wastewater sludge and in the artificial laboratory-scale systems accounting for 0.1%–3% and 0.3%–1.1% of all bacterial 16S rRNA sequences respectively (Fig. 3). Consistent with the thermophilic lifestyle of *Desulfotomaculum* (Nilsen *et al.,* 1996; Plugge *et al.,* 2002), related sequences were not detected in almost all mesophilic digesters but also not in the thermophilic system. In contrast, *Pelotomaculum* has been consistently identified in all mesophilic full-scale reactors accounting for 0.1%–1.1% of total bacterial sequences (Fig. 3) regardless of the substrate fed to the system. The PCR-based 16S rRNA gene survey well reflects the metagenomic analysis of the thermophilic propionate-degrading enrichment culture (ENR1) (Fig. 3 and Supplementary Information Fig. S1). ENR1 was dominated by *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 contributing 39.2% to all bacterial iTags, whereas the mesophilic enrichment culture (ENR2) was dominated by *Pelotomaculum* (Fig. 3). Strikingly, *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 contributed the largest fraction to all SPOB and candidate SPOB sequences in 9 of 12 full-scale reactors. Like *Pelotomaculum*, *Ca.* Syntrophosphaera is likely comprised of meso- and thermophilic representatives (Imachi *et al.,* 2002; de Bok *et al.,* 2005; Imachi *et al.,* 2007) as demonstrated by the ubiquity of their 16S rRNA genes in mesophilic AD systems along with their abundance in the thermophilic enrichment cultures.

Other groups of uncultured bacteria that were consistently identified in almost all digesters in high sequence abundances were the MBA03 (*Firmicutes*) with a largely unknown ecological function and the DTU014 (*Firmicutes*). Kirkegaard *et al*. (2017) identified the DTU014, which was formerly often misclassified as *Gelria*, as a core group of bacteria associated with AD and protein-based stable isotope probing combined with metagenomics suggested syntrophic acetate oxidation among the DTU014 (Mosbæk *et al.,* 2016).

The archaeal community was largely dominated by *Methanoculleus* accounting for up to 89% of total archaeal 16S rRNA genes. In 2 of 12 full-scale digesters acetoclastic methanogens such as the *Methanosaeta* or *Methanosarcina* were most prominent (Supplementary Information Fig. S4). In summary, the diversity of identified *Archaea* agreed well with previously reported methanogenic communities in AD (Rivière *et al.,* 2009; Ziganshin *et al.,* 2013; Kirkegaard *et al.,* 2017). However, the high-sequence abundance of *Methanoculleus* in 13 of 14 biogas reactors (Supplementary Information Fig. S4) was contrasting to studies that reported overall low abundance of *Methanoculleus* in AD (Nelson *et al.,* 2011; Narihiro *et al.,* 2014).

Cell numbers of *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 exceed those of other SPOB

Taken together the metagenomic analysis and 16S rRNA gene survey, we identified *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 as a vital component of the syntrophic community associated with AD. Novel sets of genus-specific primers were designed (Supplementary Information Tables S2 and S3) for a quantitative PCR (qPCR)

Relative abundance (%)
0 5 40

	Thermophilic		Mesophilic													
	BW	PROP	WWT	MAN	MS									MS (ls)	BW (ls)	PROP
	PFL1	ENR1	CSTR1	CSTR2	CSTR3	CSTR4	CSTR5	CSTR6	CSTR7	CSTR8	CSTR9	CSTR10	CSTR11	CSTR12	CSTR13	ENR2
Aminicenantales (*Acidobacteria*)	0	0	5.6	0	0	0	0	0	0	0	0	0	0	0	1.7	0
Candidatus Microthrix (*Actinobacteria*)	0	0	2.8	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroidales UCG-001 (*Bacteroidetes*)	0	0	0	1.1	0.5	0.2	0.5	2	0.1	0.4	1.7	0	0	6.8	0.1	0.2
Bacteroidetes vadinHA17 (*Bacteroidetes*)	0	0	1.5	1.5	0	0	0	0.5	0	0	0.3	0	0	0.1	0.6	6
Fermentimonas (*Bacteroidetes*)	0	0	0	1	0.2	0.1	0.8	0.6	0.1	0.2	1	0.1	0.2	2	0	0
Proteiniphilum (*Bacteroidetes*)	1.1	0.2	1.4	0.2	2.6	3.5	2.2	0.5	6	4.2	0.4	2.8	7.9	1.4	10.9	1.6
Dysgonomonadaceae; uncultured (*Bacteroidetes*)	0	0	1.3	0.7	0.2	0.1	0.3	0.9	0	0.1	0.9	0	0	0.8	4.8	0
M2PB4-65 termite group (*Bacteroidetes*)	0	0	0	0.1	0.3	0.1	0.1	0.4	0	0.1	2	0	0	0.1	0	0
Ruminofilibacter (*Bacteroidetes*)	0	0	0	0.5	3.3	1.2	3.3	1.1	1.8	1.6	1.7	0.6	1.5	1.3	0	0
Marinilabiliaceae; uncultured (*Bacteroidetes*)	0	0	0.7	0.4	0.5	0.6	0.3	0.4	1.2	0.7	0.9	0.6	0.3	1.1	0	0
DMER64 (*Bacteroidetes*)	0	0	0	0.2	0.1	0	0	0.2	0	0	0.3	0	0	0.3	9.1	0
Rikenellaceae RC9 gut group (*Bacteroidetes*)	0	0	0	6.7	0.2	0	0.1	1.1	0	0.1	1.9	0	0	0.7	0	0.2
Rikenellaceae; uncultured (*Bacteroidetes*)	0	0	0	1.6	0.4	0.1	0.3	1.4	0	0.1	2	0	0	13.5	0	0.2
VC2.1 Bac22 (*Bacteroidetes*)	0	0	0	0	0	0	0	0	0	0	0	0	0	1.2	0	0
Lentimicrobiaceae (*Bacteroidetes*)	12.4	0.4	14.3	0.8	8.5	11.1	4.5	2.9	6.9	8.7	0.5	4.3	9.8	0.1	1	0
ST-12K33 (*Bacteroidetes*)	0	0	0	2.5	0	0	0	3.7	0	0	0	0	0	0.1	0	0.4
Longilinea (*Chloroflexi*)	0	0	1.6	0	0	0	0	0	0	0	0	0	0	0	19.3	0
Anaerolineaceae; uncultured (*Chloroflexi*)	0	0.6	6.1	0.1	0	0	0.3	0.1	0	0	0	0	0	0.3	9.8	0
SBR1031 (*Chloroflexi*)	0	0.4	0	0	0	0	0	0	0	0	0	0	0	0	1	0
***Ca.* Cloacimonas (*Cloacimonetes*)**	0	0.6	1.6	0	0	0.1	0.3	0.2	0.2	0	0.3	0	0.2	1.3	0.5	0
***Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 (*Cloacimonetes*)**	0	39.2	1.9	2.4	0.6	0.4	3.1	1.8	1.7	0	3.5	0	1.7	0	0.2	4.9
W27 (*Cloacimonetes*)	0	0	0	4.5	0.1	0	0	1	0	0	0	0	0	4	1.2	0.2
Coprothermobacter (*Coprothermobacteraeota*)	0	9.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lysinibacillus (*Firmicutes*)	0.1	0.2	0	0	0	0	1.2	0	0.6	0.3	0	0	0	0	0	0
Streptococcus (*Firmicutes*)	0	0	0.1	0	5.5	4.8	0.1	1.5	5.5	1.3	1	0.2	0.2	0	0	0
Clostridia; unclassified (*Firmicutes*)	7.4	0	0	0.1	0.9	1	1.7	0.5	2.3	2.5	0.1	1.3	3.1	0	0	0
Caldicoprobacter (*Firmicutes*)	2	1.2	0	1	3.3	4	1.1	1.5	1.5	2.6	2.4	4.1	0.7	1.5	0	0
Clostridium sensu stricto 1 (*Firmicutes*)	0.1	0.2	0.7	0.1	2.4	2	2.2	4.1	3.1	1.9	0.8	16.8	0.6	3.3	0.1	0.2
Clostridium sensu stricto 8 (*Firmicutes*)	0.1	0	0	0	0.9	0.5	1.8	0.7	2.2	3.8	0.4	3.4	2	0	0	0
Haloimpatiens (*Firmicutes*)	0	0	0	0	0.1	0.2	0	0	0.2	1.1	0	0.4	0	0	0	0
Clostridiales vadinBB60 group (*Firmicutes*)	0.2	0	0	0.6	1.6	1.7	2.4	2.7	1.4	1.4	0.4	0.7	2	0.2	0.5	0
Defluviitalea (*Firmicutes*)	2.3	0	0	0	0.4	0.1	2.8	1	0.2	0.3	0.2	0.2	0.3	0	0	0
Gallicola (*Firmicutes*)	0	0	0.1	1.9	0	0	0	0	0	0	0	0	0	0.1	0	0.4
Keratinibaculum (*Firmicutes*)	0	0	0	0	2.7	3.2	0	0	0.4	0	0	0.2	0	0	0	0
Sedimentibacter (*Firmicutes*)	0	0	2.2	2.3	2.2	1.4	8.4	8.7	3	2.2	5.8	0.3	2.7	4.5	0.4	13.7
Tepidimicrobium (*Firmicutes*)	2.3	0	0	0	0.1	0.1	0	0	0	0	0	0.9	0	0	0	0
Tissierella (*Firmicutes*)	0	0	0	0.1	1.1	1.2	0.7	0.5	0.4	0.2	2.2	0.8	0.4	0.2	0	0
W5053 (*Firmicutes*)	0	0	0	0.1	0	0	0	0	0	0	3.9	0	0	0.1	0	0
Hydrogenispora (*Firmicutes*)	0.5	0	0	0.1	1	0.8	0.8	0.2	0.4	0.6	0.2	0.4	0.4	0	0	0
Herbinix (*Firmicutes*)	0.5	0	0	0.1	0.8	0.6	0.7	0.8	0.7	0.8	1	0.3	0.4	1.2	0	0
***Desulfotomaculum* (*Firmicutes*)**	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0
***Pelotomaculum* (*Firmicutes*)**	0	0.4	0.1	1.1	0.3	0.3	0.2	0.3	0.3	0.2	0.7	0.1	0.1	0.7	0	40.5
Peptococcaceae; uncultured (*Firmicutes*)	0.1	1	0	0.3	0.9	0.6	3.2	0.3	0.4	0.3	0.4	0.1	0.9	0.3	0.1	0.4
Intestinibacter (*Firmicutes*)	0	0	2.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Fastidiosipila (*Firmicutes*)	0	0	0	25.5	0.3	0.1	0.7	4.6	0	0	14.6	0	0	6.2	0	2.8
Pseudobacteroides (*Firmicutes*)	0	0	1.9	0	0	0	0.1	0	0	0	0	0	0	0	0	0
Ruminiclostridium 1 (*Firmicutes*)	2.1	0	0	0.2	4.1	2.6	2.7	3.1	2	1.5	2.3	2.4	2.1	0.3	0	0
Ruminiclostridium (*Firmicutes*)	2.5	0	0.1	0.6	2.8	2.1	1.9	1.7	1.8	1.4	1.9	1.7	2.1	1.2	0.1	0
Ruminococcaceae UCG-010 (*Firmicutes*)	0.5	0	0	0.3	0.7	0.8	0.9	1.5	0.4	0.5	1.8	0.3	0.6	0.7	0.2	0
Ruminococcaceae UCG-013 (*Firmicutes*)	0.1	0	0	0	0.3	0.4	0.4	1.1	0.1	0.1	0.9	0.1	0.1	0.3	0	0
Ruminococcaceae; unclassified (*Firmicutes*)	0.4	1.4	0.4	1	2.5	2	1.9	2.1	2.5	2	3.7	1.4	2.5	2.9	0.9	1.2
Ruminococcaceae coprostanoligenes group (*Firmicutes*)	0.2	0	0.1	0.5	0.5	0.4	0.9	0.7	0.5	0.4	0.4	0.2	0.5	2.2	0.4	0
Syntrophomonas (*Firmicutes*)	1.2	0	1.4	5.7	0.5	0.6	0.3	0.7	0.5	0.3	0.9	0.3	0.4	0.7	0.9	0.7
Clostridiales; uncultured/unclassified (*Firmicutes*)	2.5	0	0	0.4	0.2	0.3	0.2	0.2	0.3	0.3	0.6	0.2	0.1	1.6	0	0
D8A-2 (*Firmicutes*)	0.8	0.2	0.9	0.3	0.6	1	0.4	0.3	0.4	0.7	0.3	0.3	0.5	0.1	1.6	0
DTU014 (*Firmicutes*)	9.7	6.6	0	3.1	3	3.8	2.7	1	3.9	4.4	0.4	5	7.3	0.2	0	5.4
M55-D21 (*Firmicutes*)	0.8	0	0	0.1	0.5	0.4	0.3	0.3	0.4	0.6	0.1	0.8	0.5	0.1	0	0
MBA03 (*Firmicutes*)	23.4	0.4	0	5.2	22.3	21.8	19.1	15.3	27	30.8	11.6	34.3	31.4	7.6	0	0.2
Syntrophaceticus (*Firmicutes*)	1.7	0	0	0	1.1	2	0	0.1	1.5	0.1	0	0	0	0	0	0
Erysipelotrichaceae UCG-004 (*Firmicutes*)	0.1	0	0	1.2	0.6	0.9	1.9	1.8	0.3	0.1	0.4	0.8	1	0.8	0.1	0
Erysipelotrichaceae; uncultured (*Firmicutes*)	0	0	0	0.1	0.1	0	0.1	0.3	0	0	2.7	0	0	0	0	0
Limnochordales; uncultured (*Firmicutes*)	0.5	0	0	0	0.1	0.1	0	0	0	0.1	0	0.2	0	0	0	0
Firmicutes; uncultured/unclassified	1.2	1	0	1.4	0.9	1	0.9	1	0.8	0.8	0.7	0.6	1.4	0.2	0	1.8
Leptotrichiaceae; uncultured (*Fusobacteria*)	0	0	0.1	0	0	0	0	0	0	0	0	0	0	2.5	0	0
Halocella (*Halanaerobiaeota*)	0.7	0	0	0	0.3	0.3	0	0	0.2	0.4	0	2.2	0.7	0	0	0
Hydrogenedensaceae (*Hydrogenedentes*)	0	0	1.3	0	0	0	0	0.1	0	0	0	0	0	0	0.3	0
SAR406 clade (*Marinimicrobia*)	0	0	1.4	0	0	0	0	0	0	0	0	0	0	0	0.1	0
Rhodobacter (*Proteobacteria*)	0	0	1.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas (*Proteobacteria*)	0	0	0.1	0	0.3	0.3	1.4	0.3	0.5	0.6	0.3	0.2	0.3	0.4	0.4	0.2
***Smithella* (*Proteobacteria*)**	0	0	1	0	0	0	0	0	0	0	0	0	0	0.1	3	0
***Syntrophobacter* (*Proteobacteria*)**	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	1.1	0
Exilispira (*Spirochaetes*)	0	0	1.8	0	0	0	0	0	0	0	0	0	0	0	0	0
Spirochaetaceae; uncultured (*Spirochaetes*)	0	0	1.5	0	0	0	0	0	0	0	0	0	0	0	1.4	0
Treponema 2 (*Spirochaetes*)	0	0	0.1	0.2	0.1	0	0.1	1	0	0	1.1	0	0	0.9	0.8	0
Acetomicrobium (*Synergistetes*)	2.5	13.1	0	0.2	2.8	4.7	0.8	0.3	3.9	4.4	0.6	2.5	0.9	0.3	0	0.4
Syner-01 (*Synergistetes*)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.2	0
Synergistaceae; uncultured (*Synergistetes*)	0	0	4.4	0.5	0.2	0.1	0.7	0.6	0.1	0.1	0.6	0	0.1	0.8	2.9	5.4
Acholeplasma (*Tenericutes*)	0	0	0	5.7	0.8	0.6	1.4	1.4	0.7	0.4	0.6	0.8	0.6	1	0.1	0.7
Izimaplasmatales; unclassified (*Tenericutes*)	1	0	0	1.4	2.5	2.9	2.5	1.7	2.9	2	0.5	1.4	2.6	0.2	0.1	0
Defluviitoga (*Thermotogae*)	13	12.2	0	0	0.9	1	0.7	0.2	0.4	0.8	0	0	2.8	0	0	0
	[94]	[88]	[62]	[85,7]	[90,5]	[90,2]	[86,4]	[83]	[91,7]	[88,5]	[84]	[94,3]	[93,9]	[78,5]	[81,9]	[87,2]

Fig. 3. Legend on next page.

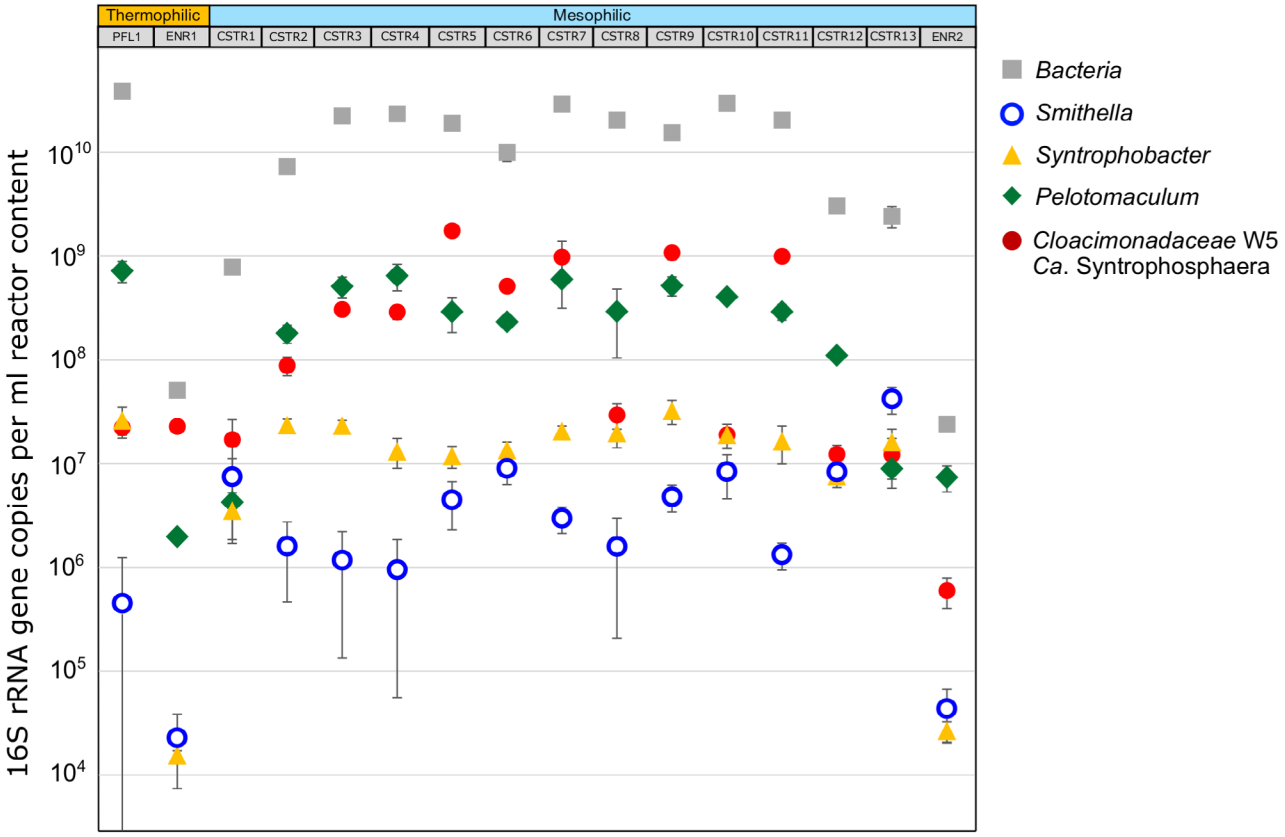

Fig. 4. 16S rRNA gene copy numbers of syntrophic propionate-oxidizing bacteria and candidate SPOB. SPOB that contributed ≥1% to total bacterial iTags in our 16S rRNA gene survey were targeted by quantitative PCR. PFL, plug flow reactor; CSTR, continuous stirred tank reactor.

assay to complement the non-quantitative iTag-approach. The genera *Smithella*, *Syntrophobacter*, *Pelotomaculum* and *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 were targeted for *in situ* quantification in all sampled biogas reactors and enrichment cultures. 16S rRNA sequences of thermophilic *Desulfotomaculum* were hardly detected (Fig. 3); therefore, those SPOB were omitted in the qPCR analysis. The novel primer sets were evaluated *in silico* (Supplementary Information Table S3), and the specificity was determined additionally by sequencing of the PCR products. Phylogenetic analysis revealed that 86%–99% of the amplicons affiliating with the respective target genus (Supplementary Information Table S3). For further evaluation, the primers were also tested on DNA extracts of selected pure cultures, whereas no amplification of non-target DNA was observed (Supplementary Information Table S2). After experimental validation of the primer sets, 16S rRNA gene copy numbers per ml reactor content were quantified. In most studied AD systems (10 of 14), copy numbers of *Syntrophobacter* and *Smithella* were substantially lower compared to *Pelotomaculum* and *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 (Fig. 4). In full-scale digesters, *Smithella* and *Syntrophobacter* reached copy numbers of 7.6×10^6 and 3.3×10^7 respectively. 16S rRNA gene copy numbers of *Pelotomaculum* were consistently high, accounting for more than 10^8 copies in almost all systems. Although more variable, the highest copy numbers were quantified for *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 (up to 1.8×10^9 copies per ml). Using the average 16S rRNA gene copy numbers per genome for the target genera, absolute cell numbers of SPOB and *Bacteria* per ml reactor content were estimated (see Supplementary Information Methods for details). The highest cell number of *Bacteria*, accounting for 9.74×10^9 cells per ml was found in the thermophilic reactor treating biowaste by dry fermentation

Fig. 3. Relative abundance of 16S rRNA amplicon sequences affiliating with *Bacteria.* The highest classification rank of groups that contributed ≥1% to all bacterial 16S rRNA sequences in at least one sample is depicted (SILVA SSU Ref NR r132). The sum of shown percentages is displayed in parentheses at the bottom. BW, biowaste; WWT, wastewater treatment plant; MAN, manure; MS maize silage; ls, laboratory-scale reactor; PFL, plug flow reactor; CSTR, continuous stirred tank reactor. Syntrophic propionate-oxidizing bacteria (SPOB) and candidate SPOB are highlighted in bold.

(Supplementary Information Fig. S5). On the contrary, in AD of wastewater sludge, we observed the lowest cell numbers accounting for 1.97×10^8 bacterial cells per ml. In line with previous quantitative detection of SPOB in methanogenic environments based on qPCR or FISH (Ariesyady *et al.,* 2007; Narihiro *et al.,* 2012; Li *et al.,* 2014; Moertelmaier *et al.,* 2014; Mathai *et al.,* 2015), comparable abundances of *Pelotomaculum*, *Syntrophobacter* and *Smithella* were identified accounting for 0.7%–7.3%, 0.1%–1.3% and 0%–6.9% of all bacterial cells respectively. Relative abundances of SPOB quantified by qPCR were in good coherence with the sequencing-based 16S rRNA gene survey (Supplementary Information Table S4). Most intriguingly, the *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 accounted for up to 85% of total SPOB in the mesophilic systems reaching an abundance of up to 8.84×10^8 cells per ml, which is the highest cell number among those four groups of SPOB detected in all 14 studied AD systems. Assuming that those identified syntrophic bacteria oxidize propionate *in situ*, *Ca.* Syntrophosphaera might play an essential role for a stable AD process by providing acetate, CO_2 and hydrogen to the methanogens from a thermodynamically challenging substrate.

Conclusions

For the first time, we provide direct evidence for syntrophic propionate oxidation by members of the candidate phylum *Cloacimonetes* and thus complementing previous studies suggesting that this uncultured phylum harbour novel SPOB beyond the usual suspects. Our results broadening the phylogenetic diversity for syntrophic propionate metabolism and provide insights into the ecological role of the *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5. High cell numbers and 16S rRNA gene abundances compared to other SPOB in anaerobic biogas reactors demonstrate their competitive success within a niche in anaerobic syntrophy. Besides the engineered ecosystems, *Ca.* Syntrophosphaera/*Cloacimonadaceae* W5 and related *Cloacimonetes* have been identified, although in some cases in fairly low abundances, in various anoxic environments including oxygen minimum zones, surface and subsurface sediments as well as soils (Derakshani *et al.,* 2001; Elshahed *et al.,* 2007; Walsh *et al.,* 2009; Hirschler-Réa *et al.,* 2012; Rinke *et al.,* 2013). Despite their ubiquity still little is known about their function in these habitats. Microbial syntrophy is largely studied by molecular tools due to the fastidiousness and often very slow growth rates of the participating organisms. However, isolation and cultivation will be irreplaceable to in-depth study their ecophysiology and metabolism. Syntrophic propionate oxidation still is an interesting field for discoveries, and future studies will pinpoint the ecological significance of other understudied but globally distributed bacteria that were proposed as SPOB such as the *Atribacteria* (Nobu *et al.,* 2015, 2016). In addition, the genus-specific qPCR assay presented in this study will be a promising tool for quick identification and quantification of SPOBs independent of the environment.

Acknowledgement

We greatly acknowledge Lena Peters and Piotr Biernacki for providing samples of laboratory-scale digesters. We are grateful to Oussama Mouileh and Nicolas Herzberg for their help with sampling. This study was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, grant number DY 132/1-1).

Conflict of interest

We declare no conflict of interest

References

Adhikari, R.Y., Malvankar, N.S., Tuominen, M.T., and Lovley, D. R. (2016) Conductivity of individual *Geobacter* pili. *RSC Adv* **6**: 8354–8357.

Amani, T., Nosrati, M., Mousavi, S.M., and Kermanshahi, R. K. (2011) Study of syntrophic anaerobic digestion of volatile fatty acids using enriched cultures at mesophilic conditions. *Int J Environ Sci Technol* **8**: 83–96.

Ariesyady, H.D., Ito, T., and Okabe, S. (2007) Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Water Res* **41**: 1554–1568.

Boone, D.R., and Bryant, M.P. (1980) Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. *Appl Environ Microbiol* **40**: 626–632.

Chen, S., Liu, X., and Dong, X. (2005) *Syntrophobacter sulfatireducens* sp. nov., a novel syntrophic, propionate-oxidizing bacterium isolated from UASB reactors. *Int J Syst Evol Microbiol* **55**: 1319–1324.

Chouari, R., Paslier, D.L., Dauga, C., Daegelen, P., Weissenbach, J., and Sghir, A. (2005) Novel major bacterial candidate division within a municipal anaerobic sludge digester. *Appl Environ Microbiol* **71**: 2145–2153.

de Bok, F.A.M., Harmsen, H.J.M., Plugge, C.M., de Vries, M. C., Akkermans, A.D.L., de Vos, W.M., and Stams, A.J.M. (2005) The first true obligately syntrophic propionate-oxidizing bacterium, *Pelotomaculum schinkii* sp. nov., co-cultured with *Methanospirillum hungatei*, and emended description of the genus *Pelotomaculum*. *Int J Syst Evol Microbiol* **55**: 1697–1703.

de Bok, F.A.M., Plugge, C.M., and Stams, A.J.M. (2004) Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res* **38**: 1368–1375.

de Bok, F.A.M., Stams, A.J.M., Dijkema, C., and Boone, D. R. (2001) Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei*. *Appl Environ Microbiol* **67**: 1800–1804.

Derakshani, M., Lukow, T., and Liesack, W. (2001) Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl Environ Microbiol* **67**: 623–631.

Elshahed, M.S., Youssef, N.H., Luo, Q., Najar, F.Z., Roe, B. A., Sisk, T.M., *et al*. (2007) Phylogenetic and metabolic diversity of *Planctomycetes* from anaerobic, sulfide- and sulfur-rich Zodletone spring, Oklahoma. *Appl Environ Microbiol* **73**: 4707–4716.

Gallert, C., and Winter, J. (2008) Propionic acid accumulation and degradation during restart of a full-scale anaerobic biowaste digester. *Bioresour Technol* **99**: 170–178.

Glissmann, K., and Conrad, R. (2000) Fermentation pattern of methanogenic degradation of rice straw in anoxic paddy soil. *FEMS Microbiol Ecol* **31**: 117–126.

Harmsen, H.J.M., Van Kuijk, B.L.M., Plugge, C.M., Akkermans, A.D.L., De Vos, W.M., and Stams, A.J.M. (1998) *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium. *Int J Syst Evol Microbiol* **48**: 1383–1387.

Hirschler-Réa, A., Cravo-Laureau, C., Casalot, L., and Matheron, R. (2012) Methanogenic octadecene degradation by syntrophic enrichment culture from brackish sediments. *Curr Microbiol* **65**: 561–567.

Imachi, H., Sakai, S., Ohashi, A., Harada, H., Hanada, S., Kamagata, Y., and Sekiguchi, Y. (2007) *Pelotomaculum propionicicum* sp. nov., an anaerobic, mesophilic, obligately syntrophic, propionate-oxidizing bacterium. *Int J Syst Evol Microbiol* **57**: 1487–1492.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Hanada, S., Ohashi, A., and Harada, H. (2002) *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. *Int J Syst Evol Microbiol* **52**: 1729–1735.

Imachi, H., Sekiguchi, Y., Kamagata, Y., Ohashi, A., and Harada, H. (2000) Cultivation and *in situ* detection of a thermophilic bacterium capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens in a thermophilic methanogenic granular sludge. *Appl Environ Microbiol* **66**: 3608–3615.

Ito, T., Yoshiguchi, K., Ariesyady, H.D., and Okabe, S. (2012) Identification and quantification of key microbial trophic groups of methanogenic glucose degradation in an anaerobic digester sludge. *Bioresour Technol* **123**: 599–607.

Kirkegaard, R.H., McIlroy, S.J., Kristensen, J.M., Nierychlo, M., Karst, S.M., Dueholm, M.S., *et al*. (2017) The impact of immigration on microbial community composition in full-scale anaerobic digesters. *Sci Rep* **7**: 9343.

Krakat, N., Schmidt, S., and Scherer, P. (2011) Potential impact of process parameters upon the bacterial diversity in the mesophilic anaerobic digestion of beet silage. *Bioresour Technol* **102**: 5692–5701.

Kreimeyer, A., Perret, A., Lechaplais, C., Vallenet, D., Médigue, C., Salanoubat, M., and Weissenbach, J. (2007) Identification of the last unknown genes in the fermentation pathway of lysine. *J Biol Chem* **282**: 7191–7197.

Li, C., Mörtelmaier, C., Winter, J., and Gallert, C. (2014) Effect of moisture of municipal biowaste on start-up and efficiency of mesophilic and thermophilic dry anaerobic digestion. *Bioresour Technol* **168**: 23–32.

Li, J., Ban, Q., Zhang, L., and Jha, A.K. (2012) Syntrophic propionate degradation in anaerobic digestion: a review. *ResearchGate* **14**: 843–850.

Liu, F., Rotaru, A.-E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., and Lovley, D.R. (2015) Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. *Environ Microbiol* **17**: 648–655.

Liu, Y., Balkwill, D.L., Aldrich, H.C., Drake, G.R., and Boone, D. R. (1999) Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. *Int J Syst Evol Microbiol* **49**: 545–556.

Mathai, P.P., Zitomer, D.H., and Maki, J.S. (2015) Quantitative detection of syntrophic fatty acid-degrading bacterial communities in methanogenic environments. *Microbiology* **161**: 1189–1197.

Melville, S., and Craig, L. (2013) Type IV pili in gram-positive bacteria. *Microbiol Mol Biol Rev* **77**: 323–341.

Moertelmaier, C., Li, C., Winter, J., and Gallert, C. (2014) Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. *Bioresour Technol* **166**: 479–484.

Mosbæk, F., Kjeldal, H., Mulat, D.G., Albertsen, M., Ward, A. J., Feilberg, A., and Nielsen, J.L. (2016) Identification of syntrophic acetate-oxidizing bacteria in anaerobic digesters by combined protein-based stable isotope probing and metagenomics. *ISME J* **10**: 2405–2418.

Müller, N., Worm, P., Schink, B., Stams, A.J.M., and Plugge, C.M. (2010) Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. *Environ Microbiol Rep* **2**: 489–499.

Narihiro, T., Nobu, M.K., Kim, N.-K., Kamagata, Y., and Liu, W.-T. (2014) The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. *Environ Microbiol* **17**: 1707–1720.

Narihiro, T., Terada, T., Ohashi, A., Kamagata, Y., Nakamura, K., and Sekiguchi, Y. (2012) Quantitative detection of previously characterized syntrophic bacteria in anaerobic wastewater treatment systems by sequence-specific rRNA cleavage method. *Water Res* **46**: 2167–2175.

Nelson, M.C., Morrison, M., and Yu, Z. (2011) A meta-analysis of the microbial diversity observed in anaerobic digesters. *Bioresour Technol* **102**: 3730–3739.

Nilsen, R.K., Torsvik, T., and Lien, T. (1996) *Desulfotomaculum thermocisternum* sp. nov., a sulfate reducer isolated from a hot North Sea oil reservoir. *Int J Syst Evol Microbiol* **46**: 397–402.

Nobu, M.K., Dodsworth, J.A., Murugapiran, S.K., Rinke, C., Gies, E.A., Webster, G., *et al*. (2016) Phylogeny and physiology of candidate phylum 'Atribacteria' (OP9/JS1) inferred from cultivation-independent genomics. *ISME J* **10**: 273–286.

Nobu, M.K., Narihiro, T., Rinke, C., Kamagata, Y., Tringe, S. G., Woyke, T., and Liu, W.-T. (2015) Microbial dark matter ecogenomics reveals complex synergistic networks in a methanogenic bioreactor. *ISME J* **9**: 1710–1722.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: assessing the quality of

microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25**: 1043–1055.

Pelletier, E., Kreimeyer, A., Bocs, S., Rouy, Z., Gyapay, G., Chouari, R., *et al*. (2008) "Candidatus Cloacamonas Acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division. *J Bacteriol* **190**: 2572–2579.

Plugge, C.M., Balk, M., and Stams, A.J.M. (2002) *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* subsp. nov., a thermophilic, syntrophic, propionate-oxidizing, spore-forming bacterium. *Int J Syst Evol Microbiol* **52**: 391–399.

Plugge, C.M., Dijkema, C., and Stams, A.J.M. (1993) Acetyl-CoA cleavage pathway in a syntrophic propionate oxidizing bacterium growing on fumarate in the absence of methanogens. *FEMS Microbiol Lett* **110**: 71–76.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., *et al*. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590–D596.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., and Lovley, D.R. (2005) Extracellular electron transfer via microbial nanowires. *Nature* **435**: 1098–1101.

Richter, M., and Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci* **106**: 19126–19131.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., *et al*. (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431–437.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., *et al*. (2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J* **3**: 700–714.

Rosenberg, H., Gerdes, R.G., and Chegwidden, K. (1977) Two systems for the uptake of phosphate in *Escherichia coli*. *J Bacteriol* **131**: 505–511.

Sasaki, K., Morita, M., Sasaki, D., Nagaoka, J., Matsumoto, N., Ohmura, N., and Shinozaki, H. (2011) Syntrophic degradation of proteinaceous materials by the thermophilic strains *Coprothermobacter proteolyticus* and *Methanothermobacter thermautotrophicus*. *J Biosci Bioeng* **112**: 469–472.

Schink, B. (1992) Syntrophism among prokaryotes. In *The Prokaryotes*, 3rd ed. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 276–299.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012) Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Annu Rev Microbiol* **66**: 429–452.

Sieber, J.R., Sims, D.R., Han, C., Kim, E., Lykidis, A., Lapidus, A.L., *et al*. (2010) The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. *Environ Microbiol* **12**: 2289–2301.

Stams, A.J.M., Grolle, K.C.F., Frijters, C.T.M., and Lier, J.B. V. (1992) Enrichment of thermophilic propionate-oxidizing bacteria in syntrophy with *Methanobacterium thermoautotrophicum* or *Methanobacterium thermoformicicum*. *Appl Environ Microbiol* **58**: 346–352.

Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S., and Lovley, D.R. (2010) Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* **330**: 1413–1415.

Tang, Y.-Q., Shigematsu, T., Morimura, S., and Kida, K. (2007) Effect of dilution rate on the microbial structure of a mesophilic butyrate-degrading methanogenic community during continuous cultivation. *Appl Microbiol Biotechnol* **75**: 451–465.

Thauer, R.K., Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**: 100–180.

van Veen, H.W. (1997) Phosphate transport in prokaryotes: molecules, mediators and mechanisms. *Antonie Van Leeuwenhoek* **72**: 299–315.

Vargas, M., Malvankar, N.S., Tremblay, P.-L., Leang, C., Smith, J.A., Patel, P., *et al*. (2013) Aromatic amino acids required for pili conductivity and long-range extracellular electron transport in *Geobacter sulfurreducens*. *MBio* **4**: e00105–e00113.

Vershinina, O.A., and Znamenskaya, L.V. (2002) The pho regulons of bacteria. *Microbiology* **71**: 497–511.

Walker, D.J., Adhikari, R.Y., Holmes, D.E., Ward, J.E., Woodard, T.L., Nevin, K.P., and Lovley, D.R. (2018) Electrically conductive pili from pilin genes of phylogenetically diverse microorganisms. *ISME J* **12**: 48–58.

Wallrabenstein, C., Hauschild, E., and Schink, B. (1995) *Syntrophobacter pfennigii* sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate. *Arch Microbiol* **164**: 346–352.

Walsh, D.A., Zaikova, E., Howes, C.G., Song, Y.C., Wright, J.J., Tringe, S.G., *et al*. (2009) Metagenome of a versatile chemolithoautotroph from expanding oceanic dead zones. *Science* **326**: 578–582.

Wang, L., Zhou, Q., and Li, F.T. (2006) Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. *Biomass Bioenergy* **30**: 177–182.

Wang, T., Zhang, D., Dai, L., Dong, B., and Dai, X. (2018) Magnetite triggering enhanced direct interspecies electron transfer: a scavenger for the blockage of electron transfer in anaerobic digestion of high-solids sewage sludge. *Environ Sci Technol* **52**: 7160–7169.

Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.-H., *et al*. (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Microbiol* **12**: 635–645.

Yoon, S.-H., Ha, S.-M., Lim, J., Kwon, S., and Chun, J. (2017) A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie Van Leeuwenhoek* **110**: 1281–1286.

Ziganshin, A.M., Liebetrau, J., Pröter, J., and Kleinsteuber, S. (2013) Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Appl Microbiol Biotechnol* **97**: 5161–5174.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information
Appendix S2: Supporting Information

Supplementary Fig. S1. Relative abundance of 16S rRNA gene fragments in the initial sample (inoculum, PFL1 T0) and after 4 month of enrichment with propionate as substrate. The enrichment was performed in duplicates (ENR1, ENR1b replicate). For sample ENR1 all 16S rRNA gene fragments extracted from the metagenome (meta) have been used for taxonomic assignment. PFL1 T0 and ENR1b replicate were only analysed by PCR-based amplicon sequencing (iTags).

Supplementary Fig. S2. Simplified scheme of carbon and energy metabolism in *Ca.* Syntrophosphaera thermopropionivorans reconstructed from the draft genome. Genes of the methylmalony-CoA pathway and lysine fermentation pathway that were lacking in the genome assembly are indicated (see also Supplementary Information S1). Fdh, formate dehydrogenase; Hyd, electron confurcating hydrogenase; Pil, type IV pili; Rnf, ferredoxin:NAD^+ oxidoreductase; SDH, succinate dehydrogenase.

Supplementary Fig. S3. Position of aromatic amino acids within pilus assembly proteins of selected conductive (e-pili) and non-conductive pili. Extracellular electron transfer or electrical conductance has been experimentally confirmed for the e-pili (Vargas *et al.,* ; Alauzet and Jumas-Bilak, 2014; Walker *et al.,*).

Supplementary Fig. S4. Relative abundance of 16S rRNA amplicon sequences affiliating with *Archaea.* The highest classification rank of groups that contributed >1% to all archaeal 16S rRNA sequences in at least one sample is depicted (SILVA SSU Ref NR r132). The sum of shown percentages is displayed in parentheses at the bottom. BW, biowaste; WWT, wastewater treatment plant; MAN, manure; MS maize silage; ls, lab-scale reactor; PFL, plug flow reactor; CSTR, continuous stirred tank reactor.

Supplementary Fig. S5. SPOB cell numbers per ml reactor content estimated by quantitative PCR.

Supplementary Table S1. Number of 16S rRNA amplicon sequences kept after quality trimming for phylogenetic classification using SILVA NGS pipeline (Quast *et al.,*).

Supplementary Table S2. Primer specificity tested with selected cultures.

Supplementary Table S3. Overview of qPCR primer used in this study. *In silico* primer specificity was evaluated using TestPrime against the SILVA SSU database Ref NR r132. Specificity was also determined by PCR amplicon sequencing.

Supplementary Table S4. Percentages of syntrophic propionate-oxidizing bacteria determined by quantitative PCR and their relative abundance of 16S rRNA gene amplicons (iTags).