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Quantitative Detection of Syntrophic Fatty Acid-degrading Bacterial Communities in Methanogenic Environments

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Introduction

Microbial degradation of complex organic matter to biogas, which contains methane and carbon dioxide, occurs in anaerobic environments that are low in external electron acceptors (Schink, 1997). Volatile fatty acids (VFA), e.g. propionate and butyrate, are major intermediates in this process and can account for a significant

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proportion of the total methane produced (Gujer & Zehnder, 1983). However, fatty acid degradation is highly endergonic under standard conditions (propionate: $\Delta G^{\circ\prime} = +72$ kJ; butyrate: $\Delta G^{\circ\prime} = +48$ kJ) (Thauer et al., 1977). Nevertheless, under methanogenic conditions, these reactions can proceed via cooperation between syntrophic fatty acid-degrading bacteria (SFAB) and methanogenic archaea, which keep the end products of VFA degradation (especially H 2 and formate) at low concentrations (Schink & Stams, 2002). These syntrophic partnerships occur in methanogenic habitats such as anaerobic digesters, rice paddy fields, freshwater sediments and wetlands.

Due to the fastidious nature of syntrophic metabolism and slow growth rates, current knowledge of SFAB is extremely limited and is based on a few pure- and co-cultures (Stams et al., 2012). To date, seven mesophilic species within three genera have been reported to degrade propionate: *Syntrophobacter* (*S. fumaroxidans, S. sulfatireducens, S. pfennigii and S. wolinii*), *Smithella* (*S. propionica*) and *Pelotomaculum* (*P. schinkii* and *P. propionicum*) while eight mesophilic species within *Syntrophomonas* (*S. bryantii, S. cellicola, S. curvata, S. erecta, S. palmitatica, S. sapnovida, S. wolfei* and *S. zehnderi*) have been reported to degrade butyrate and higher fatty acids (McInerney et al., 2008). Additionally, six thermophilic and one psychrophilic species involved in VFA degradation have been isolated (McInerney et al., 2008).

The application of molecular techniques to environmental samples has enabled the analysis of micro-organisms that are difficult to culture. Microbial diversity studies in different methanogenic habitats, based on stable isotope probing (Lueders et al., 2004 ; Chauhan & Ogram, 2006 ; Hatamoto et al., 2007 Liu et al., 2011 ; Gan et al., 2012) and enrichment culturing (Shigematsu et al., 2006 ; Sousa et al., 2007 ; Tang et al., 2007 ; Narihiro et al., 2014), have confirmed *Syntrophobacter, Smithella, Pelotomaculum* and *Syntrophomonas* to be the major bacterial genera involved in VFA degradation under mesophilic conditions. While it is important to understand SFAB diversity, it would be extremely beneficial to measure their abundance in methanogenic habitats. This is particularly important in anaerobic digesters where process upsets (e.g. substrate overload) and operational problems often cause VFA accumulation, which, in most cases, results in digester malfunction and lowered

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methane output (McCarty & Smith, 1986). VFA (especially propionate) degradation has been considered to be a rate-limiting step in anaerobic digestion (e.g. Ito et al., 2012). Despite their indispensable role in VFA degradation, little is known about the quantitative significance of SFAB, which might be a critical factor to ensure reactor stability. Therefore, monitoring the abundance of these micro-organisms would provide a much-detailed insight into reactor performance during stable and perturbed states.

Previously, probe-based molecular techniques such as membrane hybridization (Harmsen et al., 1995 ; Hansen et al., 1999 ; Scheid & Stubner, 2001 ; McMahon et al., 2004), FISH (Imachi et al., 2006 ; Ariesyady et al., 2007a) and the cleavage method with RNase H (Narihiro et al., 2012) have been used to quantify SFAB, primarily at the species level. However, using cultured species solely as targets is not ideal because known isolates represent only fraction of all 16S rRNA gene sequences deposited within a genus in Ribosomal Database Project (Cole et al., 2014). Therefore, targeting these micro-organisms at the genus level would potentially be more inclusive. Moreover, hybridization-based techniques such as FISH are labour-intensive and often display reduced sensitivity, which is a major drawback when detecting microbial populations present in low numbers (Bouvier & del Giorgio, 2003).

Quantitative PCR (qPCR) is a powerful technique that allows rapid, reproducible and sensitive detection of specific microbial populations in complex ecosystems (Smith & Osborn, 2009). From a practical standpoint, this technique has been successfully used in combination with analytical methods to relate methanogen abundance and dynamics to digester function (Hori et al., 2006 ; Yu et al., 2006 ; Morris et al., 2014). In this study, we report the development of four genus-specific qPCR assays, based on the 16S rRNA gene, for the quantification of known SFAB within the genera *Syntrophobacter*, *Smithella*, *Pelotomaculum* and *Syntrophomonas*. After validation, these novel qPCR assays were used to measure SFAB abundance in biomass samples obtained from a variety of methanogenic environments.

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Methods

Sample collection

Fourteen methanogenic biomass samples (nine engineered and five natural environments) were collected and analysed in this study. Samples from engineered habitats included one propionate enrichment culture, one pilot-scale and seven full-scale reactors. The enrichment culture was established using seed biomass from brewery sludge as described previously (Tale et al., 2011). The culture was fed calcium propionate (0.25g chemical oxygen demand, COD $l-1$-day -1) and basal nutrient medium (Schauer-Gimenez et al., 2010), once per day, continuously stirred at 35 ± 1 °C and maintained at a 15-day hydraulic retention time (HRT). After 5.5 years of operation, the feed concentration was increased from 0.25 to 1.04g COD $l-1$-day -1 and feeding frequency was modified from once per day to once per hour. Biomass samples were collected at T = 0 (seed inoculum), 2.5 and 6 years post-start-up. The pilot-scale reactor was fed daily with non-fat dry milk (2.5g COD $l-1$-day -1) and basal nutrient medium, continuously stirred at 35 ± 1 °C and maintained at a 15-day HRT. Full-scale samples were obtained from seven mesophilic municipal and industrial reactors, which included four upflow anaerobic sludge blanket (UASB) reactors (UASB-1: soft-drink bottling waste; UASB-2: food flavouring waste; UASB-3 and 4: brewery waste) and three continuous stirred-tank reactors (CSTR) (CSTR-1 and 2: municipal waste; CSTR-3: cheese processing waste). Specific methanogenic activity (SMA) tests, using propionate as sole carbon substrate, were performed as described by Sorensen & Ahring (1993) . In addition, five samples were collected from natural methanogenic habitats including cow rumen (East Lansing, MI), horse faeces (Camp Lake, WI), an experimental rice paddy soil (Milwaukee, WI), a bog stream (Cedarburg Bog, WI) and swamp sediments (Woods Hole, MA). All samples for DNA extraction were stored at − 20 °C immediately upon receipt.

DNA extraction

DNA extraction was performed on biomass samples (0.25g wet pellet weight) using a PowerSoil DNA Isolation kit according to the

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manufacturer's instructions (MO BIO). DNA integrity was confirmed on 0.8 % agarose gels stained with ethidium bromide (10 µg ml − 1). DNA extracts were purified using the PowerClean® DNA Clean-Up kit according to the manufacturer's instructions (MO BIO) and quantified spectrophotometrically (Nanodrop ND-1000; ThermoScientific). The purified DNA was stored in 10 mM Tris buffer (pH: 8) at − 80 °C until subsequent analysis.

Primer design and in silico validation

For each genus of interest, full-length or partial 16S rRNA gene sequences (≥ 1200 bp) were retrieved from the Ribosomal Database Project (RDP) – Release 11, Update 1 (Cole et al., 2014), aligned using clustalw2 (Larkin et al., 2007) and manually examined for genus-specific oligonucleotides. Probe Match function (RDP) was used to determine genus specificity and coverage of each newly designed oligonucleotide and probes previously used for hybridization-based studies. Oligonucleotides that qualified as potential primer sets (based on probe length: 18–25 bases, melting temperature: 50–65 °C, GC content: 40–65 %, low possibility of hairpin and self/hetero-dimer formation and product size: 75–300 bp) were selected for qPCR-based applications.

Experimental validation

Primer set specificity was evaluated using target and non-target bacterial DNA. Five positive DNA controls were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ): *S. fumaroxidans* (DSM 10017), S. sulfatireducens (DSM 16706), P. thermopropionicum (DSM 13744), S. curvata (DSM 15682) and S. zehnderi (DSM 17840). Genomic DNA extracts of *S. fumaroxidans* and S. wolfei were kindly provided by C.M. Plugge (Wageningen University, Netherlands) and M.J. McInerney (University of Oklahoma, USA), respectively. For *Smithella*, an environmental clone (EMBL accession number LN650407), displaying 100 % sequence similarity to S. propionica, was obtained from the propionate enrichment culture using primers designed in this study. To check for non-specific amplification, each primer set was tested against 28 non-target bacterial DNA with varying degrees of primer mismatches (data not shown). Each PCR

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mixture (50 µl) contained 100 nM of each primer, 0.2 mM dNTPs, 50 ng template DNA, 1 × Standard Taq Reaction Buffer (New England BioLabs) and 1.25U Taq Polymerase (New England BioLabs). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at either 55 °C (*Pelotomaculum*) or 60 °C (all others) for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were examined in 2 % agarose gels to confirm product presence and size.

To further verify primer set specificity, clone libraries were constructed for each genus using PCR products from DNA extracted from anaerobic biomass. PCR products were generated as described above and purified with an Ultra-Clean PCR Clean-Up kit (MO BIO). PCR products were cloned into pCR4-TOPO plasmid vector and transformed into One Shot TOP10 Chemically Competent *E. coli* cells using a TOPO TA Cloning kit according to the manufacturer's instructions (Invitrogen). Cells were spread onto LB agar plates containing ampicillin (50 µg ml − 1) and grown overnight at 37 °C. Positive transformants were randomly selected and colony PCR was performed with vector-specific primers PUC-F (5′-GTAAAACGACGGCCAG-3′) and PUC-R (5′-CAGGAAACAGCTATGAC-3′) (Invitrogen). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. For each genus, 47–50 clones with insert DNA were identified and further purified. The clones were sequenced at the DNA Sequencing and Genotyping Facility - University of Chicago Comprehensive Cancer Center (Chicago, IL). Taxonomic assignments (up to genus level) were performed for all 16S rRNA gene sequences using the Classifier function (bootstrap cut-off: 50 %) at the RDP (Wang et al., 2007). One hundred and ninety-three 16S rRNA gene sequences, representing four clone libraries, were deposited in the European Nucleotide Archive (see below).

Standard curve construction

Standard curves were constructed using 16S rRNA gene-based PCR products, derived from either pure culture DNA or environmental

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clones, using the genus-specific primers designed in this study. PCR amplification and cloning was performed as described above. Positive transformants were grown overnight at 37 °C in LB broth with ampicillin (100 µg ml − 1). Plasmids were purified with a Plasmid Mini-Prep kit according to the manufacturer's instructions (Qiagen) and quantified as described above. Plasmids were sequenced (as described above) to confirm the presence of the correct insert. Plasmid DNA was normalized to 10 10 copies per µl and diluted tenfold to obtain a dilution series ranging from 10 0 to 10 10 copies per µl. This dilution series was used to determine the linear dynamic range for each assay developed in this study.

Quantitative PCR

Quantitative PCR (qPCR), based on SYBR Green chemistry, was carried out in triplicate on a CFX Connect Real-Time PCR Detection System (Bio-Rad) according to the recommendations of Smith et al. (2006) and Smith & Osborn (2009) . Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009), as applicable to environmental samples, were followed while optimizing qPCR protocols. qPCRs were performed in triplicate in a reaction volume of 20 µl and the final mixture contained: 1 × iTaq Universal SYBR Green Supermix (Bio-Rad), 500 nM of each primer, 10 ng of template DNA and PCR-grade sterile water. Each qPCR run included a no-template control. Amplification was performed as a two-step cycling procedure: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and at 55 °C (*Pelotomaculum*) or 60 °C (all others) for 30 s. Melt-curve analysis was performed after each run to confirm reaction specificity. Baseline and threshold calculations were determined with CFX Manager software (Bio-Rad). Total Bacterial and Archaeal 16S rRNA gene copies were quantified using domain-specific primers (341F-518R and 915F-1059R, respectively) as described previously (Muyzer et al., 1993 ; Yu et al., 2005). In addition, methanogen-specific methyl coenzyme M reductase alpha-subunit, (mcrA), gene copies were quantified as described by Morris et al. (2014).

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Nucleotide sequence accession numbers

The 16S rRNA gene sequences reported in this study have been deposited in the EMBL database under accession numbers LN650256 to LN650448.

Results And Discussion

Primer design and in silico validation

Four genus-specific primer sets were designed (Table 1) based on 16S rRNA gene sequences retrieved from the RDP. In silico analysis using the RDP Probe Match function revealed that each primer set (SBC, SMI, PEL and SMS) targeted 91, 67, 84 and 83 % of all sequences (≥ 1200 bp) in the database within the genera *Syntrophobacter*, *Smithella*, *Pelotomaculum* and *Syntrophomonas*, respectively. Importantly, these primer sets displayed either comparable or greater coverage than genus-specific probes previously designed for hybridization-based studies: SYN835 (*Syntrophobacter*: 89 %; Scheid & Stubner, 2001), Synbac824 (*Syntrophobacter*: 90 %; Ariesyady et al., 2007a), GIh821m (*Pelotomaculum*: 86 %; Imachi et al., 2006), Synm700 (*Syntrophomonas*: 59 %; Hansen et al., 1999) and GSYM1240 (*Syntrophomonas*: 60 %; Narihiro et al., 2012). Additionally, all SFAB species type strains within target genera: *Syntrophobacter* (*S. fumaroxidans, S. pfennigii, S. sulfatireducens, S. wolinii*), *Smithella* (*S. propionica*), *Pelotomaculum* (*P. propionicum, P. schinkii, P. thermopropionicum*) and *Syntrophomonas* (*S. cellicola, S. erecta, S. palmitatica, S. sapovorans, S. wolfei, S. zehnderi*, except *S. curvata*) were detected only by using the respective primer sets. Primer set mismatches with all closely related non-target species type strains (within target family) are illustrated in Table S1 (available in the online Supplementary Material).

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Table 1. Characteristics of the16S rRNA gene-targeted qPCR primer sets designed in this study

Target genus	Primer*	Sequence† (5′–3′)	*E. coli* position	Tm (°C)	GC (%)	Coverage‡ (%)	Product size (bp)	Annealing temperature (°C)
Syntrophobacter	SBC-695F	ATTCGTAGAGATCGGGAGGAATACC	695–719	57.4	48.0	94.8	150	60
	SBC-844R	TGRKTACCCGCTACACCTAGTGMTC	820–844	60.6	54.0	94.0		
Smithella	SMI-732F	GRCTTTCTGGCCCDATACTGAC	732–753	57.2	53.8	86.4	100	60
	SMI-831R	CACCTAGTGAACATCGTTTACA	810–831	52.4	40.9	77.3		
Pelotomaculum	PEL-622F	CYSDBRGMSTRCCTBWGAAACYG	622–644	60.0	57.2	96.2	257	55
	PEL-877R	GGTGCTTATTGYGTTARCTAC	857–877	51.5	42.9	87.2		
Syntrophomonas	SMS-637F	TGAAACTGDDDDTCTTGAGGGCAG	637–660	57.8	47.2	89.2	121	60
	SMS-757R	CAGCGTCAGGGDCAGTCCAGDMA	735–757	63.4	61.6	93.6		

* F, Forward primer; R, reverse primer.
† R = A/G, K = G/T, M = A/C, D = A/G/T, Y = C/T, S = G/C, B = C/G/T, W = A/T.
‡ Ratio (%) of number of sequence hits within target group to the total number of target sequences.

Experimental validation

Primer set specificity was experimentally verified using DNA extracts or environmental clones representing 34 bacterial species. PCR products of expected size (SBC: 150 bp, SMI: 100 bp, PEL: 257 bp, SMS: 121 bp) were obtained from all target DNA (Fig. S1a), whereas no amplification was observed with non-target DNA (data not shown). To further confirm primer set specificity, four clone libraries (47–50 clones per genus) were constructed from DNA extracted from anaerobic biomass using the genus-specific primers designed in this study. Classifier function (RDP) designated 100, 93, 98 and 52 % of the clones as *Syntrophobacter*, *Smithella*, *Pelotomaculum* and *Syntrophomonas*, respectively. The remaining clones were below the recommended confidence threshold (bootstrap cut-off: 50 %). Though all SMS-specific clones were placed within the target family, only 52 % of the total clones could be classified down to the genus level. In silico analysis using pre-classified SMS-specific 16S rRNA gene sequences, retrieved from the RDP, revealed that the SMS-specific primers amplified a 121 bp region (*E. coli* positions 637–757) that exhibited low

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taxonomic resolution, which thereby did not allow accurate classification beyond the family level.

Standard curves

Standard curves, constructed from a series of tenfold plasmid DNA dilutions, displayed a linear dynamic range spanning eight orders of magnitude (10 9 to 10 2 copies) and a lower detection limit of 100 copies per reaction (Table 2 ; Fig. S2). The regression coefficient (R 2) of each standard curve was always above 0.99. High cycle threshold (C T) values were observed for no-template controls. Melt-curve analysis displayed a single observable peak for each genus (SBC: 82 °C, SMI: 79.5 °C, PEL: 84.5 °C and SMS: 81.5 °C) (Fig. S1b). Peaks indicative of non-specific amplification were not observed.

Table 2. Characteristics of the standard curves constructed in this study

Assay	Target genus	Linear range (copies µl− 1)	Slope	Efficiency (%)	R2	y-intercept	Clone used as standard (GenBank/EMBL accession no.)
SBC	*Syntrophobacter*	102–109	− 3.177	106.4	0.999	37.083	*S. fumaroxidans* (X82874)
SMI	*Smithella*	102–109	− 3.217	104.6	0.997	37.518	Clone SMI06 (LN650407)
PEL	*Pelotomaculum*	102–109	− 3.362	98.3	0.999	39.245	*P. thermopropionicum* (AB035723)
SMS	*Syntrophomonas*	102–109	− 3.301	100.9	0.998	39.414	*S. wolfei* (M26492)

Quantification of microbial communities

The novel qPCR assays were applied to quantify 16S rRNA gene copies of SFAB in biomass samples obtained from a variety of mesophilic methanogenic habitats. Biomass samples were determined to be methanogenic based upon the demonstration of methane production when fed propionate (SMA test; data not shown) and/or detection of the mcrA gene, which encodes the alpha-subunit of 'methyl coenzyme M reductase' an enzyme that catalyses the terminal step in methanogenesis (Fig. 1). Using the new primer sets, each SFAB genus was detected in all samples, though their abundance (Fig. 2) varied by up to four orders of magnitude. In general, total SFAB were at least an order of magnitude more abundant in anaerobic reactor samples (10 5–10 6 16S rRNA gene copies ng − 1 DNA) when compared to samples obtained from natural environments (10 2–10 4

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gene copies ng − 1 DNA) (Figs. 2 and 3). These results are in agreement with previous high-throughput sequencing- (Sundberg et al., 2013) and hybridization-based studies (Harmsen et al., 1996 ; Hansen et al., 1999 ; McMahon et al., 2004 ; Ariesyady et al., 2007a ; Narihiro et al., 2012 that estimated SFAB to generally constitute only a fraction (< 2 %) of the total microbial community in anaerobic digesters. When viewed in total, the data from this and the previous studies suggest that SFAB constitute a 'keystone' guild, i.e. organisms whose impact on community structure and function is far greater than suggested by their abundance (Power et al., 1996). A loss of SFAB function, i.e. VFA degradation, would lower pH and negatively impact the entire microbial consortia and could trigger system collapse. Moreover, Tale et al. (2011) reported enhanced recovery of upset digesters when augmented with a propionate enrichment culture, which in this study was shown to contain high numbers of known syntrophic propionate-degraders (see Fig. 4 ; T = 2.5 years).

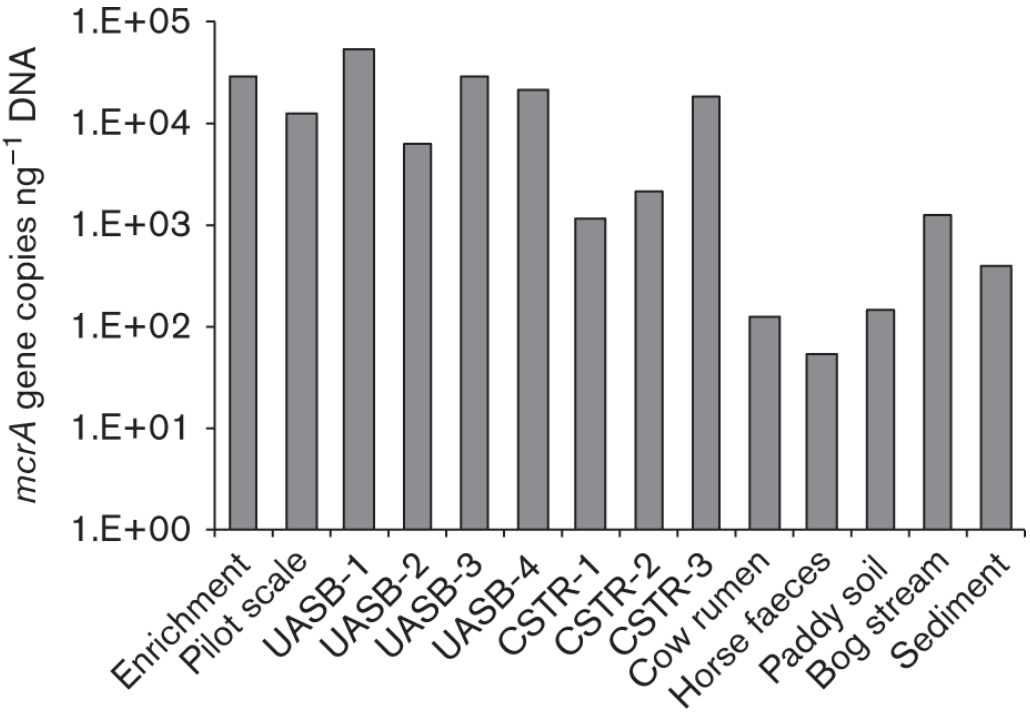

Fig. 1. Mean mcrA gene copies ng − 1 DNA in biomass samples from different methanogenic environments. Enrichment: 6 years post-start-up. UASB, upflow anaerobic sludge blanket reactor; CSTR, continuously stirred-tank reactor. UASB-1, soft-drink bottling waste; UASB-2, food flavouring waste; UASB-3 and -4, brewery waste; CSTR-1 and -2, municipal waste; CSTR-3, cheese processing waste. Standard error was less than 10 % with triplicates of each sample.

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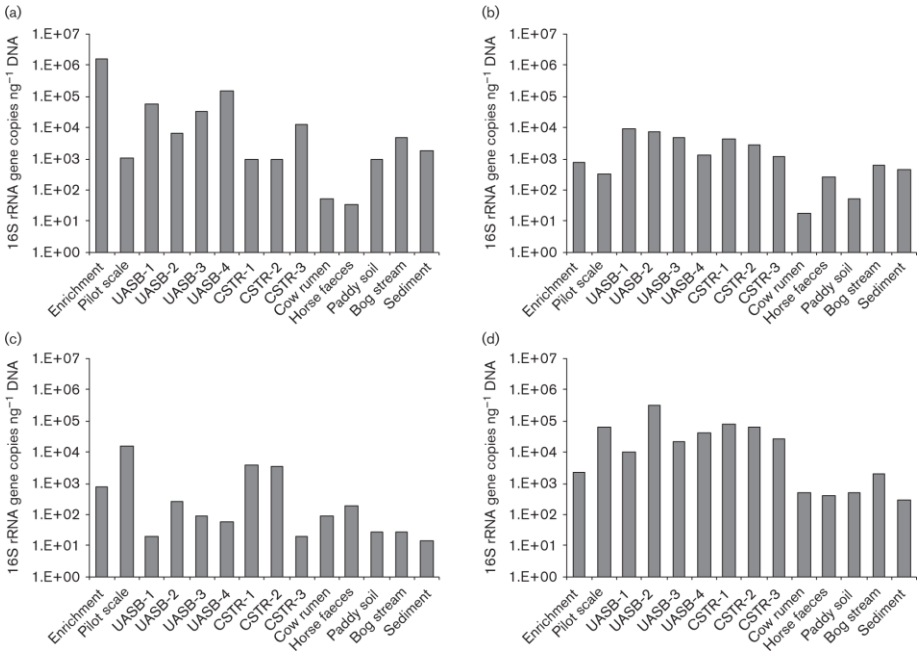

Fig. 2. Quantification of syntrophic fatty acid degraders (a) *Syntrophobacter*, (b) *Smithella*, (c) *Pelotomaculum* and (d) *Syntrophomonas*, in biomass samples from different methanogenic environments showing mean number of 16S rRNA gene copies ng − 1 DNA. Enrichment: 6 years post-start-up. UASB, upflow anaerobic sludge blanket reactor; CSTR, continuously stirred-tank reactor. UASB-1, soft-drink bottling waste; UASB-2, food flavouring waste; UASB-3 and -4, brewery waste; CSTR-1 and -2, municipal waste; CSTR-3, cheese processing waste. Standard error was less than 10 % with triplicates of each sample.

	Archaea	SBC	SMI	PEL	SMS
Enrichment	17.517	50.920	0.023	0.025	0.067
UASB-1	7.575	0.877	0.141	0.000	0.162
UASB-3	5.909	0.720	0.102	0.002	0.475
UASB-4	4.649	2.460	0.022	0.001	0.668
Pilot scale	3.955	0.016	0.005	0.235	0.954
UASB-2	3.758	0.147	0.163	0.006	7.017
Bog stream	1.984	0.155	0.021	0.001	0.071
Sediment	1.829	0.194	0.045	0.001	0.031
CSTR-2	1.634	0.029	0.080	0.103	1.895
CSTR-3	1.526	0.174	0.017	0.000	0.370
CSTR-1	0.773	0.017	0.076	0.065	1.320
Paddy soil	0.206	0.033	0.002	0.001	0.017
Cow rumen	0.099	0.001	0.000	0.001	0.007
Horse faeces	0.098	0.001	0.009	0.007	0.014

Fig. 3. Heat map displaying relative abundance (%) of various microbial groups in biomass samples from different methanogenic environments. Enrichment: 6 years post-start-up. Relative abundance = [Target group abundance/(Bacteria+Archaea abundance)] × 100. SBC, *Syntrophobacter*; SMI, *Smithella*; PEL, *Pelotomaculum*; SMS, *Syntrophomonas*. Samples are ordered according to archaeal relative abundance.

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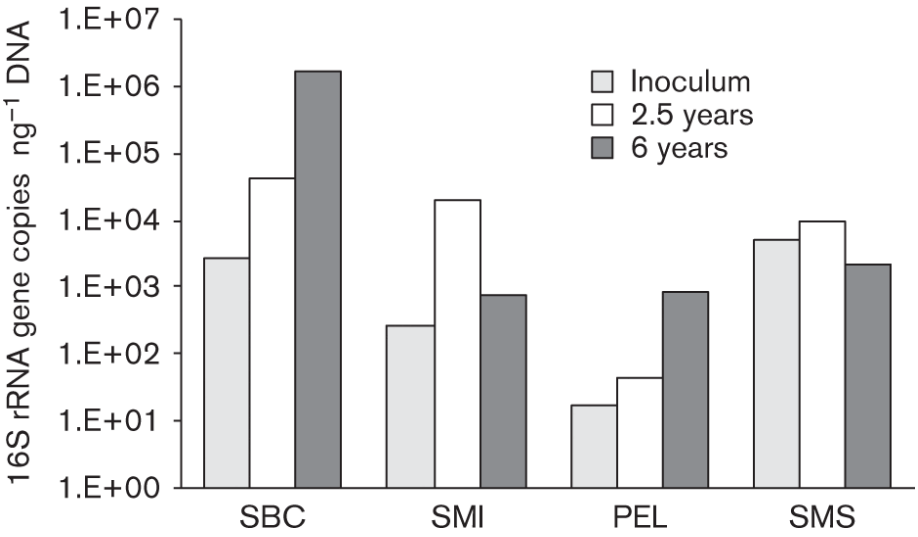

Fig. 4. Effect of the addition of propionate on the abundance of syntrophic fatty acid degraders in a long-term enrichment culture. SBC, *Syntrophobacter*; SMI, *Smithella*; PEL, *Pelotomaculum*; SMS, *Syntrophomonas*. Standard error was less than 10 % for within triplicates each sample.

Engineered environments

Amongst the full-scale reactor samples, reactor configuration and substrate identity appeared to influence SFAB and methanogen abundance. UASB reactors harboured at least tenfold more propionate-degraders than CSTR digesters (Figs 2 and 3). This result could be because UASB configuration promotes granule formation that brings SFAB and methanogens within close physical proximity, thereby facilitating efficient fatty acid degradation (Schink & Thauer, 1988). It is also noteworthy that the majority of currently identified SFAB have been isolated from full-scale UASB reactors (Stams et al., 2012). Interestingly, in municipal reactors, numbers of *Syntrophobacter* were reduced while those of *Pelotomaculum* were increased when compared with industrial reactors (Fig. 2). In addition, municipal reactors also displayed the lowest abundance of methanogens (Fig. 1) amongst all full-scale reactors. Differences in waste composition and nutrient levels may explain these observations. Industrial sludge samples have been reported to display higher methane production rates against propionate than those obtained from municipal sludge (Tale et al., 2011).

An analysis of the enrichment culture over time revealed a 20- and 534-fold increase in the abundance of total propionate-degraders

(*Syntrophobacter*+ *Smithella*+ *Pelotomaculum*), at 2.5 and 6 years post-start-up, respectively, when compared to the seed inoculum (Fig. 4). The increase in substrate concentration from 0.25 to 1.04 g COD l^{-1} day^{-1} resulted in a 27-fold increase in the abundance of total propionate-degraders. The abundance of *Syntrophobacter* and *Pelotomaculum* increased 41- and 18-fold, respectively, while that of *Smithella* decreased 28-fold in the culture after 6 years when compared with the enrichment at 2.5 years (Fig. 4). After 6 years, *Syntrophobacter* dominated the microbial community with 51 % of the total 16S rRNA gene sequences detected (Fig. 3). This result is comparable to those from a recent high-throughput sequencing study where *Syntrophobacter* accounted for up to 88 % and 52 % of the total bacterial 16S rRNA gene sequences in propionate enrichment cultures seeded sludge and swine manure, respectively (Narihiro et al., 2015). The presence of *Syntrophomonas*, a butyrate-degrader, in the propionate enrichment culture may be due to the presence of *Smithella*, which utilizes a non-randomizing pathway where propionate is first dismutated to acetate and butyrate. The butyrate then becomes available to *Syntrophomonas*, which syntrophically metabolizes it to acetate via β-oxidation (de Bok et al., 2001). Previously, stable isotope probing-based studies, using 13C-labelled propionate, identified that *Syntrophomonas*, in addition to *Syntrophobacter*, *Smithella*, and *Pelotomaculum*, was enriched in the 'heavy' 13C-labelled DNA fractions (Lueders et al., 2004 ; Chauhan & Ogram, 2006 ; Gan et al., 2012), supporting the presence of these bacteria in the propionate enrichment.

Previous studies, in agreement with our findings, have reported differences in the structure of propionate-degrading bacterial communities in (a) anaerobic sludge samples incubated at different propionate concentrations (Ariesyady et al., 2007b) and (b) propionate-fed chemostats maintained at different HRTs (Shigematsu et al., 2006). It has been suggested that the coexistence of phylogenetically diverse but functionally redundant microbial communities (i.e. parallel substrate processing) is essential to maintain a stable ecosystem function under fluctuating environmental conditions (Fernandez et al., 2000 ; Hashsham et al., 2000). These conditions are frequently observed in full-scale digesters where perturbations such as substrate overload often result in VFA accumulation. Hence, as observed within acetoclastic methanogens

(Yu et al., 2006), it is plausible that differences in growth rates and substrate affinities within members of these microbial groups help maintain low propionate concentrations.

Natural environments

Within natural samples, the highest numbers of SFAB were observed in the swamp sediment and bog samples (Figs. 2 and 3). Previous studies have reported syntrophic fatty acid degradation in freshwater sediments (Lovley & Klug, 1982 ; Scholten & Stams, 1995) and wetlands (Chauhan et al., 2004 ; Juottonen et al., 2005). In contrast, relatively lower numbers of SFAB were detected in the experimental rice paddy soil (Figs. 2 and 3). This result was unexpected because high propionate turnover rates have been reported in anoxic paddy field soil (Krylova et al., 1997 ; Glissmann & Conrad, 2000). This anomaly could be attributed to the fact that soil samples analysed in this study were obtained from an open experimental flooded rice plot maintained in a temperate region. Amongst all the samples analysed, the lowest abundance of SFAB was detected in cow rumen and horse faeces (Figs. 2 and 3). These animals use microbes to ferment cellulose to VFA, the cow in the rumen (Russell & Hespell, 1981) and the monogastric horse in its hindgut (Mackie & Wilkins, 1988). Results from the current study may not be unusual, because both animals absorb VFA via their intestinal epithelium as a major source of energy, and these acids would, therefore, not be as readily available to support SFAB growth (Bergman, 1990). Moreover, it has been suggested that SFAB, with long generation times, cannot maintain stable populations in habitats (e.g. cow rumen) that have short retention times (McInerney et al., 1981).

Conclusions

This study provides a suite of validated assays that were successfully used to quantify SFAB in biomass samples obtained from a variety of methanogenic habitats. To the best of our knowledge, this is the first qPCR-based study to detect SFAB at the genus level, and the first, using a targeted approach, to quantify these bacteria in natural environments. Our data confirm that SFAB constitute only a

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fraction of the total microbial community, and that anaerobic reactors harbour higher numbers of SFAB when compared with natural methanogenic habitats. In addition, within full-scale reactors, we report that SFAB and methanogen abundance varied with reactor configuration and substrate identity. Future studies must be performed to understand how different anaerobic digester process parameters (e.g. substrate composition, temperature, retention time and organic loading rate) affect SFAB and methanogen community dynamics. A better understanding of syntrophic microbial communities will help optimize digester technologies for enhanced biogas production and efficient waste treatment.

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