

6-1-2015

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

Prince Peter Mathai
Marquette University

Daniel Zitomer
Marquette University, daniel.zitomer@marquette.edu

James Maki
Marquette University, james.maki@marquette.edu

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

Prince P. Mathai

*Department of Biological Sciences, Marquette University,
Milwaukee, WI*

Daniel H. Zitomer

*Department of Civil, Construction and Environmental
Engineering, Water Quality Center, Marquette University,
Milwaukee, WI,*

James S. Maki

*Department of Biological Sciences, Marquette University,
Milwaukee, WI*

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

proportion of the total methane produced (Gujer & Zehnder, 1983). However, fatty acid degradation is highly endergonic under standard conditions (propionate: $\Delta G^{\circ} = +72\text{kJ}$; butyrate: $\Delta G^{\circ} = +48\text{kJ}$) (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. sapnoida*, *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

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 ; McMahan 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.

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 I – 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 I – 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 I – 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

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 10mM 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 (≥ 1200bp) 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: 75–300bp) 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

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 1.25 U 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

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 10¹⁰ copies per µl and diluted tenfold to obtain a dilution series ranging from 10⁰ to 10¹⁰ 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), 500nM of each primer, 10ng 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 3min, followed by 40 cycles at 95 °C for 10s and at 55 °C (*Pelotomaculum*) or 60 °C (all others) for 30s. 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).

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).

Table 1. Characteristics of the 16S rRNA gene-targeted qPCR primer sets designed in this study

Target genus	Primer*	Sequence† (5'-3')	<i>E. coli</i> position	Tm (°C)	GC (%)	Coverage‡ (%)	Product size (bp)	Annealing temperature (°C)
<i>Syntrophobacter</i>	SBC-695F	ATTCGTAGAGATCGGGAG GAATACC	695–719	57.4	48.0	94.8	150	60
	SBC-844R	TGRKTACCCGCTACACCT AGTGMTC	820–844	60.6	54.0	94.0		
<i>Smithella</i>	SMI-732F	GRCTTTCTGGCCCDATAC TGAC	732–753	57.2	53.8	86.4	100	60
	SMI-831R	CACCTAGTGAACATCGTT TACA	810–831	52.4	40.9	77.3		
<i>Pelotomaculum</i>	PEL-622F	CYSDBRGMSTRCCTBWGA AACYG	622–644	60.0	57.2	96.2	257	55
	PEL-877R	GGTGCTTATTGYGTTARCT AC	857–877	51.5	42.9	87.2		
<i>Syntrophomonas</i>	SMS-637F	TGAAACTGDDDDTCTTGA GGGCAG	637–660	57.8	47.2	89.2	121	60
	SMS-757R	CAGCGTCAGGGDCAGTCC AGDMA	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: 150bp, SMI: 100bp, PEL: 257bp, SMS: 121bp) 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 121bp region (*E. coli* positions 637–757) that exhibited low

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 (%)	R ²	y-intercept	Clone used as standard (GenBank/EMBL accession no.)
SBC	<i>Syntrophobacter</i>	102–109	– 3.177	106.4	0.999	37.083	<i>S. fumaroxidans</i> (X82874)
SMI	<i>Smithella</i>	102–109	– 3.217	104.6	0.997	37.518	Clone SMI06 (LN650407)
PEL	<i>Pelotomaculum</i>	102–109	– 3.362	98.3	0.999	39.245	<i>P. thermopropionicum</i> (AB035723)
SMS	<i>Syntrophomonas</i>	102–109	– 3.301	100.9	0.998	39.414	<i>S. wolfei</i> (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

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).

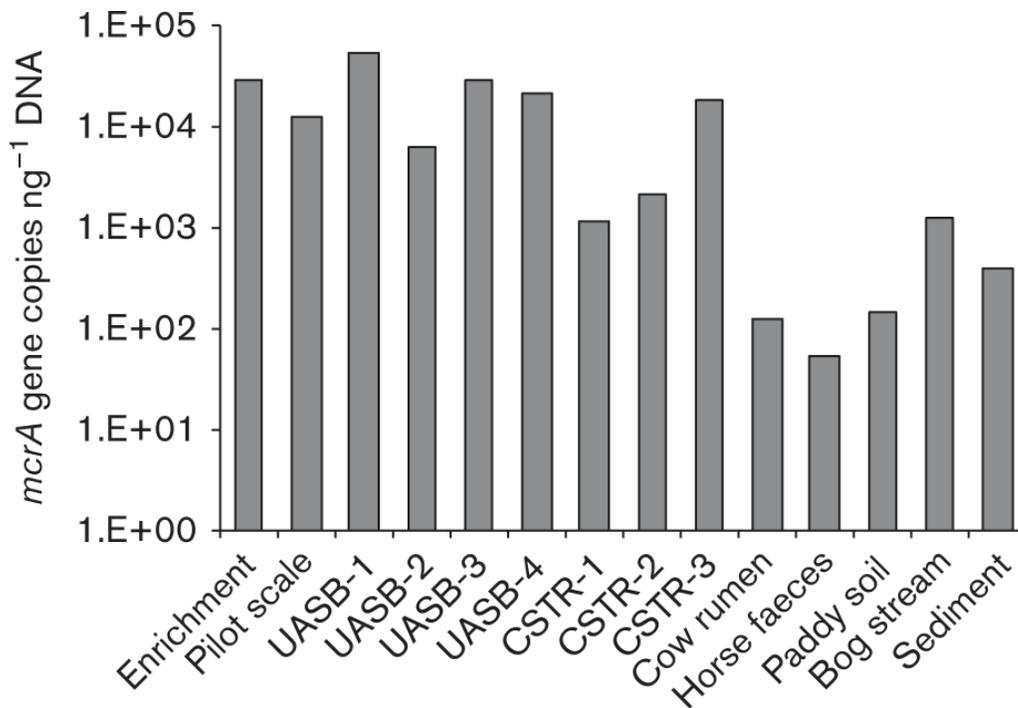


Fig. 1. Mean *mcrA* gene copies ng – 1 DNA in biomass samples from different methanogenic environments. Enrichment: 6 years post-start-up. UASB, upflow anaerobic sludge blanket reactor; CSTR, continuously stirred-tank reactor. UASB-1, soft-drink bottling waste; UASB-2, food flavouring waste; UASB-3 and -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.

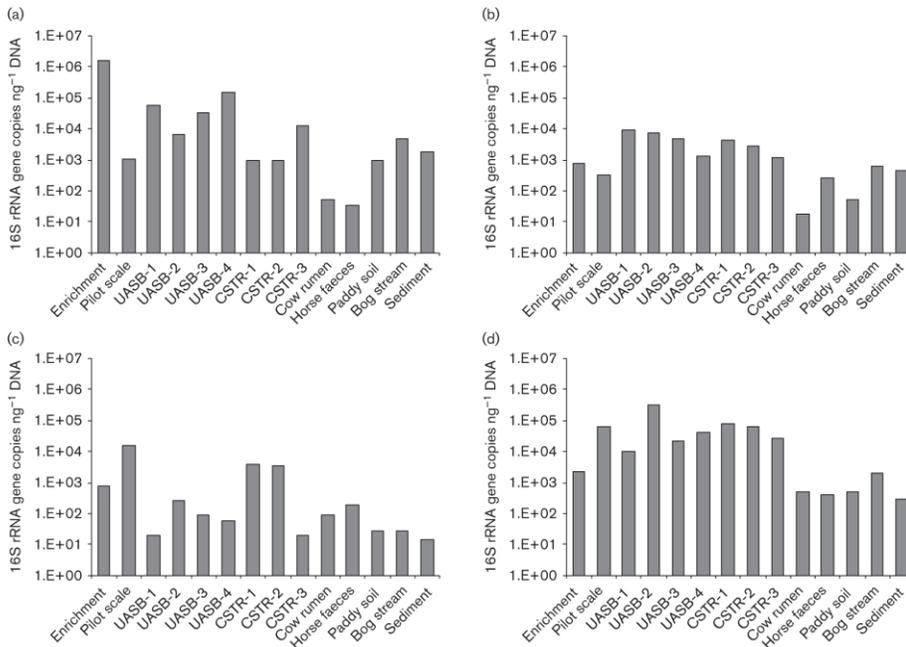


Fig. 2. Quantification of syntrophic fatty acid degraders (a) *Syntrophobacter*, (b) *Smithella*, (c) *Pelotomaculum* and (d) *Syntrophomonas*, in biomass samples from different methanogenic environments showing mean number of 16S rRNA gene copies ng⁻¹ DNA. Enrichment: 6 years post-start-up. UASB, upflow 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.

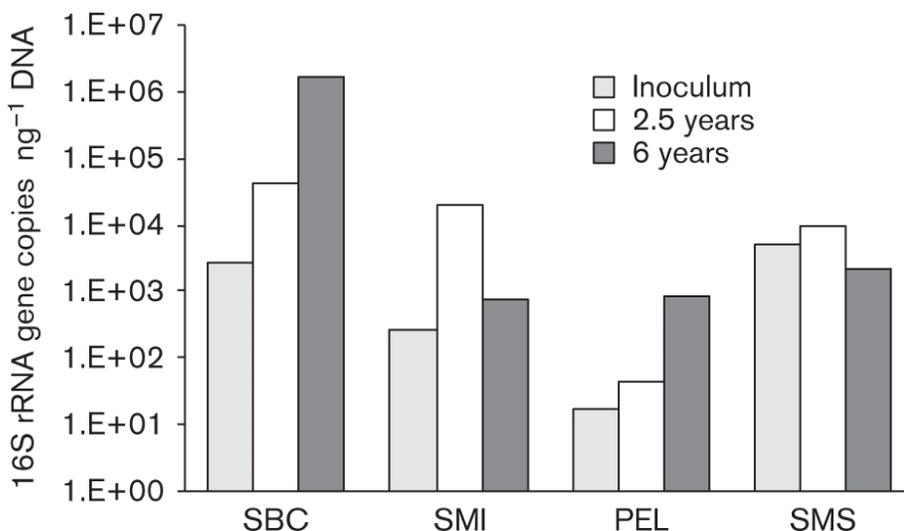


Fig. 4. Effect of the addition of propionate on the abundance of syntrophic fatty acid degraders in a long-term enrichment culture. SBC, *Syntrophobacter*; SMI, *Smithella*; PEL, *Pelotomaculum*; SMS, *Syntrophomonas*. Standard error was less than 10 % for within triplicates each sample.

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 1.04g 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

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.

Acknowledgements

The authors thank Rachel Morris (Michigan State University, USA), Michael Schläppi, Keerthi Cherukuri, Ben Bocher and Mike Dollhopf (Marquette University, USA) for their assistance, the University of Wisconsin-Milwaukee Field Station (USA) and operators of full-scale reactors for providing, or allowing the collection of, samples. The authors are also grateful to Caroline Plugge (Wageningen University, Netherlands) and Michael McInerney (University of Oklahoma, USA) for providing us with genomic DNA of *S. fumaroxidans* and *S. wolfei*, respectively. This work was funded in part by WE Energies and Marquette University.

References

- Ariesyady H. D., Ito T., Okabe S. (2007a). Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Water Res* 41, 1554–1568.
- Ariesyady H. D., Ito T., Yoshiguchi K., Okabe S. (2007b). Phylogenetic and functional diversity of propionate-oxidizing bacteria in an anaerobic digester sludge. *Appl Microbiol Biotechnol* 75, 673–683.
- Bergman E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol Rev* 70, 567–590.
- Bouvier T., del Giorgio P. A. (2003). Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): A quantitative review of published reports. *FEMS Microbiol Ecol* 44, 3–15.
- Bustin S. A., Benes V., Garson J. A., Hellems J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M. W., other authors (2009). The MIQE

- guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55, 611–622.
- Chauhan A., Ogram A. (2006). Fatty acid-oxidizing consortia along a nutrient gradient in the Florida Everglades. *Appl Environ Microbiol* 72, 2400–2406.
- Chauhan A., Ogram A., Reddy K. R. (2004). Syntrophic-methanogenic associations along a nutrient gradient in the Florida Everglades. *Appl Environ Microbiol* 70, 3475–3484.
- Cole J. R., Wang Q., Fish J. A., Chai B., McGarrell D. M., Sun Y., Brown C. T., Porras-Alfaro A., Kuske C. R., Tiedje J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* (41), D633–D642.
- de Bok F. A. M., Stams A. J. M., Dijkema C., Boone D. R. (2001). Pathway of propionate oxidation by a syntrophic culture of *Smithella propionica* and *Methanospirillum hungatei*. *Appl Environ Microbiol* 67, 1800–1804.
- Fernandez A. S., Hashsham S. A., Dollhopf S. L., Raskin L., Glagoleva O., Dazzo F. B., Hickey R. F., Criddle C. S., Tiedje J. M. (2000). Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. *Appl Environ Microbiol* 66, 4058–4067.
- Gan Y., Qiu Q., Liu P., Rui J., Lu Y. (2012). Syntrophic oxidation of propionate in rice field soil at 15 and 30 °C under methanogenic conditions. *Appl Environ Microbiol* 78, 4923–4932.
- Glissmann K., Conrad R. (2000). Fermentation pattern of methanogenic degradation of rice straw in anoxic paddy soil. *FEMS Microbiol Ecol* 31, 117–126.
- Gujer W., Zehnder A. J. B. (1983). Conversion processes in anaerobic digestion. *Water Sci Technol* 15, 127–167.
- Hansen K. H., Ahring B. K., Raskin L. (1999). Quantification of syntrophic fatty acid-beta-oxidizing bacteria in a mesophilic biogas reactor by oligonucleotide probe hybridization. *Appl Environ Microbiol* 65, 4767–4774.
- Harmsen H. J. M., Kengen H. M. P., Akkermans A. D. L., Stams A. J. M. (1995). Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichments cultures. *Syst Appl Microbiol* 18, 67–73.
- Harmsen H. J., Akkermans A. D., Stams A. J., de Vos W. M. (1996). Population dynamics of propionate-oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. *Appl Environ Microbiol* 62, 2163–2168.
- Hashsham S. A., Fernandez A. S., Dollhopf S. L., Dazzo F. B., Hickey R. F., Tiedje J. M., Criddle C. S. (2000). Parallel processing of substrate correlates with greater functional stability in methanogenic bioreactor

- communities perturbed by glucose. *Appl Environ Microbiol* 66, 4050–4057.
- Hatamoto M., Imachi H., Ohashi A., Harada H. (2007). Identification and cultivation of anaerobic, syntrophic long-chain fatty acid-degrading microbes from mesophilic and thermophilic methanogenic sludges. *Appl Environ Microbiol* 73, 1332–1340.
- Hori T., Haruta S., Ueno Y., Ishii M., Igarashi Y. (2006). Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. *Appl Environ Microbiol* 72, 1623–1630.
- Imachi H., Sekiguchi Y., Kamagata Y., Loy A., Qiu Y. L., Hugenholtz P., Kimura N., Wagner M., Ohashi A., Harada H. (2006). Non-sulfate-reducing, syntrophic bacteria affiliated with desulfotomaculum cluster I are widely distributed in methanogenic environments. *Appl Environ Microbiol* 72, 2080–2091.
- Ito T., Yoshiguchi K., Ariesyady H. D., 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.
- Juottonen H., Galand P. E., Tuittila E. S., Laine J., Fritze H., Yrjälä K. (2005). Methanogen communities and Bacteria along an ecohydrological gradient in a northern raised bog complex. *Environ Microbiol* 7, 1547–1557.
- Krylova N. I., Janssen P. H., Conrad R. (1997). Turnover of propionate in methanogenic paddy soil. *FEMS Microbiol Ecol* 23, 107–117.
- Larkin M. A., Blackshields G., Brown N. P., Chenna R., McGettigan P. A., McWilliam H., Valentin F., Wallace I. M., Wilm A., other authors (2007). clustal w and clustal_x version 2.0. *Bioinformatics* 23, 2947–2948.
- Liu P., Qiu Q., Lu Y. (2011). Syntrophomonadaceae-affiliated species as active butyrate-utilizing syntrophs in paddy field soil. *Appl Environ Microbiol* 77, 3884–3887.
- Lovley D. R., Klug M. J. (1982). Intermediary metabolism of organic matter in the sediments of a eutrophic lake. *Appl Environ Microbiol* 43, 552–560.
- Lueders T., Pommerenke B., Friedrich M. W. (2004). Stable-isotope probing of micro-organisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. *Appl Environ Microbiol* 70, 5778–5786.
- Mackie R. I., Wilkins C. A. (1988). Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract. *Appl Environ Microbiol* 54, 2155–2160.
- McCarty P. L., Smith D. P. (1986). Anaerobic wastewater treatment. *Environ Sci Technol* 20, 1200–1206.

- McInerney M. J., Mackie R. I., Bryant M. P. (1981). Syntrophic association of a butyrate-degrading bacterium and methanosarcina enriched from bovine rumen fluid. *Appl Environ Microbiol* 41, 826–828.
- McInerney M. J., Struchtemeyer C. G., Sieber J., Mouttaki H., Stams A. J., Schink B., Rohlin L., Gunsalus R. P. (2008). Physiology, ecology, phylogeny, and genomics of micro-organisms capable of syntrophic metabolism. *Ann N Y Acad Sci* 1125, 58–72.
- McMahon K. D., Zheng D., Stams A. J., Mackie R. I., Raskin L. (2004). Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. *Biotechnol Bioeng* 87, 823–834.
- Morris R., Schauer-Gimenez A., Bhattad U., Kearney C., Struble C. A., Zitomer D., Maki J. S. (2014). Methyl coenzyme M reductase (mcrA) gene abundance correlates with activity measurements of methanogenic H₂/CO₂-enriched anaerobic biomass. *Microb Biotechnol* 7, 77–84.
- Muyzer G., de Waal E. C., Uitterlinden A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59, 695–700.
- Narihiro T., Terada T., Ohashi A., Kamagata Y., Nakamura K., 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.
- Narihiro T., Nobu M. K., Kim N. K., Kamagata Y., Liu W. T. (2015). The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. *Environ Microbiol* 17, 1707–1720.
- Power M. E., Tilman D., Estes J. A., Menge B. A., Bond W. J., Mills L. S., Daily G., Castilla J. C., Lubchenco J., Paine R. (1996). Challenges in the quest for keystones. *Bioscience* 46, 609–620.
- Russell J. B., Hespell R. B. (1981). Microbial rumen fermentation. *J Dairy Sci* 64, 1153–1169.
- Schauer-Gimenez A. E., Zitomer D. H., Maki J. S., Struble C. A. (2010). Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. *Water Res* 44, 3555–3564.
- Scheid D., Stubner S. (2001). Structure and diversity of Gram-negative sulfate-reducing bacteria on rice roots. *FEMS Microbiol Ecol* 36, 175–183.
- Schink B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 61, 262–280.
- Schink B., Stams A. J. M. (2002). Syntrophism among Prokaryotes. *The Prokaryotes* 2, 309–335.

- Schink B., Thauer R. K. (1988). Energetics of syntrophic methane formation and the influence of aggregation. In *Granular Anaerobic Sludge, Microbiology and Technology*, pp. 5–17. Edited by Lettinga G., Zehnder A. J. B., Grotenhuis J. T. C., Hulshoff-Pol L. W.. Wageningen, The Netherlands: Pudoc.
- Scholten J. C., Stams A. J. (1995). The effect of sulfate and nitrate on methane formation in a freshwater sediment. *Antonie van Leeuwenhoek* 68, 309–315.
- Shigematsu T., Era S., Mizuno Y., Ninomiya K., Kamegawa Y., Morimura S., Kida K. (2006). Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. *Appl Microbiol Biotechnol* 72, 401–415.
- Smith C. J., Osborn A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol* 67, 6–20.
- Smith C. J., Nedwell D. B., Dong L. F., Osborn A. M. (2006). Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ Microbiol* 8, 804–815.
- Sorensen A. H., Ahring B. K. (1993). Measurements of the specific methanogenic activity of anaerobic digester biomass. *Appl Microbiol Biotechnol* 40, 427–443.
- Sousa D. Z., Pereira M. A., Stams A. J., Alves M. M., Smidt H. (2007). Microbial communities involved in anaerobic degradation of unsaturated or saturated long-chain fatty acids. *Appl Environ Microbiol* 73, 1054–1064.
- Stams A. J., Sousa D. Z., Kleerebezem R., Plugge C. M. (2012). Role of syntrophic microbial communities in high-rate methanogenic bioreactors. *Water Sci Technol* 66, 352–362.
- Sundberg C., Al-Soud W. A., Larsson M., Alm E., Yekta S. S., Svensson B. H., Sørensen S. J., Karlsson A. (2013). 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol* 85, 612–626.
- Tale V. P., Maki J. S., Struble C. A., Zitomer D. H. (2011). Methanogen community structure-activity relationship and bioaugmentation of overloaded anaerobic digesters. *Water Res* 45, 5249–5256.
- Tang Y. Q., Shigematsu T., Morimura S., 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., Decker K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 41, 100–180.

- Wang Q., Garrity G. M., Tiedje J. M., Cole J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73, 5261–5267.
- Yu Y., Lee C., Kim J., Hwang S. (2005). Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* 89, 670–679.
- Yu Y., Kim J., Hwang S. (2006). Use of real-time PCR for group-specific quantification of aceticlastic methanogens in anaerobic processes: population dynamics and community structures. *Biotechnol Bioeng* 93, 424–433.