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Research Article

RESEARCH ARTICLE

# Monitoring the dynamics of syntrophic β-oxidizing bacteria during anaerobic degradation of oleic acid by quantitative PCR

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One sentence summary: The development and application of quantitative PCR assays targeting long-chain fatty acid (LCFA)-degrading syntrophic bacteria in anaerobic bioreactors indicated that higher rates of LCFA mineralization correlated with syntrophic bacteria abundance.
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## ABSTRACT

The ecophysiology of long-chain fatty acid-degrading syntrophic β-oxidizing bacteria has been poorly understood due to a lack of quantitative abundance data. Here, TaqMan quantitative PCR (qPCR) assays targeting the 16S rRNA gene of the known mesophilic syntrophic β-oxidizing bacterial genera *Syntrophomonas* and *Syntrophus* were developed and validated. Microbial community dynamics were followed using qPCR and Illumina-based high-throughput amplicon sequencing in triplicate methanogenic bioreactors subjected to five consecutive batch feedings of oleic acid. With repeated oleic acid feeding, the initial specific methane production rate significantly increased along with the relative abundances of *Syntrophomonas* and methanogenic archaea in the bioreactor communities. The novel qPCR assays showed that *Syntrophomonas* increased from 7 to 31% of the bacterial community 16S rRNA gene concentration, whereas that of *Syntrophus* decreased from 0.02 to less than 0.005%. High-throughput amplicon sequencing also revealed that *Syntrophomonas* became the dominant genus within the bioreactor microbiomes. These results suggest that increased specific mineralization rates of oleic acid were attributed to quantitative shifts within the microbial communities toward higher abundances of syntrophic β-oxidizing bacteria and methanogenic archaea. The novel qPCR assays targeting syntrophic β-oxidizing bacteria may thus serve as monitoring tools to indicate the fatty acid β-oxidization potential of anaerobic digester communities.

**Keywords:** syntrophy; β-oxidization; anaerobic digestion; quantitative PCR (qPCR); high-throughput sequencing; *Syntrophomonas*; *Syntrophus*; methanogenesis; long-chain fatty acids (LCFA)

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## INTRODUCTION

Anaerobic digestion (AD) is an attractive biotechnology used to treat organic wastes and produce renewable energy as methane. Co-digesting energy-rich wastes with municipal sludge or manure has gained increased interest due to observed improvements in bioreactor methane production (Davidsson *et al.* 2008; Luostarinen, Luste and Sillanpää 2009; Wang, Aziz and de los Reyes 2013). Lipid-rich wastes are desirable co-substrates for AD due to their relatively high specific methane yield (Cavaleiro, Pereira and Alves 2008; Palatsi *et al.* 2010). However, the long-chain fatty acids (LCFA) produced during lipid hydrolysis have been shown to inhibit anaerobic microorganisms (Hanaki, Matsuo and Nagase 1981; Koster and Cramer 1987; Angelidaki and Ahring 1992; Rinzema *et al.* 1994; Lalman and Bagley 2000, 2002). Achieving efficient degradation of LCFA by anaerobic microorganisms is therefore critical to obtain stable and elevated methane production during the AD of lipid-rich wastes.

Under anaerobic conditions, LCFA are primarily degraded through $\beta$-oxidation to yield hydrogen and acetate (Weng and Jeris 1976). Methane formation from LCFA occurs through a syntrophic partnership between LCFA $\beta$-oxidizing acetogenic bacteria and methanogenic archaea, which are responsible for maintaining low concentrations of acetate and hydrogen (Schink 1997; Sousa *et al.* 2009). Currently, all of the bacterial species that have been characterized to grow on LCFA in syntrophic partnership with methanogens belong to the families of *Syntrophomonadaceae* within the class of *Clostridia* (McInerney 1992; Zhao *et al.* 1993; Hatamoto *et al.* 2007a; Sousa *et al.* 2007b, 2009; Wu, Dong and Liu 2007), or *Syntrophaceae* within the subclass of *Deltaproteobacteria* (Jackson *et al.* 1999).

The effect of LCFA exposure on methanogenic activity has been observed to vary with contact time and has been attributed to either microbial acclimation to LCFA or adaptation through changes in the microbial community composition. Despite early reports of bactericidal (Rinzema *et al.* 1994) and permanent toxic effects of LCFA (Angelidaki and Ahring 1992), LCFA inhibition was shown to be reversible with anaerobic microorganisms capable of mineralizing biomass-sorbed LCFA after a lag period (Pereira *et al.* 2003, 2004, 2005). Methanogenic communities were also observed to develop an increased tolerance toward LCFA after pulse (Nielsen and Ahring 2006; Cavaleiro, Pereira and Alves 2008; Palatsi *et al.* 2009) and continuous feeding (Pereira *et al.* 2005; Cavaleiro *et al.* 2009; Baserba, Angelidaki and Karakashev 2012). However, the use of culture-independent molecular fingerprinting techniques (Hatamoto *et al.* 2007b; Sousa *et al.* 2007a; Palatsi *et al.* 2010; Baserba, Angelidaki and Karakashev 2012) led to contradictory results regarding the changes in microbial community structure during LCFA degradation. Sousa *et al.* (2007a) used PCR-denaturing gradient gel electrophoresis (DGGE) to study the microbial communities of an anaerobic bioreactor fed oleate (C18:1) and palmitate (C16:0) followed by batch degradation. The bacterial communities shifted toward more *Syntrophomonadaceae*-affiliated organisms that were not detectable in the initial inoculum. Conversely, Palatsi *et al.* (2010) found no significant changes in the bacterial community structure using PCR-DGGE on sludge from an anaerobic bioreactor subjected to repeated pulses of LCFA, even though improvements in hydrogenotrophic and $\beta$-oxidation activities were observed. Thus, it remains uncertain whether the increased tolerance of anaerobic communities to LCFA is a result of the proliferation of specialized-degrading populations (Hatamoto *et al.* 2007b; Sousa *et al.* 2007a; Baserba, Angelidaki and Karakashev 2012), or rather to changes in the physiology of existing populations (Palatsi *et al.* 2010). Additional ecological understanding of the microorganisms responsible for degrading LCFA in methanogenic communities is thus needed.

The ability to quantify syntrophic $\beta$-oxidizing bacteria could offer new insight into their ecophysiology during the degradation of LCFA. Membrane hybridization (Hansen, Ahring and Raskin 1999) and cleavage (Narihiro *et al.* 2012) probes have been previously developed targeting the mesophilic syntrophic $\beta$-oxidizing genera *Syntrophomonas* and *Syntrophus*. Yet, poor detection has been reported when employing these methods due to the potentially low concentrations of $\beta$-oxidizing bacteria in anaerobic bioreactors (Menes and Travers 2006; Narihiro *et al.* 2012), thus calling for a more sensitive quantification technique. Quantitative PCR (qPCR) is a widely employed method that uses DNA primer sets to obtain rapid and highly sensitive detection of specific microorganisms (Bustin *et al.* 2009), and has proven to be a powerful tool for monitoring microbial populations in the complex communities of anaerobic bioreactors (Yu *et al.* 2005; Hori *et al.* 2006; Lee *et al.* 2009; Westerholm *et al.* 2011). The use of a dual-labeled fluorogenic hydrolysis probe in the qPCR assay (TaqMan qPCR) may offer a higher level of specificity in comparison to SYBR Green assays (Wittwer *et al.* 1997). Currently, there have been no qPCR primer and TaqMan hydrolysis probe sets designed to quantify mesophilic syntrophic LCFA $\beta$-oxidizing bacteria.

The objective of this study was to develop and validate TaqMan qPCR assays targeting the known mesophilic syntrophic LCFA $\beta$-oxidizing genera *Syntrophomonas* and *Syntrophus*, and to apply the newly developed qPCR assays to monitor changes in the abundance of the syntrophic $\beta$-oxidizing groups in methanogenic bioreactors that were batch fed with oleic acid. High-throughput amplicon sequencing of the bacterial 16S rRNA gene was also conducted on time-series samples from the bioreactors to further examine microbial community dynamics.

## MATERIALS AND METHODS

### Batch-fed methanogenic bioreactors degrading oleic acid

Triplicate bioreactors (330 mL Pyrex glass serum bottles) were initially purged with $N_2/CO_2$ (80:20, 1atm) and sealed with butyl rubber septa. The bioreactors were seeded with sludge (21 g VS $L^{-1}$) from a mesophilic laboratory-scale complete-mix AD bioreactor co-digesting grease waste with primary and waste activated sludge. The seed sludge was diluted 50:50 with anaerobic basal medium prepared according to Karlsson *et al.* (1999). The bioreactors were initially incubated at 37°C for 7 days until methane production ceased from the background substrate in the seed sludge. After this period, sludge samples for DNA extraction (10 mL), total solids and volatile solids (TS/VS) (8 mL), volatile fatty acids (VFA) (1 mL) and LCFA (3 mL) analyses were withdrawn to characterize the initial conditions.

The bioreactors were incubated without shaking in a 37°C environmental chamber during five consecutive batch feedings of oleic acid. The bioreactors were fed with oleic acid (80% purity, VWR) every seventh day using a needle and syringe to obtain the target initial concentrations given in Table 1. The liquid volume that was removed for liquid sampling throughout the batch degradation period (42 mL) was replaced with anaerobic basal medium during each subsequent batch feeding to maintain an initial working volume of 120 mL. This operation resulted in the replacement of approximately two reactor liquid volumes throughout the experimental period, and thus, promoted the washout of the initial inoculum over time. Liquid samples were withdrawn with a needle and syringe for analysis of VFA (1 mL)

**Table 1.** Operating conditions and performance characteristics of methanogenic bioreactors degrading oleic acid. ($n = 3$).

| | Batch cycle | | | | |
|---|---|---|---|---|---|
| Parameter | 1 | 2 | 3 | 4 | 5 |
| VS ($g\ L^{-1}$)[a] | 7.8 ± 0.3 | 4.9 ± 0.2 | 3.5 ± 0.1 | 2.1 ± 0.2 | 1.2 ± 0.1 |
| pH | 7.3 ± 0.03 | 7.3 ± 0.01 | 7.2 ± 0.03 | 7.2 ± 0.01 | 7.1 ± 0.01 |
| Oleic acid Added (mM) | 2.9 | 4.0 | 5.0 | 5.0 | 6.6 |
| Initial oleic acid to solids ratio ($mg\ g\ VS^{-1}$)[b] | 100 ± 4 | 230 ± 10 | 410 ± 7 | 670 ± 65 | 1570 ± 130 |
| Initial specific methane production rate ($mL\ CH_4\ g\ VS^{-1}\ d^{-1}$) | 57 ± 3 | 85 ± 2 | 130 ± 1 | 170 ± 7 | 260 ± 24 |

[a]Average of the levels at the beginning and end of each batch degradation period.
[b]Based on the amount of oleic acid added and the VS concentration in each vial, since the LCFA did not accumulate above 0.15 mM at the end of each batch period (see Table S2, Supporting Information).

and LCFA (3 mL) six times during each batch degradation period. Sludge samples for DNA extraction (10 mL) and TS/VS and pH (8 mL) were withdrawn at the end of each batch period before the subsequent feeding. The bioreactor headspace was purged with $N_2/CO_2$ (80:20, 1 atm) prior to the addition of new substrate at the start of each batch-feeding period. The bioreactor conditions during each batch degradation period are summarized in Table 1.

Biogas volume and composition were measured daily throughout the bioreactor operation. The initial specific methane production rates in each batch period were determined by dividing the initial slope of the methane production curve (in $mL\ CH_4\ L^{-1}\ d^{-1}$) by the average volatile solids (in $g\ VS\ L^{-1}$) of each vial and were expressed as $mL\ CH_4\ g\ VS^{-1}\ d^{-1}$.

## Extraction and quantification of genomic DNA

Bioreactor samples for DNA extraction were immediately centrifuged at 10 000 × g at 4°C for 10 min, decanted and stored at –20°C. Genomic DNA was isolated from approximately 0.2 g of wet solids using the PowerSoil RNA/DNA Isolation Kit (MO-BIO, Inc., Carlsbad, California) according to the manufacturer's instructions. This DNA extraction protocol was chosen due to its use of phenol—-chloroform, which can help to ensure efficient recovery of high-quality archaeal and bacterial DNA (Urakawa, Martens-Habbena and Stahl 2010). The concentration of extracted DNA was immediately measured with the Quant-IT dsDNA High-Sensitivity Assay Kit with a Qubit 2.0 flourometer, according to the manufacturer's instructions (Invitrogen, Carlsbad, California). Extracted DNA was stored in nuclease-free water at –20°C.

## Design and validation of qPCR primer and probe sets

16S rRNA gene sequences of strains belonging to the genera *Syntrophomonas* and *Syntrophus* were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/genbank/, retrieved July 2013) and separately aligned using the Clustal package in Jalview software (v. 2.8) (Waterhouse *et al.* 2009). Sequence regions of high similarity within the multiple gene alignments were identified, and were used to design potential hybridization sites for primers and probes. Primer sequences were designed to meet the following criteria: minimal dimer formation, a guanine + cytosine (GC) content of 40–70%, minimal repeats of guanine and cytosine, an amplicon fragment length less than 150 bp and the fewest degenerate nucleotides possible. Hydrolysis (TaqMan) probes were designed based on: a 5′ location as close to the forward primer as possible, less than 30 nucleotides in length, minimal self- and cross-annealing, no terminal guanine base at the 5′ end, a melting temperature 5–10°C above the associated primer pair and minimal degenerate nucleotides. All hydrolysis probes were labeled with 6-FAM at the 5′end and BHQ-1 at the 3′ end.

The optimal qPCR primer and probe sets were chosen based on their adherence to the above criteria, as well as their target group specificity and coverage. The specificity and group coverage of each primer and probe set was tested *in silico* by conducting searches with: BLAST of the GenBank Database (http://blast.ncbi.nlm.nih.gov/); the Ribosomal Database Project (RDP) ProbeMatch software (Cole *et al.* 2009) and the SILVA TestPrime 1.0 and TestProbe 3.0 programs (Klindworth *et al.* 2013). Potential false-positive targets were identified by increasing the number of allowed sequence mismatches in the *in silico* database searches. Organisms with three or more mismatches within each of the three oligonucleotide sequences (≥9 mismatches total) were excluded from the analysis due to their low probability of amplification (Yu *et al.* 2005). The optimal primer/probe sets targeting *Syntrophomonas* and *Syntrophus* 16S rRNA genes are given in Table 2 along with their respective target group coverages.

In addition to the *in silico* analysis, the specificities of the qPCR assays were tested empirically using genomic DNA from pure cultures. Positive controls included genomic DNA from *Syntrophomonas wolfei* subsp. *saponavida* (DSM 4212), *S. palmitatica* (DSM 18709), *S. zehnderi* OL-4, *Syntrophus aciditrophicus* (DSM 26646) and *Sy. gentianae* (DSM 8423). Negative controls to test for non-specific amplification included DNA from *Syntrophobacter wolinii*, *Syn. pfennigii*, *Pelotomaculum propionicicum*, *Desulfotomaculum kuznetsovii* (DSM 6115), *Desulfomonile tiedjei* (DSM 6799), *Desulfovibrio desulfuricans* (DSM 642), *Clostridium ultunense*, *C. acidurici* (DSM 604), *Enterococcus faecalis*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Methanosaeta concilii* (DSM 3671), *Methanospirillum hungatei* (DSM 864), *Methanococcus voltae* (DSM 1537), *Methanosarcina barkeri* (DSM 800) and *Methanobacterium formicicum* (DSM 1535). Most genomic DNA samples of pure-culture isolates were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Additional genomic DNA samples from isolated strains were obtained from collections at Wageningen University (Wageningen, the Netherlands), Linköping University (Linköping, Sweden) and the Swedish University of Agricultural Sciences (Uppsala, Sweden). Approximately 0.5–2 ng DNA of each pure-culture DNA sample was loaded in the qPCR reaction for specificity testing.

**Table 2.** Characteristics of qPCR primers and hydrolysis probes developed to target *Syntrophomonas* and *Syntrophus* genera.

| Oligo. name[a] | Target group | Position[b] | Oligo. sequence (5′ to 3′) | Expected length (bp) | $T_m$ (°C)[c] | Target group coverage (%)[d] |
|---|---|---|---|---|---|---|
| Synm-678 (F) | *Syntrophomonas* | 678–691 | CCWGGTGTAGCGGT | 75 | 60.3 | 97 |
| Synm-696 (P) | | 696–722 | TGCGTAGAAATCAGGAGGAAYACCAGT | | 70.0 | 90 |
| Synm-738 (R) | | 738–752 | TCAGGGYCAGTCCAG | | 61.5 | 97 |
| Syntr-441 (F) | *Syntrophus* | 441–459 | GGTGGGAAGAAATGTATKGA | 137 | 61.6 | 88 |
| Syntr-462 (P) | | 462–487 | TTAAYAGCCTTTGTACTTGACGGTAC | | 66.8 | 88 |
| Syntr-559 (R) | | 559–576 | CTCTTTACGCCCAATGAT | | 60.1 | 88 |

[a]F = forward primer; P = hydrolysis probe; R = reverse primer.
[b]According to *E. coli* J01695 numbering.
[c]Calculated using nearest-neighbor method with the OligoAnalyzer 3.1 program (http://www.idtdna.com), using the model inputs: 200nM probe, 500nM primer and 200 mM monobasic salt.
[d]Calculated as the ratio of matches with sequences from isolated organisms within the target group over the total number of sequences from isolated organisms in that group. The ratios were calculated based on available sequences in the RDP using the Probe Match program (http://rdp.cme.msu.edu/probematch) with inputs: Quality = 'Good', Size ≥ 1200bp, Source = 'Isolates', Mismatches = 0.

## Quantitative PCR

qPCR targeting partial 16S rRNA gene sequences was performed using a LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany). Each 20 $\mu$L reaction mix contained 5 $\mu$L DNA template, 2 $\mu$L PCR-grade water (Roche Diagnostics GmbH), 500 nM of each forward and reverse primers, 200 nM of hydrolysis probe and 10 $\mu$L LightCycler 480 Probes Master mix (2X, Roche Diagnostics GmbH). All samples were analyzed in duplicate. No-template controls were included with each qPCR run to ensure that no contamination occurred.

In addition to the *Syntrophomonas* and *Syntrophus* genus-level qPCR assays developed in this study (Table 2), qPCR analysis was also conducted targeting 16S rRNA genes of the Bacteria domain, the methanogenic archaeal orders of *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales* and the methanogenic archaeal families of *Methanosarcinaceae* and *Methanosaetaceae* using previously developed primer/probe sets (Yu *et al.* 2005). The details of all primer/probe sets used for qPCR in this study are provided in Table S1 (Supporting Information). The amplification procedure for all qPCR assays consisted of an initial denaturation at 95°C for 10 min, followed by 42 cycles of 95°C for 10 s, and annealing and extension at 60°C for 30 s. The only exception was the *Methanomicrobiales* assay, which had the annealing and extension step at 63°C for 30 s (Yu *et al.* 2005).

DNA samples for calibration standards were prepared by cloning the target PCR amplicon fragment of each primer set into a pUC57 plasmid (GeneScript, Piscataway, New Jersey). The strains from which 16S rRNA gene sequences were used to construct the calibration standards for each target group are given in Table S1 (Supporting Information). Plasmids containing the target PCR amplicon sequence were linearized with XbaI (FastDigest, Thermo Scientific, Waltham, Massachusetts) according to the manufacturer's instructions and were quantified by Qubit. Gene copy numbers were calculated from the measured DNA concentration and the molecular weight of the ligated plasmid containing the PCR amplicon insert. Calibration standards for each qPCR primer/probe set were constructed from the linearized plasmids in a dilution series ranging from 25 to $10^8$ gene copies (nine calibration standard concentrations total). Calibration standards were run in duplicate, and were included in each qPCR run for all target groups. The average slopes and intercepts of the qPCR calibration standard curves for the various targets are given in Table S1 (Supporting Information).

Extracted DNA from the bioreactors was diluted 1:10 in nuclease-free water to prevent PCR inhibition. A 4-fold series of 1:10 dilutions of a randomly selected experimental DNA sample was included with each qPCR run to ensure that the PCR efficiency of the experimental samples was similar to the calibration standards (efficiency values were within 10% of standards). The gene copy numbers were normalized to the amount of DNA loaded in the PCR reaction (ng) in order to correct for any variation in DNA extraction efficiencies between samples.

## High-throughput amplicon sequencing of bacterial 16S rRNA genes

All DNA extracts were processed for high-throughput amplicon sequencing on the Illumina MiSeq platform. Two-step nested PCR was conducted prior to sequencing to improve PCR sensitivity. The primers used in the initial PCR were a modified 341F (CCTAYGGGRBGCASCAG) and a modified 806R (GGACTACNNGGGTATCTAAT) (Sundberg *et al.* 2013), which targeted a 465 bp DNA fragment flanking the V3 and V4 regions of the 16S rRNA gene of Bacteria and Archaea. The initial PCR was performed in a total volume of 20 $\mu$L containing 5 $\mu$L of sample DNA extract (diluted 1:10), 500 nM of each of the primers 341F and 806R, 0.2 mM dNTPs mixture, 0.4 U of Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland), 1X Phusion High-Fidelity Buffer (Finnzymes) and PCR grade water. The PCR amplification was performed with an initial incubation at 98°C for 30 s, followed by 35 cycles of 98°C for 5 s, annealing at 60°C for 20 s, extension at 72°C for 20 s and a final extension step at 72°C for 5 min. PCR was run in duplicate for each DNA sample and then subsequently pooled. PCR products were purified using the High-Pure PCR Cleanup Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA concentrations of the purified PCR product were subsequently quantified using the Quant-IT dsDNA High-Sensitivity Assay Kit and a Qubit fluorometer, and amplicon size was verified by gel electrophoresis. Addition of adapters and indices to DNA fragments was done in a second PCR using the modified primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R. The second PCR was performed in a total volume of 20 $\mu$L containing 2 $\mu$L AccuPrime PCR Buffer (10X, Invitrogen), 0.24 U AccuPrime *Taq* DNA Polymerase High-Fidelity (Invitrogen), 500 nM each of the fusion primers 515F and 806R with indexes, 2 $\mu$L of 1:10 diluted PCR product from the first PCR and PCR grade water. The second PCR was run with the following conditions: 94°C for 2 min, followed by 15 cycles of 94°C for 20 s, 56°C for 30 s and 68°C for 40 s, and a final extension at 68°C for 5 min. PCR amplicons were purified using Agencourt AMPure

XP (Agencourt Bioscience Corporation, MA, USA), and the concentration was measured using PicoGreen staining (Invitrogen), according to the manufacturer's protocols. The sample amplicons were pooled at equimolar concentrations, and subjected to high-throughput sequencing on the Illumina MiSeq platform at the University of Copenhagen Molecular Microbial Ecology Lab according to MiSeq Reagent Kit Preparation Guide (Illumina, Inc., San Diego, CA, USA). Sequences were submitted to the NCBI Sequence Read Archive as BioProject PRJNA262832.

Sequences were processed and analyzed using QIIME version 1.8.0 (Caporaso *et al.* 2010b). Paired-end sequences were joined using the fastq-join method (Aronesty 2013) within the QIIME script *join_paired_ends.py* with a minimum overlap of 100 bp and a zero percent difference allowed in the overlap region. Sequences shorter than 220 bp and with quality scores less than 20 were filtered using the *split_libraries_fastq.py* script in QIIME. Sequence chimeras were identified and filtered with USEARCH61 (Edgar 2010; Edgar *et al.* 2011). Filtered sequences were clustered into operational taxonomic units (OTUs) based on 3% sequence divergence using USEARCH61 (Edgar 2010). Representative sequences of each OTU were identified based on the cluster seed and were classified using a naïve Bayesian algorithm with the RDP Classifier program version 2.2 (Wang *et al.* 2007). Archaeal sequences were removed from the sequence libraries prior to further analysis of the bacterial community. The representative sequences were aligned to the Greengenes core reference alignment (DeSantis *et al.* 2006) using PyNAST (Caporaso *et al.* 2010a). Weighted UniFrac distances (Lozupone and Knight 2005) were calculated with QIIME to determine changes in phylogenetic community structure over time. Community richness was determined by rarefying each sample based on the lowest number of sequences observed in a single sample, and the community evenness was estimated with the Gini coefficient (Wittebolle *et al.* 2009).

### Analytical methods

The total gas production was measured based on the pressure increase in the bioreactors using a handheld pressure transducer (Testo 3123, Testo, Sparta, New Jersey). The change in headspace volume over time within the batch vials due to liquid sampling was accounted for when determining the total gas production. The methane content of the headspace was measured in triplicate by GC-FID (Hewlett Packard 5880 A) at each gas pressure sampling point. All measured gas volumes are reported at standard temperature and pressure (1 atm pressure and 0°C). The pH of bioreactor samples was measured using an Inolab pH 7310 meter (WTW, Weilheim, Germany). TS and VS were determined according to Standard Methods (Eaton and Franson 2005). VFA (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, capronate and iso-capronate) were analyzed by GC-FID (HP 6890, Hewlett Packard), and were separated with a BP21 (FFAP) column (30 m × 0.32 mm × 0.25 $\mu$m, SGE Analytical Science) as described previously (Jonsson and Borén 2002).

The protocol for LCFA extraction and analysis was adapted from Lalman and Bagley (2000). Bioreactor sludge samples were stored at –20°C and thawed at 4°C prior to LCFA extraction. 1 mL of sample was transferred to a 10 mL glass vial and the following were added: 100 $\mu$L of 10 g $L^{-1}$ pentadecanoic acid (>99% purity, Sigma Aldrich, St. Gallen, Switzerland) in methanol as in internal standard (IS), 200 $\mu$L of 250 g $L^{-1}$ sodium chloride in MilliQ water, two drops of 50% sulfuric acid and 2 mL of 1:1 hexane:methyl-tert-butyl ether (MTBE). Samples were then sealed with Teflon-lined caps, vortexed to mix, placed on an orbital shaker at 250 rpm for 20 min before centrifuging at 4500 × g for 10 min. The organic supernatant was decanted for analysis by GC-FID (Clarus 580, Perken-Elmer) with a split/splitless injector. LCFA were separated with a BP21 (FFAP) column (30 m × 0.32 mm × 0.25 $\mu$m, SGE Analytical Science) with He as the carrier gas at a flow of 2 mL $min^{-1}$ and a split ratio of 1:10. The injector temperature was 250°C, and the injection volume was 1 $\mu$L. The detector temperature was 280°C, and the detector flow was comprised of 45 mL $min^{-1}$ $H_2$ and 450 mL $min^{-1}$ air. The oven temperature was initially 160°C with a 20°C $min^{-1}$ ramp to 225°C, isothermal for 15 min, and then a final 5°C $min^{-1}$ ramp to 240°C. Calibration standards included 10, 25, 50, 100, 250, 500 and 1000 mg $L^{-1}$ of palmitic, stearic and oleic acids (>99% purity, Sigma Aldrich), each containing 500 mg $L^{-1}$ of pentadecanoic acid (IS) dissolved in 1:1 hexane:MTBE. The average recovery of palmitic acid, stearic acid and oleic acid was 88, 87 and 102%, respectively.

### Statistical analysis

Changes in abundance based on qPCR data were determined using two-tailed *t*-tests with biological ($n = 3$) and technical replicates ($n = 2$). Changes in abundance and microbial community structure parameters (i.e. Unifrac distances, evenness, richness) based on high-throughput amplicon sequencing data were determined using two-tailed *t*-tests with biological replicates ($n = 3$).

## RESULTS

### Validation of *Syntrophomonas* and *Syntrophus* genus-level qPCR assays

The qPCR primer and probe sets developed for *Syntrophomonas* and *Syntrophus* successfully detected all species belonging to the respective target groups during the specificity testing with pure-culture DNA (Figs S1 and S2, Supporting Information). Moreover, the *in silico* analysis of the *Syntrophus* and *Syntrophomonas* qPCR assays showed that between 88 and 97% of target 16S rRNA gene sequences in the RDP database matched the corresponding primers and probe sets (Table 2), indicating that false-negative results were minimized due to the high target group coverage of the assays. Slopes of the calibration dilution series were calculated by performing linear regressions on plots of $C_t$ values versus the $\log_{10}$ of the template concentrations. The reproducibility of the qPCR assays was tested by generating eight standard calibration curves over approximately four months, yielding average slopes of –3.30 and –3.32 for the *Syntrophomonas* and *Syntrophus* sets, respectively. These average qPCR calibration slopes for the *Syntrophomonas* and *Syntrophus* assays corresponded to average PCR efficiencies of 101 and 100%, respectively. The relative standard deviations of the slopes and intercepts of standard calibration curves were less than 3%. The linear dynamic range of both primer/probe sets was between 25 to $10^8$ gene copies (Figs S3 and S4, Supporting Information).

The *in silico* testing of the *Syntrophomonas* qPCR assay identified potential false-positive 16S rRNA gene sequences from non-target organisms. Potentially interfering sequences with four to five total mismatches within the primer and probe oligonucleotide sequences aligned to the genera *Desulfotomaculum, Desulfitibacter, Syntrophothermus, Thermosyntropha, Thermoanaerobacter, Halomonas* and *Chromohalobacter*. Sequences from the genus *Pelospora* were also identified as potential false-positives with less than four total mismatches in the primer

and probe sequences of the *Syntrophomonas* qPCR assay. Yet, *Syntrophothermus, Thermosyntropha* and *Thermoanaerobacter* are thermophilic (Svetlitshnyi, Rainey and Wiegel 1996; Sekiguchi *et al.* 2000; Zhang *et al.* 2012), while *Halomonas* and *Chromohalobacter* species thrive in hypersaline environments (Mata *et al.* 2002; Arahal and Ventosa 2006), and are thus not expected to be present in the study bioreactors. Moreover, no sequences aligning to *Desulfotomaculum, Halomonas, Chromohalobacter* and *Thermoanaerobacter* were detected by high-throughput amplicon sequencing of the bioreactor samples, while sequences aligning to *Desulfitibacter* and *Syntrophothermus* were detected below 0.1% relative abundance and *Thermosyntropha* and *Pelospora* sequences remained below 0.5% relative abundance. It is therefore unlikely that DNA from the above groups interfered with the specificity of the *Syntrophomonas* qPCR assay applied to the mesophilic bioreactors. Moreover, the specificity of the *Syntrophomonas* qPCR assay was verified with no observed amplification of *Desulfitibacter alkalitolerans* DNA (five total mismatches) and minimal amplification of *Desulfotomaculum kuznetsovii* DNA (four total mismatches) (Fig. S1, Supporting Information). The potential for interference with the *Syntrophomonas* qPCR assay due to the presence of non-target DNA was further negated by demonstrating that the addition of *D. kuznetsovii* DNA (0.6 ng) to each standard in the calibration curve had no significant impact on the slope of the standard curve between 100 and $10^8$ gene copies (Fig. S5, Supporting Information). The *Syntrophomonas* 16S rRNA gene concentration in the bioreactor DNA samples also showed no significant change when spiked with $10^6$–$10^7$ 16S rRNA gene copies of *D. kuznetsovii* (Fig. S6, Supporting Information) ($P > 0.10$), and testing of all other pure-culture isolates with the *Syntrophomonas* qPCR assay showed no amplification of non-target DNA (Fig. S1, Supporting Information). The *Syntrophomonas* qPCR assay was therefore highly specific for *Syntrophomonas* 16S rRNA genes.

No potential non-target 16S rRNA gene sequences were found with less than two mismatches in each of the three oligonucleotides in the *Syntrophus* primer/probe set (up to six mismatches total) during the *in silico* database searches. Furthermore, the specificity testing showed that the *Syntrophus* primer/probe set did not amplify DNA from *Desulfomonile tiedjei* (Fig. S2, Supporting Information), a species that also belongs to the family *Syntrophaceae*, nor was amplification observed with other non-target DNA samples (Fig. S2, Supporting Information). The effect of non-target DNA on the efficiency of the *Syntrophus* primer/probe set was assessed by spiking each standard in the calibration curve with *De. tiedjei* DNA (0.5 ng DNA), which had no significant impact on the PCR slope or linear dynamic range between 25 and $10^8$ gene copies (Fig. S7, Supporting Information). Thus, false-positive results were minimized by the high target specificity of the *Syntrophus* qPCR assay.

## Specific mineralization rates of oleic acid in methanogenic bioreactors

The amount of oleic acid added to the bioreactors during batch feeding was more than doubled by the fifth batch degradation cycle (Table 1), and for all degradation cycles most of the oleic acid was metabolized (Table S2, Supporting Information). The detection of stearic, palmitic and acetic acids as intermediates from oleic acid (Table S2, Supporting Information) indicated that $\beta$-oxidation was the primary degradation pathway for oleic acid metabolism (Weng and Jeris 1976; Lalman and Bagley 2001; Pereira *et al.* 2002). While the VS concentration decreased in each consecutive batch cycle due to the replacement of a fraction of the liquid volume with anaerobic basal medium during feeding, the initial specific methane production rate (normalized to VS) increased after each batch feeding (Table 1). This observation suggested an increase in the fraction of LCFA $\beta$-oxidizing consortia, as discussed below.

## Quantitative dynamics of syntrophic $\beta$-oxidizing bacteria by qPCR

The newly developed qPCR assays targeting the $\beta$-oxidizing genera of *Syntrophomonas* and *Syntrophus* were applied to the triplicate bioreactors degrading oleic acid. Changes in the 16S rRNA gene concentration of the syntrophic bacterial groups are shown in Fig. 1A and B. The *Syntrophomonas* 16S rRNA gene concentration significantly increased from $4.2 \times 10^4$ to $22 \times 10^4$ copies $ng^{-1}$ DNA during the bioreactor operation ($P = 1E\text{-}11$; Fig. 1A), while the *Syntrophus* 16S rRNA gene concentration significantly decreased from 90 to 9 copies $ng^{-1}$ DNA ($P = 2E\text{-}10$; Fig. 1B). The 16S rRNA gene concentration of the Bacteria domain slightly increased throughout the bioreactor operation from $5.9 \times 10^5$ to $7.1 \times 10^5$ copies $ng^{-1}$ DNA ($P = 0.002$). The gene copy levels of the two syntrophic $\beta$-oxidizing groups were normalized to that of the Bacteria domain to assess the relative abundances of the groups over time (Fig. 1C and D). Fig. 1C illustrates the significant increase in *Syntrophomonas* abundance from 7% of the bacterial 16S rRNA gene concentration initially to approximately 31% after the last batch feeding of oleic acid. The average relative abundance of *Syntrophomonas* in each batch degradation period correlated strongly with the initial specific methane production rate from oleic acid (Pearson's coefficient = 0.99; Fig. 2A). In contrast, the *Syntrophus* 16S rRNA gene was quantified at an initial relative abundance of 0.015% and decreased to 0.001% of the bacterial 16S rRNA gene concentration by the end of bioreactor operation (Fig. 1D). These results indicated that *Syntrophomonas* species, but not *Syntrophus* species, were actively growing from the degradation of oleic acid in the bioreactors.

## Quantitative dynamics of methanogenic archaea by qPCR

The sum of the 16S rRNA gene concentrations of all methanogenic archaeal target groups increased 2.8-fold throughout the bioreactor operation, from $1.4 \times 10^5$ to $3.9 \times 10^5$ copies $ng^{-1}$ DNA ($P = 0.001$; Fig. 3A). Correspondingly, the ratio of the total methanogenic archaea 16S rRNA gene concentration to that plus the Bacteria domain (representing the total prokaryotic community) significantly increased from 19 to 35% throughout bioreactor operation ($P = 0.0004$). The relative abundance of the total methanogenic archaea 16S rRNA gene concentration in the prokaryotic community correlated strongly with the initial specific methane production rate from oleic acid (Pearson's coefficient = 0.97; Fig. 2B). The change in 16S rRNA gene concentration of each targeted methanogenic group is shown in Fig. S8 (Supporting Information). The 16S rRNA gene concentration of each methanogenic archaea group was normalized to the sum of all methanogenic archaea targets to infer their relative abundances over time, as shown in Fig. 3B. The methanogenic archaeal community composition stayed relatively stable over time, with the hydrogenotrophic order of *Methanomicrobiales* accounting for 70% of the methanogenic archaeal 16S rRNA gene concentration and the acetoclastic family of *Methanosaetacea* accounting for 30% (Fig. 3B). The order of *Methanobacteriales* and family of *Methanosarcinaceae* comprised less than 1% of the methanogenic archaeal 16S rRNA

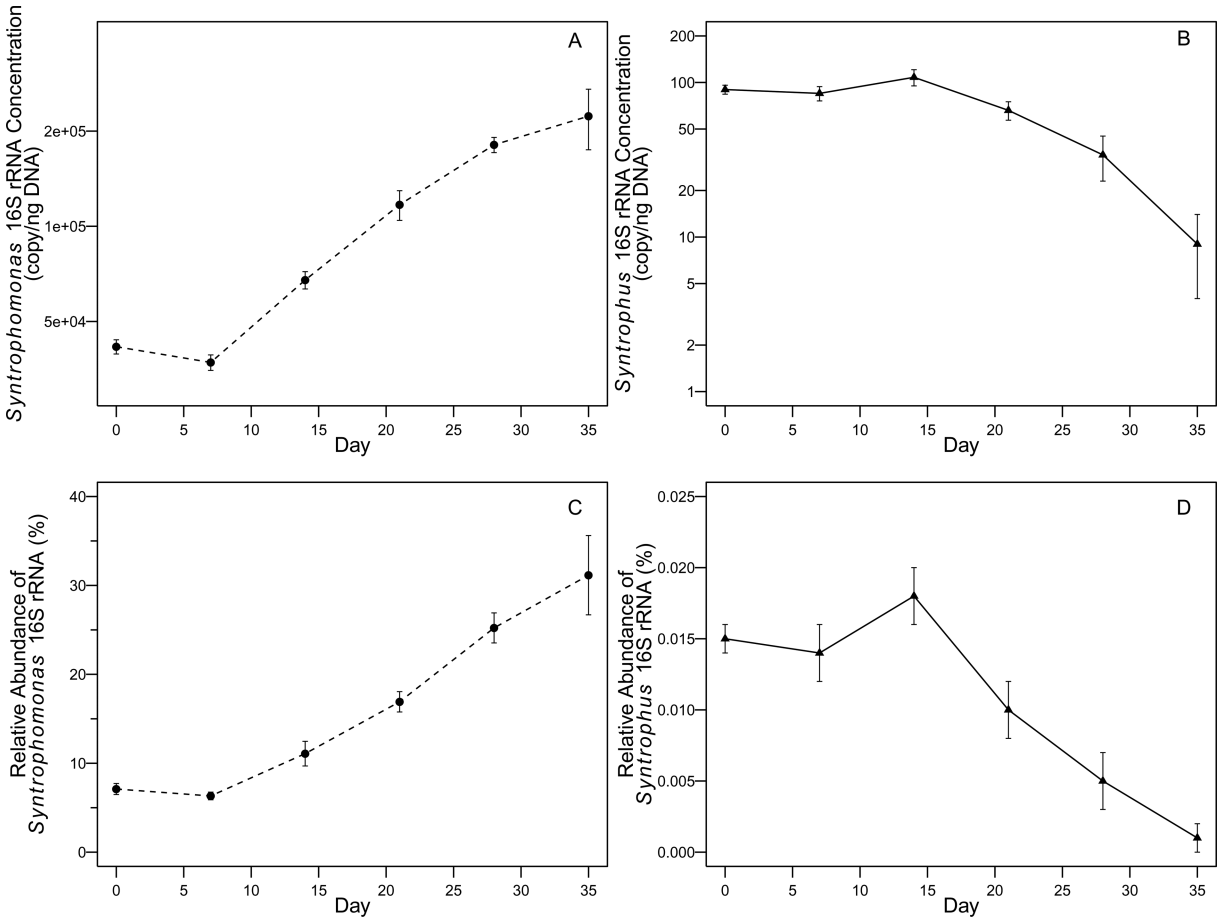


**Figure 1.** Changes in 16S rRNA gene concentrations of (**A**) *Syntrophomonas* and (**B**) *Syntrophus* in methanogenic bioreactors; ratio of 16S rRNA gene concentrations of (**C**) *Syntrophomonas* and (**D**) *Syntrophus* to that of total Bacteria in bioreactors. Error bars indicate one standard deviation based on biological ($n = 3$) and technical replicates ($n = 2$).

gene concentration throughout bioreactor operation, while the order of *Methanococcales* was not detected by the qPCR assay (Fig. 3B).

## Dynamics of bacterial community by high-throughput amplicon sequencing of 16S rRNA genes

Bacterial community dynamics in the triplicate bioreactors were monitored using Illumina-based high-throughput amplicon sequencing of 16S rRNA genes for 18 biomass samples, which produced a total of 1180 000 non-chimeric bacterial sequence reads and an average of 68 000 sequences per sample. The bacterial community structure of the bioreactors significantly changed over time, while similar community structures were maintained between the triplicate bioreactors. This result was apparent by the significant increase in phylogenetic structure differences within the bioreactor microbiomes (determined by weighted UniFrac distances) relative to the initial conditions ($P = 0.0002$; Fig. S9, Supporting Information), and by the similar dynamics within the OTU sets of the triplicate bioreactors (Fig. 4). The richness of the bacterial community significantly decreased throughout the bioreactor operation ($P = 0.005$), as did the community evenness ($P = 0.007$; Fig. S9, Supporting Information). These observed changes in the diversity and phylogenetic structure within the bacterial communities indicated that a specialized population of bacteria developed during the degradation of oleic acid and its by-products, during which non-growing bacterial groups were washed out of the bioreactors.

The growth dynamics of all OTUs representing more than 1% relative abundance are shown in Fig. 4. The bacterial communities were dominated primarily by the orders *Clostridiales* and *Anaerolineales*, which together accounted for over 55% of the population by the end of bioreactor operation (Fig. 5A) and were comprised mainly of the genera *Syntrophomonas* and *Levilinea*, respectively (Fig. 5B). *Syntrophomonas* became the most abundant genus within the bacterial communities by the end of the experiment, increasing in relative abundance from 6 to 19% of sequence reads (Fig. 5B). *Syntrophomonas*-affiliated OTUs that were detected above 1% relative sequence abundance (seven OTUs total) all showed general patterns of increased abundance within the bioreactor microbiomes over time (Fig. 4). Other bacterial orders that experienced growth in the bioreactor microbiomes included *Synergistales* and *Enterobacteriales*, which were comprised mainly of the genera *Synergistes* and *Escherichia/Shigella*, respectively (Figs 4 and 5A). Notably, no *Syntrophus* sequences were detected above 0.3% relative abundance throughout the bioreactor operation (data not shown), which is consistent with the results of the qPCR assay.

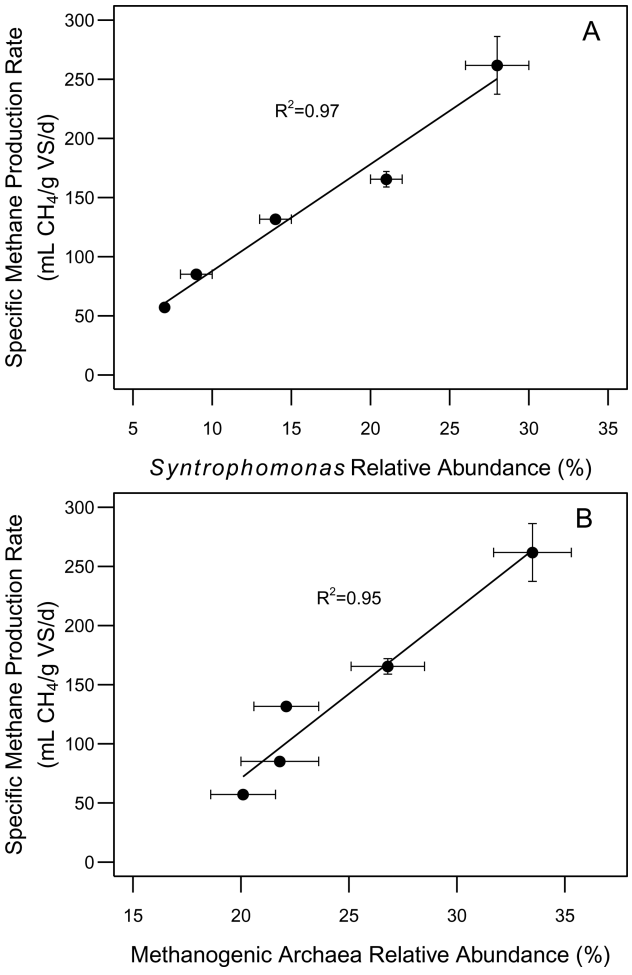


**Figure 2.** Correlation between the initial specific methane production rate during the oleic acid batch degradation periods and the average relative abundance of (**A**) *Syntrophomonas* 16S rRNA genes in bacterial community and (**B**) methanogenic archaea 16S rRNA genes in prokaryotic community. The total methanogenic archaea gene concentration was determined as the sum of all methanogen target groups, and the prokaryotic gene concentration was determined as the sum of methanogenic archaea and total Bacteria gene counts. Error bars for the specific methane production rate indicate one standard deviation based on biological replicates ($n = 3$), and error bars for relative abundance values indicate one standard deviation based on biological ($n = 3$) and technical replicates ($n = 2$).

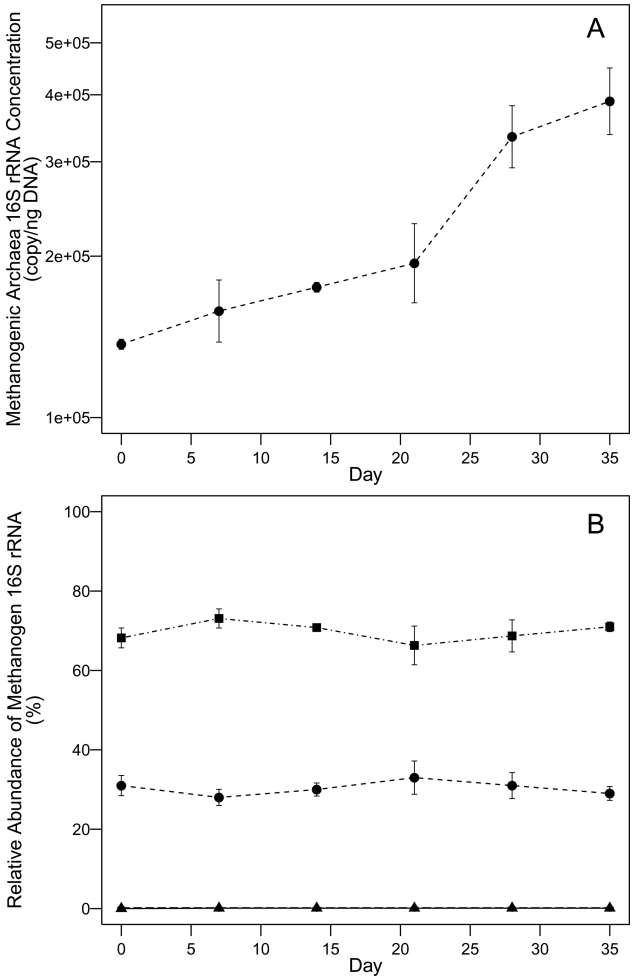


**Figure 3.** (**A**) Change in the 16S rRNA gene concentration of the sum of all methanogenic archaea targets in bioreactors degrading oleic acid; (**B**) relative abundance of the 16S rRNA gene concentration of each methanogenic target group relative to the sum of methanogenic archaea targets; (filled squares) = *Methanomicrobiales*; (filled circles) = *Methanosaetaceae*; (filled rhombus) = *Methanobacteriales*; (filled triangles) = *Methanosarcinaceae*. *Methanobacteriales* and *Methanosarcinaceae* remained below 1% relative abundance, while *Methanococcales* was not detected by qPCR of the bioreactor samples. Error bars indicate one standard deviation based on biological ($n = 3$) and technical replicates ($n = 2$).

## DISCUSSION

The dynamics and ecological role of syntrophic $\beta$-oxidizing bacteria in methanogenic bioreactors has been poorly understood due to a relative lack of information regarding the concentration of these groups within anaerobic communities (Hansen, Ahring and Raskin 1999; McMahon 2001; McMahon *et al.* 2004; Ariesyady, Ito and Okabe 2007; Narihiro *et al.* 2012). The application of the newly developed TaqMan qPCR assays in combination with high-throughput amplicon sequencing of bacterial 16S rRNA genes proved to be a valuable approach to monitor the quantitative dynamics of syntrophic $\beta$-oxidizing communities in bioreactors mineralizing oleic acid. Thus, the combination of these assays may provide a platform for analyzing and surveying microorganisms in anaerobic digesters degrading lipid-rich wastes.

The increase in the initial specific methane production rate by 4.6 times throughout the experimental period showed that the bioreactor communities developed a greater capacity to convert oleic acid into methane, which was likely caused by the growth of LCFA-degrading consortia within the community. The increase in the relative abundance of *Syntrophomonas* species in the bioreactors to over 30% of the bacterial community determined by qPCR (Fig. 1C) showed that this group was actively growing through oleic acid $\beta$-oxidation. The strong correlation between the relative abundance of *Syntrophomonas* species and the initial specific methane production rate from oleic acid (Fig. 2A) further suggested that *Syntrophomonas* species contributed to the increase in mineralization rates of the LCFA observed in the bioreactors at higher oleic acid loadings. High-throughput amplicon sequencing corroborated the observed growth of *Syntrophomonas* species by predicting that *Syntrophomonas*-affiliated sequences increased to approximately 20% of the bioreactor communities (Fig. 5B). The various extent of growth observed within the *Syntrophomonas*-affiliated OTUs (Fig. 4) suggested that different *Syntrophomonas* ecotypes may

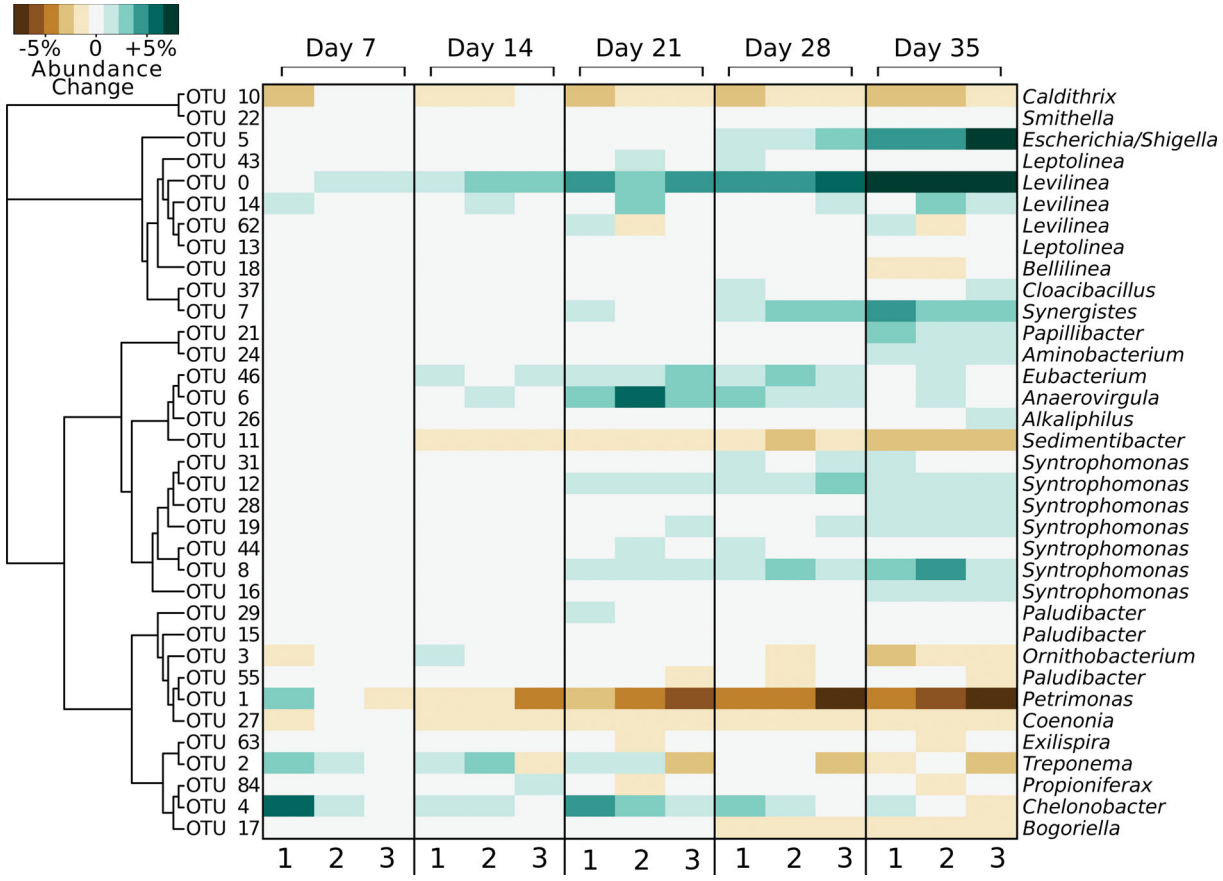


**Figure 4.** Change in the relative abundance of each OTU that accounted for 1% or more of the bacterial community during the bioreactor operation relative to initial conditions, as determined by high-throughput amplicon sequencing of 16S rRNA genes. The left axis shows a phylogenetic clustering of the OTUs based on sequence alignment with MUSCLE (http://www.ebi.ac.uk). The right axis shows the genus level classification of each representative OTU sequence. The scale on the color key is relative to the entire community (e.g. an increase in relative abundance from 1 to 5% would be shown as +4%). The relative abundance change of each OTU is shown for all reactor replicates (e.g. 1, 2 and 3) at days 7, 14, 21, 28 and 35.

express different physiological capacities to degrade oleic acid. A phylogenetic comparison of 16S rRNA gene sequences of characterized *Syntrophomonas* species with that of the most abundant *Syntrophomonas*-affiliated OTUs indicated that the enriched OTUs were distributed throughout the genus (Fig. S10, Supporting Information). The importance of *Syntrophomonadaceae* species in anaerobic communities degrading LCFA has been implicated in prior studies based on molecular fingerprinting techniques (Sousa *et al.* 2007a; Baserba, Angelidaki and Karakashev 2012). Yet, the quantitative results provided by the newly developed qPCR assays, in combination with high-throughput amplicon sequencing, further showed that increased oleic acid degradation rates could be obtained with higher relative abundances of $\beta$-oxidizing *Syntrophomonas* species. Thus, the abundance of syntrophic $\beta$-oxidizing bacteria, as measured by the novel qPCR assays, may be used to indicate the capacity of anaerobic digester communities to $\beta$-oxidize fatty acids, in part involving LCFA.

In contrast to *Syntrophomonas*, the relative abundance of the *Syntrophus* genus was much lower in the initial seed and decreased throughout the bioreactor operation as measured by qPCR. The high-throughput amplicon sequencing results also indicated that *Syntrophus*-affiliated sequences decreased in relative abundance. These results suggested that *Syntrophus* species were not growing through oleic acid $\beta$-oxidation either due to out-competition by other populations or to LCFA inhibition. The ability of *Syntrophus* species to degrade oleate remains to be determined (Sousa *et al.* 2009), and their inability to do so could have also contributed to their observed washout. The disappearance of *Proteobacteria*-related species has been previously reported based on PCR-DGGE profiles of anaerobic communities degrading oleic acid in batch (Sousa *et al.* 2007a) and continuous (Baserba, Angelidaki and Karakashev 2012) modes. It has also been suggested that *Deltaproteobacteria* were more strongly associated with the degradation of saturated versus unsaturated LCFA (Hatamoto *et al.* 2007b; Sousa *et al.* 2009). The high-throughput amplicon sequencing indicated that *Smithella*, a genus also within the *Syntrophaceae* family that is phylogenetically closely related to *Syntrophus* (Gray *et al.* 2011), maintained a stable abundance in the bacterial microbiomes over time (Figs 4 and 5B). *Smithella* species have been associated with syntrophic degradation of long-chain alkanes (Gray *et al.* 2011; Embree *et al.* 2014), yet it is unclear if members of *Smithella* take part in $\beta$-oxidizing LCFA. However, a partial genome of an uncultured *Smithella* species obtained from an enriched methanogenic hexadecane-degrading community showed that the bacterium contained genes involved in LCFA $\beta$-oxidization (Embree *et al.* 2014). The saturated LCFA produced during oleic acid degradation (Table S2, Supporting Information) therefore may have supported the population of *Smithella* in the bioreactors. Thus, despite the high target group coverage of the *Syntrophus* qPCR assay developed in this study (Table 2), it is possible that other

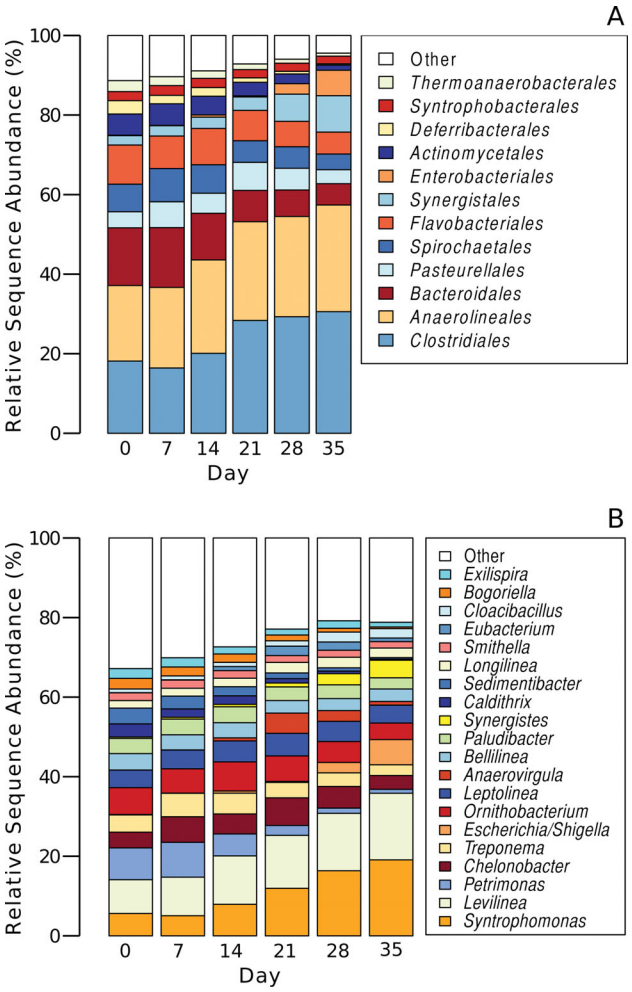


**Figure 5.** Relative abundance of bacterial groups based on high-throughput amplicon sequencing of 16S rRNA genes from bioreactor samples taken on days 0, 7, 14, 21, 28 and 35 showing (**A**) the 12 most abundant bacterial orders and (**B**) the 20 most abundant genera. The groups were ranked based on their maximum observed relative abundances, and all other groups were combined and are shown as 'Other'. The abundances of groups shown at each time point represent an average of the triplicate bioreactors.

members of the *Syntrophaceae* family not targeted by the qPCR assay, such as *Smithella*, were involved in LCFA degradation within the bioreactors.

While previous studies have hypothesized a toxicity of LCFA toward methanogenic archaea (Hanaki, Matsuo and Nagase 1981; Koster and Cramer 1987; Angelidaki and Ahring 1992), the observed increase in the abundance of hydrogenotrophic and acetoclastic methanogens (Fig. 3) suggested that oleic acid and its intermediate degradation products did not exhibit a permanent toxic effect on the methanogenic groups targeted by qPCR. The strong correlation between the initial specific methane production rate and the abundance of methanogenic archaea (Fig. 2B) further showed that higher concentrations of syntrophic $\beta$-oxidizing consortia resulted in more efficient LCFA mineralization in the anaerobic bioreactors. The stable abundance of *Methanosaetaceae* in the bioreactors revealed that this acetoclastic group was growing on the acetate produced through oleic acid $\beta$-oxidation (Table S2, Supporting Information), even though oleic acid has been shown to inhibit acetoclastic methanogens (Hanaki, Matsuo and Nagase 1981; Koster and Cramer 1987; Hwu and Lettinga 1997; Sousa *et al.* 2013). Salvador *et al.* (2013) showed with PCR-DGGE that *Methanosaeta* was the dominant acetoclastic genus in continuously fed bioreactors treating oleate-based waste with LCFA loadings greater than 10 g COD $L^{-1}$ $day^{-1}$. Yet, it was recently shown that oleate was more toxic than palmitate to the acetoclastic methanogens *Methanosaeta concilii* and *Methanosarcina mazei* (Sousa *et al.* 2013). The results of this study provide quantitative evidence suggesting that both hydrogenotrophic and acetoclastic methanogenic groups remained active during oleic acid degradation.

To the best of our knowledge, this is the first application of a high-throughput amplicon sequencing approach to characterize anaerobic LCFA-degrading bacterial communities. The changes in relative abundance of *Syntrophomonas* and *Syntrophus* sequences correlated with the levels predicted by the respective qPCR assays (Pearson's coefficients of 0.99, 0.93, respectively; Fig. S11, Supporting Information), implying that this technique can be used to compliment novel qPCR assays applied to complex environmental samples. The significant difference in phylogenetic structure (weighted UniFrac distances) in the bacterial microbiomes over time (Fig. S9, Supporting Information) showed that growth on oleic acid selected for specialized bacterial populations, which in this case were dominated by *Syntrophomonas* species (Figs 1, 4 and 5). The high-throughput sequencing also detected the growth of bacterial groups not previously characterized to participate in syntrophic LCFA $\beta$-oxidization (Fig. 4). For instance, *E. coli* is known to $\beta$-oxidize LCFA anaerobically with nitrate as a terminal electron acceptor (Campbell, Morgan-Kiss and Cronan 2003); yet, the cause of the increase in relative abundance of *Escherichia/Shigella*-affiliated sequences to 5% in these methanogenic bioreactors (Fig. 5B) is not known. Moreover, increases in the relative abundance of sequences aligning with *Synergistes* and *Levilinea* (Figs 4 and 5), members of which have been respectively associated with syntrophic short-chain fatty acid (Ito *et al.* 2011) and protein degradation (Yamada and Sekiguchi 2009), suggested that the microbial oleic acid-degrading food web was potentially complex. The increased abundance of these groups might be attributable to growth on endogenous decay products and/or on byproducts of LCFA $\beta$-oxidization. However, the ecological role of these groups in oleic acid-degrading communities warrants further investigation.

Collectively, the results of this study contributed new evidence to show that increased mineralization rates of LCFA were a result of quantitative shifts within the microbiome toward a higher abundance of $\beta$-oxidizing *Syntrophomonas* bacteria and methanogenic archaea. The newly developed qPCR assays may thus serve as a novel method to determine the fatty acid $\beta$-oxidization potential of AD reactors based on the concentration of syntrophic $\beta$-oxidizing bacteria within the community. Such an approach may lead to better anaerobic digester feeding strategies that result in stable elevated methane production from waste fats, oils and grease.

## SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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***Conflict of interest.*** None declared.

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