Detection of active, potentially acetate-oxidizing syntrophs in an anaerobic digester by flux measurement and formyltetrahydrofolate synthetase (FTHFS) expression profiling

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Syntrophic oxidation of acetate, so-called reversed reductive acetogenesis, is one of the most important degradation steps in anaerobic digesters. However, little is known about the genetic diversity of the micro-organisms involved. Here we investigated the activity and composition of potentially acetate-oxidizing syntrophs using a combinatorial approach of flux measurement and transcriptional profiling of the formyltetrahydrofolate synthetase (FTHFS) gene, an ecological biomarker for reductive acetogenesis. During the operation of a thermophilic anaerobic digester, volatile fatty acids were mostly depleted, suggesting a high turnover rate for dissolved H₂, and hydrogenotrophic methanogens were the dominant archaeal members. Batch cultivation of the digester microbiota with ¹³C-labelled acetate indicated that syntrophic oxidation accounted for 13.1–21.3% of methane production from acetate. FTHFS genes were transcribed in the absence of carbon monoxide, methoxylated compounds and inorganic electron acceptors other than CO₂, which is implicated in the activity of reversed reductive acetogenesis; however, expression itself does not distinguish whether biosynthesis or biodegradation is functioning. The mRNA- and DNA-based terminal RFLP and clone library analyses indicated that, out of nine FTHFS phylotypes detected, the FTHFS genes from the novel phylotypes I–IV in addition to the known syntroph *Thermacetogenium phaeum* (i.e. phylotype V) were specifically expressed. These transcripts arose from phylogenetically presumed homoacetogens. The results of this study demonstrate that hitherto unidentified phylotypes of homoacetogens are responsible for syntrophic acetate oxidation in an anaerobic digester.

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Abbreviations: HS score, homoacetogen similarity score; IC, inorganic carbon; SIM, selected ion monitoring; SS, suspended solids; TOC, total organic carbon; T-RF, terminal restriction fragment; T-RFLP, terminal RFLP; VFAs, volatile fatty acids.

The GenBank/EMBL/DDBJ accession numbers for the sequences discussed in this study are AB523737 and AB523738 for the archaeal 16S rRNA gene, AB353082–AB353096 for environmental clones of the FTHFS genes and transcripts, and AB523739 for the FTHFS homologue from *Thermacetogenium phaeum*.

INTRODUCTION

In methanogenic environments, the metabolic connection of micro-organisms is essential for the mineralization of organic matter. Methanogenic conversion occurs by a stepwise degradation process consisting of hydrolysis, fermentation, syntrophic oxidation and methanogenesis. Acetate is the most significant precursor of CH₄ and/or CO₂ within this process (Lovley & Klug, 1982; Mountfort & Asher, 1978; Rothfuss & Conrad, 1993; Sansone & Martens, 1981). Two genera of the methanogenic archaea,

Methanosarcina and *Methanosaeta* (formerly called *Methanothrix*), have been determined to be acetoclastic (Jetten *et al.*, 1992). In addition, syntrophic acetate oxidizers associated with hydrogenotrophic methanogens are involved in the degradation of acetate. This syntrophic activity has been found in both natural and engineered environments (Nüsslein *et al.*, 2001; Petersen & Ahring, 1991; Schnürer *et al.*, 1999; Shigematsu *et al.*, 2004).

Anaerobic digesters have been widely used for the treatment of industrial and municipal wastewaters (Lettinga, 1995; Speece, 1996). Up to now, microbial communities that drive anaerobic digestion have been investigated using a variety of molecular approaches (Narihiro & Sekiguchi, 2007; Talbot *et al.*, 2008). Using isotope-tracing assays, syntrophic oxidation and acetoclastic cleavage have been identified as the main mechanisms of acetate degradation in anaerobic digesters (Ahring, 1995; Karakashev *et al.*, 2006; Petersen & Ahring, 1991). A low concentration of acetate and the absence of the acetoclastic methanogen *Methanosaeta* have been reported to be crucial environmental factors for the occurrence of syntrophic acetate oxidation. Furthermore, recent molecular studies have indicated that syntrophic oxidation may play an important role in the activity of thermophilic anaerobic digesters (Goberna *et al.*, 2009; Hori *et al.*, 2006a; Krakat *et al.*, 2010; Ryan *et al.*, 2010). Yet very little is known about the genetic diversity of the micro-organisms involved. Only five syntrophic acetate-oxidizing bacteria have been isolated to date: the strain AOR, *Clostridium ultunense*, *Thermacetogenium phaeum*, *Thermotoga lettingae* and *Syntrophoaceticus schinkii* (Balk *et al.*, 2002; Hattori *et al.*, 2000; Lee & Zinder, 1988b; Schnürer *et al.*, 1996; Westerholm *et al.*, 2010). Also, the complete lack of ecological biomarkers for acetate-oxidizing syntrophs has greatly limited our understanding of these micro-organisms.

Which metabolic pathway is associated with syntrophic oxidation of acetate? Biochemical studies have demonstrated that the homoacetogenic bacterium *T. phaeum* may drive the acetyl-CoA pathway reversibly for syntrophic acetate oxidation (Hattori *et al.*, 2005; Hattori, 2008). Likewise, other syntrophs isolated so far, the strain AOR and *C. ultunense*, could use the acetyl-CoA pathway for both homoacetogenic and acetotrophic reactions (Lee & Zinder, 1988c; Schnürer *et al.*, 1997). Based on these findings, it is expected that syntrophic oxidation depends at least partially on the driving force of the acetyl-CoA pathway (Zinder, 1994). Formyltetrahydrofolate synthetase (FTHFS) is one of the key enzymes for reductive acetogenesis, and the coding gene has been used as a molecular biomarker for studying the ecology and activity of functional consortia (Henderson *et al.*, 2010; Leaphart *et al.*, 2003; Lovell & Leaphart, 2005; Pester & Brune, 2006). *T. phaeum* employs almost the same enzymic apparatus in both syntrophic acetate oxidation and reductive acetogenesis; the FTHFS enzyme is expressed more highly during syntrophic growth than during chemoheterotrophic growth (Hattori *et al.*, 2005). Therefore, FTHFS likely

engages in not only reductive acetogenesis but also syntrophic acetate oxidation.

Thermodynamics indicate that the amount of energy synthesized from reductive acetogenesis ($\Delta G^{\circ} = -104.6 \text{ kJ mol}^{-1}$) is lower than that synthesized from hydrogenotrophic methanogenesis ($\Delta G^{\circ} = -135.6 \text{ kJ mol}^{-1}$); the Gibbs free energy values are calculated under standard conditions (298 K, pH 7, solute at 1 M, gases at 10^5 Pa) (Lee & Zinder, 1988a). Furthermore, thermophilic temperatures are recognized as being unfavourable for reductive acetogenesis (Akutsu *et al.*, 2009). Theoretically, hydrogenotrophic methanogens can metabolize H_2 and CO_2 more under substrate-limiting thermophilic conditions than can homoacetogens (Zehnder & Stumm, 1988). The objective of this study was to investigate the activity and composition of potentially acetate-oxidizing syntrophs, with a focus on reversed reductive acetogenesis, using a combination approach of [^{13}C]acetate flux measurement and expression profiling of the bacterial FTHFS gene.

METHODS

Operation of the thermophilic anaerobic digester. A starter culture was obtained from a thermophilic anaerobic digester treating garbage slurry from restaurants (Hori *et al.*, 2006b; Ueno *et al.*, 2001). The methanogenic microbiota were cultivated at 55°C in a 2.4 l stirred tank reactor (MDL-100, B. E. Marubishi) supplemented with synthetic wastewater at a dilution rate of 0.1 day^{-1} [hydraulic retention time (HRT): 10 days]. The wastewater contained glucose (1 %, w/v) as sole carbon and energy source [organic loading rate (OLR): $400 \text{ mg total organic carbon (TOC) l}^{-1} \text{ day}^{-1}$], and the composition was (g l^{-1}): KH_2PO_4 , 0.908; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.39; NH_4Cl , 0.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.18; yeast extract (Difco), 2.0 and glucose, 10.0; as well as 10 ml vitamin solution containing (mg l^{-1}): biotin, 2.0; folic acid, 2.0; pyridoxine HCl, 10.0; thiamine HCl, 5.0; riboflavin, 5.0; nicotinic acid, 5.0; DL-calcium pantothenate, 5.0; vitamin B_{12} , 0.1; *p*-aminobenzoic acid, 5.0 and lipoic acid, 5.0. The minerals were injected directly into the reactor once per 2 days at the following final concentrations (mg l^{-1}): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.53; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.48; ZnCl_2 , 0.67; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.59; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.16; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.02; H_3BO_3 , 0.063; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0045; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.65. The pH was kept at 7.1 by automatic titration with 5 M NaOH. The reactor was operated stably under steady-state conditions for more than 3 months with little accumulation of volatile fatty acids (VFAs). The reproducibility of reactor performance and microbial community structure was confirmed by the operation of parallel reactors. The analytical methods for monitoring reactor performance have been described previously (Hori *et al.*, 2006a). Briefly, gas production was measured on the basis of liquid displacement. The concentration of VFAs was determined using an HPLC (Alliance 2695, Waters) equipped with an OAPak-A column (Tosoh) and a photodiode array (2996, Waters). Total carbon (TC) and inorganic carbon (IC) were determined using a total carbon analyser (TOC-V CSN, Shimadzu). Total suspended solids (SS) were determined as described previously (Ueno *et al.*, 2001). The culture sample removed from the anaerobic digester under steady-state conditions was used for flux measurement and microbial community analyses.

Flux measurement using batch cultivation with ^{13}C -labelled acetate. The batchwise gas generation test with ^{13}C -labelled acetate reported by Shigematsu *et al.* (2004) was improved to take into

account the effect of the background pool of dissolved IC on the degradation pathway of acetate. Briefly, 15 ml methanogenic culture was removed from the bioreactor and anaerobically placed in 25 ml serum vials, which were then sealed with butyl rubber septa. The headspace of incubation vials was flushed with N₂. The methanogenic slurry was supplemented with [1-¹³C]sodium acetate, [2-¹³C]sodium acetate, or [U-¹³C]sodium acetate (99 atom %, Sigma-Aldrich) at a final concentration of 0.5 mM. Each treatment was performed in duplicate with shaking at 55 °C. After the supplemented [¹³C]acetate had mostly been converted (2 days of incubation), the headspace CH₄ and CO₂ were analysed by a GC-MS (GC17A-QP5050, Shimadzu) equipped with a CP-PoraPLOT Q column (25 m × 0.32 mm, GL Science). Helium gas was used as carrier at a flow rate of 1.5 ml min⁻¹. The column temperature was 35 °C. The CO₂ assay revealed a high background production of unlabelled CO₂ during the incubation, which made it impossible to precisely determine fractions of ¹³CO₂ and ¹²CO₂ from [¹³C]acetate. In the CH₄ analysis using the selected ion monitoring (SIM) mode, a peak at *m/z* 17 was regarded as a fragment ion for ¹³CH₄. A peak at *m/z* 15 included the signals from ¹²CH₃⁺ and ¹³CH₂⁺. On the basis of the MS spectrum of ¹³CH₄, the signal from ¹³CH₂⁺ represented 22.85 % of the peak intensity of *m/z* 17 (data not shown). The calculated signal intensity of ¹³CH₂⁺ was subtracted from the peak intensity of *m/z* 15; this corrected signal that indicates ¹²CH₃⁺ was regarded as fragment ion for ¹²CH₄. The average peak intensities of these fragment ions for duplicate experiments are presented. The production of CH₄ and/or CO₂ from acetate is a direct consequence of the cleavage pathway; in acetoclastic cleavage the carbon of the methyl group in acetate is degraded to CH₄ and that of the carboxyl group is degraded to CO₂, whereas in syntrophic acetate oxidation the carbons of both the groups in acetate are converted to CO₂ (Ferry, 1993; Zinder & Koch, 1984). In addition, the background pool of dissolved IC affects CH₄ production via the syntrophic pathway; ¹³CO₂ formed from syntrophic oxidation of [¹³C]acetate is diluted in the background IC pool present in anaerobic digesters. Taken together, the ratio of ¹³CH₄ to ¹²CH₄ generated from the supplemented [¹³C]acetate was used for quantifying the degree of syntrophic acetate oxidation. A schematic view of the procedure used to determine the mass balance of ¹³CH₄ and ¹²CH₄ is shown in Fig. 1, in which 100 µM ¹³CH₃COOH was degraded via acetoclastic methanogenesis (50 %) and syntrophic oxidation (50 %), and the background pool of dissolved IC was 900 µM.

Extraction of total nucleic acids from the digester microbiota.

Total nucleic acids were extracted from the methanogenic culture using a direct lysis procedure, including bead beating (Noll *et al.*, 2005). Total DNA was prepared by digestion with RNase (type II-A, Sigma-Aldrich) and total RNA was obtained by DNase digestion (RQ1, Promega). The DNA was used for the archaeal 16S rRNA gene-based and FTHFS gene-based analyses. The RNA was used for transcriptional assay of the FTHFS gene.

PCR and RT-PCR. PCR and RT-PCR were conducted for terminal RFLP (T-RFLP) (Liu *et al.*, 1997) and clone library analyses. The primer set Ar109f/Ar912rt was used for the archaeal 16S rRNA gene fingerprinting (Lueders & Friedrich, 2002). This primer set offers good coverage of the 16S rRNA genes from almost all methanogenic archaea, including cultured acetoclastic methanogens. The primer set for the FTHFS gene has been described elsewhere (Lovell & Leaphart, 2005). For T-RFLP, primer Ar912rt and the FTHFS-targeted forward primer were covalently labelled with 6-carboxyfluorescein (FAM). PCR amplification was performed using AmpliTaqGold (Applied Biosystems). The thermal conditions of the 16S rRNA gene-based PCR were as described previously (Hori *et al.*, 2007), except that a total of 20 cycles were employed. The thermal conditions of the FTHFS gene-based PCR have been described elsewhere (Lovell & Leaphart, 2005). RT-PCR of the FTHFS-coding mRNA was carried out using SuperScript III One-Step RT-PCR with Platinum Taq

Fig. 1. Schematic view of the method used to determine the mass balance of ¹³CH₄ and ¹²CH₄. One situation is shown: 100 µM ¹³CH₃COOH was degraded via acetoclastic methanogenesis (50 %) and syntrophic oxidation (50 %), and the background pool of dissolved IC was 900 µM.

(Invitrogen). The thermal conditions of the FTHFS RT-PCR were as reported elsewhere (Pester & Brune, 2006) with slight modifications: we conducted reverse transcription at 55 °C for 45 min and employed 25 cycles in the standard PCR program, during which the annealing temperature was 55 °C. A high temperature (55 °C) for the reverse-transcription and annealing steps was used to avoid the formation of non-specific amplicons. The cycle number used here was the minimal number needed to obtain a sufficient amount of the RT-PCR amplicons for further examination. RT-PCR without reverse transcriptase was used to confirm the absence of DNA contamination in the transcriptional assay.

T-RFLP. The PCR and RT-PCR amplicons were purified by a Wizard SV Gel and PCR Clean-Up system (Promega). The archaeal 16S rRNA gene fragments were digested with the restriction enzyme TaqI (New England Biolabs). The FTHFS gene amplicons were digested simultaneously with MspI and RsaI (New England Biolabs). The digests were suspended in Hi-Di formamide (Applied Biosystems) containing GeneScan 500 or 1000 (Applied Biosystems) as the internal size standard. Prior to electrophoresis, the mixture was denatured at 95 °C for 3 min and then cooled on ice. Terminal restriction fragments (T-RFs) were separated by electrophoresis with a 3130xl genetic analyser (Applied Biosystems), and the T-RF length was determined by comparison with the size standard. T-RFLP profiles were analysed on the basis of the peak size and height using GeneMapper software, version 3.7 (Applied Biosystems). A T-RF with more than 1 % abundance to the total peak height was recognized as being distinct (Dunbar *et al.*, 2001). T-RFLP was conducted for the

same DNA or RNA, and the preparation of T-RF samples was performed in triplicate. A representative profile of the triplicate T-RFLP analyses was used to present a consensus result.

Clone library analysis. The PCR and RT-PCR products were purified using a QIAquick PCR purification kit (Qiagen) and then subjected to ligation with a pGEM-T Easy vector (Promega). The ligation mixture was used to transform *Escherichia coli* JM109 supercompetent cells (TOYOBO) according to the manufacturer's protocol. We randomly selected 29 clones for the archaeal 16S rRNA gene, 43 clones for the FTHFS gene and 46 clones for the FTHFS gene transcripts. The gene segments were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and a 3130xl Genetic Analyzer (Applied Biosystems). The absence of chimeric structure was checked by phylogenetically analysing the terminal stretches at the 5' and 3' ends (Ludwig *et al.*, 1998, 2004). For the FTHFS-based library, clones with >93 % amino acid sequence identity were treated as belonging to an identical phylotype and clones with at least 99.5 % gene sequence identity were defined as having the same genotype (Pester & Brune, 2006). The closest cultured representative was determined by comparing the gene sequence with those in the DDBJ nucleotide sequence database using the BLAST program. A representative clone from each genotype was sequenced from both strands, and the obtained sequences were deposited in the database.

PCR and direct sequencing of the FTHFS homologue from *T. phaeum*. *T. phaeum* (DSM 12270), a thermophilic bacterium that has been positively identified as a syntrophic acetate oxidizer, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection. This bacterium was cultivated under appropriate conditions. DNA extraction from the cell culture and PCR amplification of the FTHFS homologue are described above. The FTHFS gene-based PCR yielded the expected ~1.1 kb gene fragment. The PCR product was purified using a QIAquick PCR purification kit (Qiagen). The DNA segment was used as a template for direct sequencing from both strands with the BigDye Terminator v3.1 Cycle Sequencing kit and a 3130xl Genetic Analyzer.

Phylogenetic analysis of the FTHFS genes. The reference and obtained sequences of the FTHFS amino acids were aligned using CLUSTAL_X software, version 2.00 (Thompson *et al.*, 1997). After the alignment had been corrected manually (e.g. the alignment gaps were ruled out) on the basis of conserved or functionally important areas of FTHFS (Henderson *et al.*, 2010; Leaphart *et al.*, 2003; Lovell & Leaphart, 2005), phylogenetic trees were constructed by the neighbour-joining and maximum-likelihood methods using PHYLIP software, version 3.68 (Felsenstein, 2005), and by the neighbour-joining, maximum-parsimony and maximum-evolution methods using MEGA software, version 4.0.2 (Kumar *et al.*, 2008; Tamura *et al.*, 2007). Use of the different tree construction algorithms resulted in similar dendrogram topology. Bootstrap values were obtained from 1000 replications.

RESULTS

Reactor performance and archaeal population in the anaerobic digester

Under steady-state conditions during the operation of the thermophilic anaerobic digester, acetate concentrations were 0.49–0.60 mmol l⁻¹, and other VFAs, such as propionate and butyrate, were not detected. The TOC and IC concentrations of the culture supernatant were approximately 110 and 560 mg l⁻¹, respectively. The gas production rate was around 580 ml day⁻¹; CH₄ represented more than 80 % of the gas produced.

The amount of total SS as the cell mass in the culture was determined to be 1.34 g l⁻¹. From these chemical parameters, it was determined that approximately 70 % of the supplemented carbon within synthetic wastewater was recovered as the microbial cells and metabolites (e.g. VFAs and gases).

The archaeal population was examined by 16S rRNA gene-based T-RFLP. A total of three peaks were detected in the profile. An 84 bp T-RF accounted for 91 % of the total peak height. A T-RF of 92 bp accounted for 8 % of the total. An archaeal clone library was constructed to phylogenetically characterize these T-RFs. The most frequently detected clone (93 % of the total) was related to the hydrogenotrophic methanogen *Methanoculleus thermophilus* (AJ862839; 100 % sequence identity), which corresponded to the 84 bp T-RF. The remaining clones in the library were affiliated with the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* (AY196660; 99 % sequence identity), corresponding to the 92 bp T-RF. In the T-RFLP profile, the 187 bp T-RF, corresponding to the aceticlastic methanogen *Methanosarcina* sp., was detected but represented less than 1 % relative abundance. The long operation for over 1 year of the anaerobic digester under steady-state conditions resulted in a small increase of *Methanosarcina* sp. to ~5 % of the total archaeal population (data not shown).

Acetate degradation pathway driven by the digester microbiota

In order to characterize the acetate degradation pathway in the anaerobic digester, batchwise cultivation of the slurry micro-organisms was carried out using three different forms of ¹³C-labelled acetate at a concentration of 0.5 mM (Table 1). Fractions of ¹³CH₄ and ¹²CH₄ produced from [1-¹³C]acetate or [2-¹³C]acetate were determined using the GC-MS SIM method. Amendment with [U-¹³C]acetate was done to assay the background production of unlabelled CH₄. The background pool of dissolved IC was 560 mg l⁻¹ (i.e. 46.7 mM), which was taken into account for the calculation of the ratio of ¹³CH₄ and ¹²CH₄ produced from [¹³C]acetate. In the [1-¹³C]acetate treatment, ¹³CH₄:¹²CH₄ was 0.151, indicating that 13.1 % of methane production from acetate was mediated via the syntrophic oxidative pathway. On the other hand, ¹²CH₄:¹³CH₄ was 0.269 in the [2-¹³C]acetate treatment, which indicates that 21.3 % of the supplemented acetate was degraded by syntrophic oxidation. Overall, syntrophic acetate oxidation accounted for 13.1–21.3 % of the total acetate degradation in the anaerobic digester.

Expression profile of the FTHFS genes using mRNA and DNA fingerprinting

The expected fragment (size: approximately 1.1 kb) of the FTHFS gene was successfully amplified using RT-PCR and PCR with total RNA and DNA, respectively, extracted from the digester slurry. T-RFLP of the FTHFS genes and transcripts was conducted (Fig. 2). The pattern of the

Table 1. Distribution of $^{13}\text{CH}_4$ and $^{12}\text{CH}_4$ converted from three different forms of ^{13}C acetate

Substrate*	Peak intensity			Ratio of ¹³ CH ₄ to ¹² CH ₄ [†]	Syntrophic acetate oxidation (%)	
	<i>m/z</i> 15 (¹² CH ₄)		<i>m/z</i> 17 (¹³ CH ₄) actual			
	Actual	¹³ CH ₂ ⁺ subtracted‡				Background subtracted§
¹³ CH ₃ ¹² COONa	2 574 258 (± 110 261)	2 453 832	141 791	527 029 (± 14 883)	0.269	21.3
¹² CH ₃ ¹³ COONa	2 718 708 (± 179 38)	2 705 138	393 097	59 387 (± 5327)	0.151	13.1
¹³ CH ₃ ¹³ COONa	2 449 741 (± 523 94)	2 312 041		602 626 (± 5085)		

*The supplemented ^{13}C acetate was mostly converted to gaseous products.

†The values indicate $^{12}\text{CH}_4$: $^{13}\text{CH}_4$ for the $[2\text{-}^{13}\text{C}]$ acetate treatment and $^{13}\text{CH}_4$: $^{12}\text{CH}_4$ for the $[1\text{-}^{13}\text{C}]$ acetate treatment, which were used for quantifying the degree of syntrophic acetate oxidation.

‡The signal from $^{13}\text{CH}_2^+$ represented 22.85 % of the peak intensity of m/z 17.

§Peak intensity at m/z 15 from $[U\text{-}^{13}\text{C}]$ sodium acetate was regarded as arising from the background production of unlabelled CH_4 .

||All values are averages from duplicate experiments. Values in parentheses represent the variations between the duplicate measurements (i.e. the range).

mRNA fingerprint was different from the DNA pattern, indicating that specific lineages expressed the FTHFS genes. In both the profiles, a T-RF of 138 bp in length was predominant, followed by a 329 bp T-RF. Furthermore, a few peaks, such as that corresponding to a 319 bp T-RF, were detected by the mRNA profiling but not by the DNA analysis. Phylogenetic identification of the T-RFs was performed using the mRNA- and DNA-based clone analyses (Table 2). A total of nine FTHFS phylotypes were obtained from these two libraries. The composition of the mRNA library was quite distinct from that of the DNA library. Phylotype I was a dominant constituent of both the mRNA library and the DNA library, accounting for 32 and 39 % of the total clones, respectively. The expected T-RF length was 138 bp, which corresponded to the primary peak in the T-RFLP fingerprints (Fig. 2). The phylotype was only distantly related to cultured representatives. The sequence identity of amino acids was 76.0–77.1 % to the homoacetogen *Moorella thermoacetica* (Drake & Daniel, 2004). Phylotypes II and III were observed in the mRNA library, whereas no clone belonging to these phylotypes was detected in the DNA library. The T-RF size of phylotype III was predicted to be 319 bp, and only the mRNA T-RFLP detected this phylotype. The phylotype was novel, and the sequence identity of amino acids was 79.0 % to the homoacetogen *Oxobacter pfennigii* (formerly *Clostridium pfennigii*) (Krumholz & Bryant, 1985). Phylotype IV, corresponding to the major peak of 329 bp in the T-RFLP profiles, was detected in both the mRNA library and the DNA library. A small portion (2 %) of the mRNA library originated from phylotype V. Phylotypes VI to IX were recovered only from the DNA library.

Relationship of the FTHFS phylotypes to cultivated acetate-oxidizing syntrophs

The FTHFS gene homologue from the isolated acetate-oxidizing syntroph *T. phaeum* was determined using PCR

and direct sequencing. The FTHFS gene sequence from *T. phaeum* was completely matched with that from phylotype V, one of the phylotypes that transcribed the FTHFS genes, which indicates the phylogenetic identity of these micro-organisms. A phylogenetic tree of the FTHFS amino acids was constructed to characterize the relationship among FTHFS genes obtained from the digester microbiota (i.e. phylotypes I–IX), *T. phaeum* and reference micro-organisms (including homoacetogens and non-homoacetogens) (Fig. 3). Phylotypes I–V that transcribed the FTHFS genes were all affiliated with phylogenetic branches that mainly consist of true homoacetogenic bacteria. In earlier reports (Leaphart & Lovell, 2001; Leaphart *et al.*, 2003; Lovell & Leaphart, 2005), phylogenetic placement of the FTHFS amino acids was used to determine whether or not these bacteria are homoacetogens. However, it has also been reported that the FTHFS gene might be subject to lateral gene transfer (Salmassi & Leadbetter, 2003). More recently, Henderson *et al.* (2010) have proposed a 'homoacetogen similarity' (HS) score based on the conserved FTHFS residues that are diagnostic for homoacetogens. The characteristic amino acids in the homoacetogen FTHFS were improved according to the FTHFS sequence determined from the homoacetogen *T. phaeum*. The HS scores from phylotypes I, II, III and IV that expressed the FTHFS genes were 88, 85, 98 and 79 %, respectively (Fig. 3). These HS scores were rather higher than those from phylotypes VI–IX (i.e. 53–75 %) that did not transcribe the FTHFS genes.

DISCUSSION

In this study, we investigated the metabolic activity and genetic composition of potentially acetate-oxidizing syntrophs, which are responsible for reversed reductive acetogenesis, in a thermophilic anaerobic digester. Applying

Fig. 2. Expression profile of the bacterial FTHFS genes in the bioreactor as revealed by T-RFLP. (a) Profile of the gene transcripts; (b) profile of the genes. T-RFs with >1 % abundance with respect to the sum of all peak heights were considered distinct. The detected T-RF (bp) is given. T-RFs assignable to the FTHFS phylotypes in the clone libraries (see Table 2) are indicated by Roman numerals in bold type. T-RFLP was performed in triplicate, and a representative profile is shown.

^{13}C -labelled acetate at an environmentally relevant concentration (i.e. 0.5 mM) and tracing the fate of the stable isotope using the GC-MS SIM method directly revealed that 13.1–21.3 % of methane production from acetate was mediated by syntrophic oxidation (Table 1). Two different treatments of [$1\text{-}^{13}\text{C}$]acetate or [$2\text{-}^{13}\text{C}$]acetate resulted in similar ratios of syntrophic to non-syntrophic reactions. The percentage of acetate degradation mediated by the syntrophic oxidative pathway is consistent with data obtained elsewhere for other thermophilic processes (Karakashev *et al.*, 2006; Petersen &

Ahring, 1991). The remainder of acetate degradation might be due to aceticlastic cleavage by *Methanosarcina* sp., which has been detected as a minor archaeal population and expresses 16S rRNA in the anaerobic digester (Akuzawa *et al.*, 2011; Hori *et al.*, 2006a).

Inorganic electron acceptors (e.g. nitrate, ferric iron and/or sulfate) were not supplied to the anaerobic digester as the main components of synthetic wastewater. Only a small amount of sulfate was added concurrently with the

Table 2. Frequency and relative abundance of the bacterial FTHFS phylotypes in clone libraries originating from mRNA and DNA

Phylotype*	Number of clones		Expected T-RF (bp)†	Genotype‡	Number of clones§	
	mRNA	DNA			mRNA	DNA
I	15	17	138	G11	13	16
				G12		1
				G13	1	
				G14	1	
II	4		138	G21	4	
III	24		319	G31	24	
IV	2	12	329	G41	2	7
				G42		3
				G43		1
				G44		1
V	1	8	200	G51	1	8
VI		2	117	G61		2
VII		2	138	G71		2
VIII		1	114	G81		1
IX		1	150	G91		1
Sum	46	43				

*Clones with >93 % amino acid sequence identity.

†Expected length (bp) of the T-RF generated from digestion with restriction enzymes *MspI* and *RsaI*.

‡Clones with at least 99.5 % gene sequence identity.

§Distribution of the genotypes within each phylotype.

Fig. 3. Phylogenetic tree showing the relationship of FTHFS phylotypes I–IX. The expected T-RF lengths (bp) and the HS scores (expressed as percentages) of the obtained phylotypes are indicated in parentheses as follows: (T-RF length, HS score). The reference bacteria that have been positively identified as true homoacetogens are presented in bold type. The deduced amino acid sequences were used to construct the tree by the neighbour-joining method using PHYLIP software. No significant effect on the dendrogram topology using the other algorithms was seen. Bootstrap values were obtained from 1000 replications, and >90, 70–89 and 50–69 % are indicated by black, grey and open circles, respectively. Bar, 10 % sequence divergence. GenBank accession numbers of the reference sequences are shown.

supplementation of mineral elements, but the reduction product H_2S was not detectable in the gaseous phase of the reactor operated. VFAs were mostly depleted in the bioreactor. Because the concentration of VFAs has been reported to be positively correlated with the concentration of dissolved H_2 (Cord-Ruwisch *et al.*, 1997; Schink, 1997), the direct substrate H_2 for homoacetogens and hydrogenotrophic methanogens might be turned over at a high rate. Homoacetogenic bacteria can utilize carbon monoxide as an electron donor to obtain reducing equivalents. Methoxylated compounds such as methanol are other possible substrates for homoacetogens. In this anaerobic digester, carbon monoxide and methoxylated compounds

were never detected. Under these substrate-limiting thermophilic conditions, reversed reductive acetogenesis (i.e. syntrophic acetate oxidation) is considered to occur preferentially with respect to reductive acetogenesis. It has been reported that thermophilic temperatures are unfavourable for reductive acetogenesis (Akutsu *et al.*, 2009), and that reversed reductive acetogenesis functions to degrade acetate under thermophilic and methanogenic conditions (Ryan *et al.*, 2010), which supports the hypothesis that reversed reductive acetogenesis, rather than reductive acetogenesis, was the dominant pathway in our anaerobic digester. Taken together, the FTHFS transcription in this study is implicated as the activity of reversed

reductive acetogenesis. It has been demonstrated that the acetyl-CoA pathway also functions in acetate assimilation in a broad range of bacteria such as acetogens and sulfate reducers (Drake, 1994), and thereby FTHFS expression can reflect the outgrowth of such bacteria. However, in this study, the FTHFS genes were transcribed by specific phylotypes (e.g. I and III) (Fig. 2, Table 2). The abundant population of the FTHFS phylotypes did not necessarily transcribe the FTHFS genes, which strongly suggests that FTHFS expression was not accounted for by assimilative metabolism.

The archaeal 16S rRNA gene analyses revealed the dominance of the hydrogenotrophic methanogens *Methanococcus thermophilus* and *Methanothermobacter thermautotrophicus*. These methanogens were possibly fuelled by H₂ produced by fermentative degradation of glucose, the sole substrate during the reactor operation. In addition, from the framework determined by ¹³C flux measurement, these methanogens were possible scavengers of H₂ produced by syntrophic acetate oxidation. Subsequently, the FTHFS expression profiling was able to identify active potentially acetate-oxidizing syntrophic bacteria. Interestingly, phylotypes I–IV (particularly I and III) that transcribed the FTHFS genes were predicted to be homoacetogenic bacteria according to the high HS score (Fig. 3). The results are considered reasonable because most syntrophic acetate oxidizers are also responsible for reductive acetogenesis (Hattori, 2008). Although the sulfate reducer *Thermotoga lettingae* (NC_009828) does not have homoacetogenic activity, it is capable of oxidizing acetate syntrophically with H₂-scavenging methanogens (Balk *et al.*, 2002). This bacterium might use a different oxidative pathway, such as the citric acid cycle, as shown in earlier biochemical studies (Gebhardt *et al.*, 1983; Schauder *et al.*, 1987; Thauer, 1988). In addition, it is also noteworthy that most of the phylotypes that expressed the FTHFS genes were not similar to the isolated homoacetogenic bacteria; only phylotype V was identical to the acetate-oxidizing syntroph *T. phaeum*. These results indicate that as-yet-unidentified or phylogenetically predicted homoacetogenic bacteria played a decisive role in syntrophic acetate oxidation under the steady-state conditions in the anaerobic digester.

T-RFLP and clone library analyses in the FTHFS expression profiling showed that phylotypes I and III transcribed the FTHFS genes (Fig. 2, Table 2). Here a degenerate primer set was used for RT-PCR amplification of the FTHFS-coding mRNA and thus we were not able to precisely estimate the expression level. Also, there was a discrepancy between the peak height in the T-RFLP profiles and the relative sequence abundance in the clone libraries; this might be caused by a toxic effect of the FTHFS gene amplicons on *E. coli* competent cells, as reported elsewhere (Henderson *et al.*, 2010) and/or an insufficient number of the FTHFS gene clones obtained in this study. However, the complementary use of these two methods provided the same information in terms of the existence or nonexistence

of the phylotypes detected. The expression characteristics of phylotypes I and III seemed to be distinct from each other. Phylotype III was detected in the mRNA profiles but not in the DNA profiles, suggesting that a minor population of phylotype III expressed the FTHFS gene. The ecophysiological details and niche differentiation of these phylotypes are presently unknown and require further investigation.

The results of this study demonstrate for the first time, to our knowledge, the functional activity and molecular ecology of potentially acetate-oxidizing syntrophs that are dependent on reversed reductive acetogenesis in the thermophilic anaerobic digester. The combination of ¹³C flux measurement and expression profiling of the FTHFS genes indicates that the hitherto unidentified and phylogenetically presumed homoacetogens are involved in syntrophic oxidation of acetate. Direct identification of the active syntrophic acetate oxidizers by stable isotope probing will provide deeper insight into the microbial phylogeny and ecophysiology (Hori *et al.*, 2007, 2010). The metabolic versatility and survival strategy of the newly identified bacteria should be addressed to clarify their physiological significance for the anaerobic digestion of organic matter.

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REFERENCES

- Ahring, B. K. (1995). Methanogenesis in thermophilic biogas reactors. *Antonie van Leeuwenhoek* **67**, 91–102.
- Akutsu, Y., Li, Y.-Y., Harada, H. & Yu, H.-Q. (2009). Effects of temperature and substrate concentration on biological hydrogen production from starch. *Int J Hydrogen Energy* **34**, 2558–2566.
- Akuzawa, M., Hori, T., Haruta, S., Ueno, Y., Ishii, M. & Igarashi, Y. (2011). Distinctive responses of metabolically active microbiota to acidification in a thermophilic anaerobic digester. *Microb Ecol* **61**, 595–605.
- Balk, M., Weijma, J. & Stams, A. J. (2002). *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. *Int J Syst Evol Microbiol* **52**, 1361–1368.
- Cord-Ruwisch, R., Mercz, T. I., Hoh, C.-Y. & Strong, G. E. (1997). Dissolved hydrogen concentration as an on-line control parameter for the automated operation and optimization of anaerobic digesters. *Biotechnol Bioeng* **56**, 626–634.
- Drake, H. L. (1994). *Acetogenesis*. New York: Chapman & Hall.
- Drake, H. L. & Daniel, S. L. (2004). Physiology of the thermophilic acetogen *Moorella thermoacetica*. *Res Microbiol* **155**, 869–883.
- Dunbar, J., Ticknor, L. O. & Kuske, C. R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl Environ Microbiol* **67**, 190–197.

- Felsenstein, J. (2005).** PHYLIP (phylogeny inference package). Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, WA.
- Ferry, J. G. (1993).** Fermentation of acetate. In *Methanogenesis – Ecology, Physiology, Biochemistry & Genetics*, pp. 304–334. Edited by J. G. Ferry. New York: Chapman & Hall.
- Gebhardt, A., Linder, D. & Thauer, R. K. (1983).** Anaerobic acetate oxidation to CO₂ by *Desulfobacter postgatei* 2. Evidence from ¹⁴C-labelling studies for the operation of the citric acid cycle. *Arch Microbiol* **136**, 230–233.
- Goberna, M., Insam, H. & Franke-Whittle, I. H. (2009).** Effect of biowaste sludge maturation on the diversity of thermophilic bacteria and archaea in an anaerobic reactor. *Appl Environ Microbiol* **75**, 2566–2572.
- Hattori, S. (2008).** Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ* **23**, 118–127.
- Hattori, S., Kamagata, Y., Hanada, S. & Shoun, H. (2000).** *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. *Int J Syst Evol Microbiol* **50**, 1601–1609.
- Hattori, S., Galushko, A. S., Kamagata, Y. & Schink, B. (2005).** Operation of the CO dehydrogenase/acetyl coenzyme A pathway in both acetate oxidation and acetate formation by the syntrophically acetate-oxidizing bacterium *Thermacetogenium phaeum*. *J Bacteriol* **187**, 3471–3476.
- Henderson, G., Naylor, G. E., Leahy, S. C. & Janssen, P. H. (2010).** Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Appl Environ Microbiol* **76**, 2058–2066.
- Hori, T., Haruta, S., Ueno, Y., Ishii, M. & Igarashi, Y. (2006a).** 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.
- Hori, T., Haruta, S., Ueno, Y., Ishii, M. & Igarashi, Y. (2006b).** Direct comparison of single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) to characterize a microbial community on the basis of 16S rRNA gene fragments. *J Microbiol Methods* **66**, 165–169.
- Hori, T., Noll, M., Igarashi, Y., Friedrich, M. W. & Conrad, R. (2007).** Identification of acetate-assimilating microorganisms under methanogenic conditions in anoxic rice field soil by comparative stable isotope probing of RNA. *Appl Environ Microbiol* **73**, 101–109.
- Hori, T., Müller, A., Igarashi, Y., Conrad, R. & Friedrich, M. W. (2010).** Identification of iron-reducing microorganisms in anoxic rice paddy soil by ¹³C-acetate probing. *ISME J* **4**, 267–278.
- Jetten, M. S. M., Stams, A. J. M. & Zehnder, A. J. B. (1992).** Methanogenesis from acetate: a comparison of acetate metabolism in *Methanotherix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Rev* **88**, 181–198.
- Karakashev, D., Batstone, D. J., Trably, E. & Angelidaki, I. (2006).** Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanoaetaceae*. *Appl Environ Microbiol* **72**, 5138–5141.
- Krakat, N., Westphal, A., Schmidt, S. & Scherer, P. (2010).** Anaerobic digestion of renewable biomass: thermophilic temperature governs methanogen population dynamics. *Appl Environ Microbiol* **76**, 1842–1850.
- Krumholz, L. R. & Bryant, M. P. (1985).** *Clostridium pfennigii* sp. nov. uses methoxyl groups of monobenzenoids and produces butyrate. *Int J Syst Bacteriol* **35**, 454–456.
- Kumar, S., Nei, M., Dudley, J. & Tamura, K. (2008).** MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.
- Leaphart, A. B. & Lovell, C. R. (2001).** Recovery and analysis of formyltetrahydrofolate synthetase gene sequences from natural populations of acetogenic bacteria. *Appl Environ Microbiol* **67**, 1392–1395.
- Leaphart, A. B., Friez, M. J. & Lovell, C. R. (2003).** Formyltetrahydrofolate synthetase sequences from salt marsh plant roots reveal a diversity of acetogenic bacteria and other bacterial functional groups. *Appl Environ Microbiol* **69**, 693–696.
- Lee, M. J. & Zinder, S. H. (1988a).** Hydrogen partial pressures in a thermophilic acetate-oxidizing methanogenic coculture. *Appl Environ Microbiol* **54**, 1457–1461.
- Lee, M. J. & Zinder, S. H. (1988b).** Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H₂–CO₂. *Appl Environ Microbiol* **54**, 124–129.
- Lee, M. J. & Zinder, S. H. (1988c).** Carbon monoxide pathway enzyme activities in a thermophilic anaerobic bacterium grown acetogenically and in a syntrophic acetate-oxidizing coculture. *Arch Microbiol* **150**, 513–518.
- Lettinga, G. (1995).** Anaerobic digestion and wastewater treatment systems. *Antonie van Leeuwenhoek* **67**, 3–28.
- Liu, W. T., Marsh, T. L., Cheng, H. & Forney, L. J. (1997).** Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63**, 4516–4522.
- Lovell, C. R. & Leaphart, A. B. (2005).** Community-level analysis: key genes of CO₂-reductive acetogenesis. *Methods Enzymol* **397**, 454–469.
- 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.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998).** Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004).** ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Lueders, T. & Friedrich, M. W. (2002).** Effects of amendment with ferrihydrite and gypsum on the structure and activity of methanogenic populations in rice field soil. *Appl Environ Microbiol* **68**, 2484–2494.
- Mountfort, D. O. & Asher, R. A. (1978).** Changes in proportions of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. *Appl Environ Microbiol* **35**, 648–654.
- Narihiro, T. & Sekiguchi, Y. (2007).** Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. *Curr Opin Biotechnol* **18**, 273–278.
- Noll, M., Matthies, D., Frenzel, P., Derakshani, M. & Liesack, W. (2005).** Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environ Microbiol* **7**, 382–395.
- Nüsslein, B., Chin, K. J., Eckert, W. & Conrad, R. (2001).** Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). *Environ Microbiol* **3**, 460–470.
- Pester, M. & Brune, A. (2006).** Expression profiles of *fhs* (FTHFS) genes support the hypothesis that spirochaetes dominate reductive

- acetogenesis in the hindgut of lower termites. *Environ Microbiol* **8**, 1261–1270.
- Petersen, S. P. & Ahring, B. K. (1991).** Acetate oxidation in a thermophilic anaerobic sewage-sludge digester: the importance of non-aceticlastic methanogenesis from acetate. *FEMS Microbiol Ecol* **86**, 149–158.
- Rothfuss, F. & Conrad, R. (1993).** Thermodynamics of methanogenic intermediary metabolism in littoral sediment of Lake Constance. *FEMS Microbiol Ecol* **12**, 265–276.
- Ryan, P., Forbes, C., McHugh, S., O'Reilly, C., Fleming, G. T. & Colleran, E. (2010).** Enrichment of acetogenic bacteria in high rate anaerobic reactors under mesophilic and thermophilic conditions. *Water Res* **44**, 4261–4269.
- Salmassi, T. M. & Leadbetter, J. R. (2003).** Analysis of genes of tetrahydrofolate-dependent metabolism from cultivated spirochaetes and the gut community of the termite *Zootermopsis angusticollis*. *Microbiology* **149**, 2529–2537.
- Sansone, F. J. & Martens, C. S. (1981).** Methane production from acetate and associated methane fluxes from anoxic coastal sediments. *Science* **211**, 707–709.
- Schauder, R., Widdel, F. & Fuchs, G. (1987).** Carbon assimilation pathways in sulfate-reducing bacteria II. Enzymes of a reductive citric acid cycle in the autotrophic *Desulfobacter hydrogenophilus*. *Arch Microbiol* **148**, 218–225.
- Schink, B. (1997).** Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* **61**, 262–280.
- Schnürer, A., Schink, B. & Svensson, B. H. (1996).** *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. *Int J Syst Bacteriol* **46**, 1145–1152.
- Schnürer, A., Svensson, B. H. & Schink, B. (1997).** Enzyme activities in and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe *Clostridium ultunense*. *FEMS Microbiol Lett* **154**, 331–336.
- Schnürer, A., Zellner, G. & Svensson, B. H. (1999).** Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. *FEMS Microbiol Ecol* **29**, 249–261.
- Shigematsu, T., Tang, Y., Kobayashi, T., Kawaguchi, H., Morimura, S. & Kida, K. (2004).** Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. *Appl Environ Microbiol* **70**, 4048–4052.
- Speece, R. E. (1996).** *Anaerobic Biotechnology for Industrial Wastewaters*. Nashville, TN: Archae Press.
- Talbot, G., Topp, E., Palin, M. F. & Massé, D. I. (2008).** Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Res* **42**, 513–537.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007).** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thauer, R. K. (1988).** Citric-acid cycle, 50 years on. Modifications and an alternative pathway in anaerobic bacteria. *Eur J Biochem* **176**, 497–508.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Ueno, Y., Haruta, S., Ishii, M. & Igarashi, Y. (2001).** Changes in product formation and bacterial community by dilution rate on carbohydrate fermentation by methanogenic microflora in continuous flow stirred tank reactor. *Appl Microbiol Biotechnol* **57**, 65–73.
- Westerholm, M., Roos, S. & Schnürer, A. (2010).** *Syntrophaceticus schinkii* gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. *FEMS Microbiol Lett* **309**, 100–104.
- Zehnder, A. J. B. & Stumm, W. (1988).** Geochemistry and biochemistry of anaerobic habitats. In *Biology of Anaerobic Microorganisms*, pp. 1–38. Edited by A. J. B. Zehnder. New York: Wiley Interscience.
- Zinder, S. H. (1994).** Syntrophic acetate oxidation and “reversible acetogenesis”. In *Acetogenesis*, pp. 387–415. Edited by H. L. Drake. New York: Chapman & Hall.
- Zinder, S. H. & Koch, M. (1984).** Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch Microbiol* **138**, 263–272.

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