Changes in the Acetogenic Population in a Mesophilic Anaerobic Digester in Response to Increasing Ammonia Concentration

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Changes in the acetogenic population were investigated in an experimental laboratory-scale biogas reactor (37°C) subjected to gradually elevated ammonia levels (0.8 to 6.9 g NH₄⁺-N L⁻¹). A shift from aceticlastic acetate degradation to syntrophic acetate oxidation had previously been confirmed in this reactor. In a parallel control reactor, operating at constant ammonia levels (0.65–0.90 g NH₄⁺-N L⁻¹), acetate degradation proceeded via the aceticlastic pathway throughout the operating period (660 d). The acetogenic populations in the reactors were analysed using degenerated primers designed to target the functional gene encoding a key enzyme of the acetyl-CoA pathway, 10-formyltetrahydrofolate synthetase (FTHFS). The analysis consisted of terminal restriction fragment length polymorphism (T-RFLP) analysis coupled with the construction of clone libraries, and quantitative PCR (qPCR) analysis. The T-RFLP data obtained were statistically analysed by non-metric multidimensional scaling. The most abundant FTHFS genes recovered in the clone libraries were assigned to terminal restriction fragments of the T-RFLP profile. The results of the investigation clearly indicated that increased ammonia concentration substantially influenced the putative acetogenic population structure and caused two distinct shifts of the most abundant members; however, the identity of the dominating species remains unknown, as none of the genes had been identified previously. Despite the shifts in the population, the qPCR analysis revealed a relatively stable abundance of the acetogenic population throughout the operation.

Key words: acetogens, methanogenesis, syntrophic acetate oxidation, 10-formyltetrahydrofolate synthetase

Ammonia produced during the anaerobic degradation of proteins is a concern due to its potential inhibitory effects on the acetogenic microbial consortia. In the anaerobic degradation process, ammonia primarily affects methanogens (2), but acetogenic bacteria can also be inhibited (14, 20). Acetogens play a significant role in the anaerobic mineralisation of organic material, where they compete with primary and secondary fermenters for the degradation of monomeric compounds or for fermentation products such as organic acids, ethanol and hydrogen (4). A decline in the activity of methanogens and acetogenic bacteria induces the accumulation of organic acids in the process, causing a decrease in pH, and may be accompanied by process failure (30).

In biogas processes operating under mesophilic conditions (37°C), high ammonia concentrations have been shown to be an important factor in regulating the main pathway of methane formation from aceticlastic to syntrophic acetate oxidation (23, 25). Syntrophic acetate oxidation involves the conversion of acetate to hydrogen and carbon dioxide by syntrophic acetate-oxidising bacteria (SAOB), followed by the reduction of carbon dioxide to methane by hydrogen-utilizing methanogens (8, 32). The majority of the SAOB characterised to date have been identified as acetogens, i.e., Thermacetogenium phaeum, Clostridium ultunense, Syntrophacecticus schinkii, and Tepidanaerobacter acetatoxydans (7, 13, 22, 29).

Acetogens are a very phylogenetically diverse group, but their one common characteristic is utilisation of the acetyl-CoA pathway for assimilation of CO₂ into cell carbon and for the conservation of energy (3). Degenerated primers, designed to target a functional gene encoding a key enzyme in this pathway, 10-formyltetrahydrofolate synthetase (FTHFS) (11), have been used in several investigations to study acetogenic populations in different environments, such as salt marshes (12), hindguts (19), anaerobic sludge (21), human faeces (17) and microbial electrolysis cells (18).

The objective of the present investigation was to examine changes in the acetogenic population structure in an ammonia-stressed, methanogenic process operating under mesophilic conditions. In order to profile the dynamics of the acetogenic population, the degenerated primers mentioned above were used to amplify the partial FTHFS genes. The amplicons were subsequently analysed using terminal restriction fragment length polymorphism (T-RFLP) analysis or preserved as clone libraries for sequence determinations. The putative acetogenic population was also quantified using quantitative PCR (qPCR) analysis.

Materials and Methods

Reactor samples

The samples originated from two anaerobic reactors described by Schnürer and Nordberg (25). These laboratory-scale reactors (4.25 L) were processed semi-continuously and fed daily with source-sorted organic municipal waste (24). The two processes operated at moderate temperature (37°C) for a period of 660 d at an organic loading rate (OLR) of 3 g VS L⁻¹ day⁻¹ (g volatile solids per liter reactor volume and day) and at a hydraulic retention time (HRT) of 30 d. A portion of the feed to the experimental reactor
was replaced with egg albumin powder (Källbergs Industries, Sweden), resulting in a gradual increase in ammonia levels in the process from 0.8 to 6.9 g NH₄⁺-N L⁻¹ (Fig. 1). The control reactor operated at a stable ammonia concentration of 0.65–0.90 g NH₄⁺-N L⁻¹, pH between 7.2 and 7.4, and a volatile fatty acid (VFA) concentration below 0.1 g L⁻¹, respectively. At this point, the methane yield was 0.40 L gVS⁻¹. Previous labelling analysis had established a shift from aceticlastic acetate degradation to syntrophic acetate oxidation in the experimental process when the ammonia-nitrogen concentration had reached 5.5 g NH₄⁺-N L⁻¹ (Fig. 1). In the control reactor, acetate degradation was primarily through syntrophic acetate oxidation in the experimental process when the ammonia-nitrogen concentration was replaced with egg albumin powder (Källbergs Industries, Sweden). The data have been re-plotted from Schnirrer and Nordberg (22).

DNA extraction

Samples of digester sludge were withdrawn from the control and the experimental reactor after 70, 142, 225, 442 and 642 d of operation, and stored at -20°C. Triplicate total genomic DNA samples were extracted from each reactor and sampling point, using the FastDNA Spin kit (Qbiogene, Illkirch, France). Genomic DNA of pure culture was extracted as described previously (27).

Primers and PCR conditions

To study the acetogenic population structure, the degenerated primer pair FTHFSf and fsh1 (5'-GTWTGGCGWARGGGYGGMGAAGG-3') was used with the protocol described previously (31). To quantify the 220 bp T-RF, highly abundant in the experimental reactor at day 442 and 642, the primer pair fsh220f (5'-AAATGTTGTCGCAAGGGAGATGGG-3') and fsh220r (5'-CATAAGTACCGCAA CAGCACCTTC-3') was constructed using Geneious Pro version 4.8.5 (Biomatters, Auckland, New Zealand). The primer specificity was evaluated against the GenBank database using BLAST (1). The PCR program consisted of an initial step at 95°C for 3 min, 30 cycles of 30 s at 95°C, 1 min at 65°C and 30 s at 72°C, followed by a final step of 10 min at 72°C. PCR amplifications were conducted with the Ready-To-Go PCR kit (GE Healthcare, Buckinghamshire, UK) and the PCR products were visualised with ethidium bromide staining.

Bacterial strains used for primer evaluation

To complement previous comprehensive evaluations of the primer pair FTHFSf/FTHFSr (11) and fsh1/FTHFSr (31), and the primer pair constructed in the present study (fsh220f/fsh220r), representative acetogens known as syntrophic acetate oxidisers were used to test primer specificity: *Thermacetogenium phaeum* (DSM 12270) obtained from DSMZ (Braunschweig, Germany); *Clostridium ultunense* (DSM 10521), *Syntrophaceticus schinkii* (JCM 16669), and *Tepidanaerobacter acetatoxidans* (DSM 21804) obtained from the Department of Microbiology, Swedish University of Agricultural Sciences (Uppsala, Sweden).

**T-RFLP and NMS analysis**

FTHFS genes were amplified using T-RFLP analysis using the PCR conditions described above. The 5' end of the FTHFSr primer was labelled with 6-carboxyfluorescein (FAM). Each 25 μL PCR reaction contained 25 pmol of the FTHFSf and FTHFSr primer pair and 70 ng genomic DNA, extracted from the reactor samples. Triplicate PCR reactions of each DNA preparation were performed and the corresponding PCR products were pooled to reduce potential bias. The FAM-labelled PCR products of anticipated length (~1,100 bp) were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and digested at 37°C overnight by the restriction enzyme *Ddel* (New England Biolabs, Herts, UK). Fluorescently labelled terminal restriction fragment lengths (T-RFs) were separated and detected with an ABI3730XL capillary sequencer (Applied Biosystems, Carlsbad, CA, USA). The size of the fluorescently labelled T-RFs was established by comparison with the GS ROX 500 internal size standard (Applied Biosystems). T-RFLP profiles were analyzed with the software Peak Scanner (Applied Biosystems), including peaks ranging from 50 to 664 bp. The relative abundance of the individual T-RFs was calculated by dividing the peak area by the total area of all peaks. T-RFs constituting less than 0.5% of the total peak area were excluded.

Correlations between T-RFLP data and concentration of NH₄⁺-N in the processes were analysed by NMS (Non-metric Multidimensional Scaling) using PC-ORD v. 5.10 (MJM Software, Oregon, USA). Bray-Curtis distance measurement was used to produce distance matrices, which were based on arcsin-transformed relative abundance values of T-RFs and the NH₄⁺-N data, relativised by column totals. NMS ordination was performed with a random starting configuration, a maximum of 250 iterations, and an instability criterion of 0.00001. A two-dimensional solution was recommended for the data and was shown to explain 84% of the variation in the species composition (cumulative R²=0.836). To maximise the variance of the different data points included in the ordination, it was rotated using Varimax (9). Potential correlations between NH₄⁺-N values and community structures were evaluated and visualised as vectors in joint plots. In addition, Mantel’s test (15) with 999 randomised Monte Carlo simulations was applied to evaluate the null hypothesis, assuming no relationship between the two distance matrices.
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Construction of FTHFS clone libraries and phylogenetic analysis

In order to identify specific acetogenic species, clone libraries were constructed using DNA extracted from the control reactor (day 442 and 642) and the experimental reactor (day 225, 442 and 642). PCR products from triplicate DNA extractions from the reactor samples were retrieved using the PCR protocol specified above and the FTHFSf and FTHFSr primer pair. The triplicate amplicons were pooled and gel-purified as described above. Clone libraries were constructed using PCR 4-TOPO vector and Escherichia coli TOP10 cells (Invitrogen, Carlsbad, CA, USA), or the pGEMT easy vector and JM 109 cells (Promega, Madison, WI, USA). Clones were analysed by colony PCR using the PCR conditions described above and screened by restriction fragment length polymorphism (RFLP) analysis, using the restriction enzymes DdeI or HhaI and HaeIII (1 U μL−1; New England Biolabs). The clones were grouped according to their restriction pattern and the recovery of the clone libraries was calculated according to the formula given by Good (6). Plasmids from representatives of each group were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced by the Uppsala Genome Center using the M13 forward (5'-CCCAGTCAG GACGTTGTAAAACG-3') and M13 reverse (5'-AGGGATAA CAATTTCACAGG-3') primers. The sequences obtained were edited using Geneious Pro version 4.8.5 (Biomatters) and compared with sequences available in the Blast database (1). Sequences of close relatives and reference sequences were recovered from GenBank. Deduced amino acid sequences of the obtained and reference sequences were aligned, and a phylogenetic tree was constructed by the neighbour-joining method, using MEGA software version 4 (26), with complete deletion of gaps and missing data. The stability of the tree branches was assessed by the bootstrap method (1,000 replicates).

qPCR analysis

Standard for quantification of the total putative acetogenic population was constructed using amplicons generated from genomic DNA of T. acetatoxydans with the primer pair FTHFSf and fsh1 and the protocol described previously (31). Standard for quantification of the 220 bp T-RF consisted of amplicons generated from the partial FTHFS gene corresponding to the 220 bp fragment, retrieved from the clone library of the experimental reactor (day 442 and 642), and the primer pair fsh220. The PCR products were gel-purified and cloning, transformation and isolation of plasmids were performed as described above. DNA concentration in the purified standards was determined with a Nanodrop spectrophotometer (GE Healthcare). The standards were prepared to a concentration of 10^8 molecules μL−1 and were then 10-fold serially diluted and standard concentrations from 10^8 molecules μL−1 down to 10^4 molecules μL−1 were run in duplicate or triplicate alongside the qPCR assay. Quantitative PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For quantification of the total acetogenic population, a previously described protocol was used (31). qPCR analysis of the 220 bp T-RF was performed applying the following conditions: 7 min at 95°C; 40 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 1 min, and extension at 72°C for 30 s. At the end of each qPCR assay, a temperature melt curve was performed to monitor artefacts such as primer dimer formation and to assess whether non-specific amplification had occurred (55–95°C, ΔT=0.1°C s−1). The 20 μL reaction contained 3 μL DNA template, 3.5 μM of each primer and 10 μL IQ Supermix PCR reagent (Bio-Rad) with SYBR-Green 1 for fluoroscent DNA intercalation. To assess levels of background contamination, triplicate wells containing a reaction mix without template DNA were included in each assay. Logarithmic values of the concentration of the gene were plotted against the threshold cycle (Ct) number and used for estimation of gene abundance in unknown samples. Results were reported as average gene abundances per milliliter of reactor sludge.

Nucleotide sequence accession numbers

The sequences of the FTHFS clones obtained in this study have been deposited in GenBank under the following accession numbers: HM365334–HM365337 from the control reactor (days 442 and 642 of operation); HQ156223, HM365339 and HM365340 from the experimental reactor (days 225, 442 and 642 of operation).

Results

PCR

PCR amplification with the FTHFSf and FTHFSr primers and the primer pair fsh1 and FTHFSr yielded specific products of anticipated length (~1,100 bp and 250 bp, respectively) from all reactor samples. A second PCR product of ~1,200 bp was retrieved with the FTHFSf and FTHFSr primers from samples extracted from the control reactor on days 442 and 642 of operation. Cloning and sequencing analysis of the unspecific products revealed high identity to eukaryotic FTHFS genes and this product was thus not included in the T-RFLP analysis or in the later construction of clone libraries. In evaluation of the specificity of the FTHFSf and FTHFSr primers for SAOB, PCR products of 1,100 bp were retrieved from pure culture of C. ultunense; however, no product above the detection limit of ethidium bromide staining was generated from T. phaeum, S. schincki, or T. acetatoxydans. The fsh1 and FTHFSr primer pair generated products of 250 bp from C. ultunense, T. acetatoxydans, T. phaeum and from the partial FTHFS gene corresponding to the 220 bp T-RF in the experimental reactor (day 442 and 642). No product was retrieved from any of the analyzed syntrophic acetate-oxidising bacteria using the primer pair fsh220.

T-RFLP analysis

The results of T-RFLP analysis revealed a relatively stable acetogenic population structure in the control reactor during the entire period of operation (Fig. 2). Four major T-RFs with relative abundances of at least 4% were detected in the control reactor (days 70–442). The fragment of 531 bp predominated, whereas the other T-RFs consisted of 79, 141 and 255 bp. The final sampling point (day 642) of the control reactor showed a profile with reduced 79 and 531 bp fragment peaks and instead the appearance of a highly abundant 203 bp fragment. The profile of the control reactor included the 203 bp T-RF already at days 225 and 442 of operation, but with clearly lower relative abundance.

At the first sampling point, the experimental reactor had a comparable profile to the control reactor, including a highly abundant 531 bp fragment and minor T-RFs of 79, 141 and 255 bp. A highly abundant T-RF of 662 bp, which was present in the control reactor at low relative abundance on days 442 and 642 of operation, was also detected in the first sampling of the experimental reactor. When the ammonia-nitrogen content increased from 0.8 to 1.9 g NH₄⁺-N L⁻¹ (from day 70 to 142) in the experimental reactor, a discernible shift in the most abundant acetogenic population members occurred. The relative abundance of the 79 bp fragment increased considerably, whereas the 533 and 662 bp T-RFs were reduced in abundance, or vanished completely. The minor 141 and 255 bp T-RFs remained practically unchanged. An additional change in the dominance of the population became...
apparent when the ammonia concentration increased from 3.3 to 5.5 g NH$_4$$^+$-N L$^{-1}$ (from day 225 to 442). At this point, a dominant T-RF of 220 bp accounted for about 90% of the total fluorescence intensity.

Correlations between acetogenic population dynamics and ammonia concentrations in the processes were further analysed by NMS (Fig. 3). The ordination demonstrated a significant correlation between the T-RF pattern and increasing NH$_4$$^+$-N concentration in the experimental reactor on days 442 and 642 of operation. The T-RFs of the experimental reactor on days 142 and 225 were grouped together and moderately correlated to increasing NH$_4$$^+$-N concentration. The samples from the control reactor on days 70–642 and from the experimental reactor on day 70 clustered together and had no significant correlation to NH$_4$$^+$-N concentration. The differences in acetogenic community structure between the samples and dissimilarities in NH$_4$$^+$-N ($R=0.55$, $P<0.001$) were found to be correlated by Mantel’s test.

**FTHFS gene libraries and phylogenetic analysis**

Samples used for construction of clone libraries originated from the control reactor on days 442 and 642 and the experimental reactor on days 225, 442 and 642. The sequences encoding partial FTHFS homologues were aligned and compared. Sequences that shared identity of more than 99.5% were considered to represent the same genotype.

The most abundant FTHFS genes recovered in the clone libraries from the control reactor (day 442 and 642) were assigned to terminal restriction fragments of the T-RFLP profile. The clone libraries from the control reactor had a recovery of 92–93%. The partial $fhs$ sequences representing the T-RFs of 141 bp, 255 bp, 531 bp and 662 bp were retrieved from the control reactor on day 442 at rates of 3%, 61%, 22% and 14%, respectively. The sequences corresponding to these T-RFs were revealed to have relatively low maximal identity (~70%) to available FTHFS gene sequences in the database. The partial FTHFS sequence representing the relatively highly abundant 203 bp fragment in the control reactor on day 642 of operation proved to be slightly longer than the other sequences obtained and had close identity to eukaryotic FTHFS gene sequences. From the experimental reactor, the recovery of the clone libraries was 94–100%. On day 225, 83% of the clones corresponded to the highly abundant 79 bp T-RF and the sequence had low identity (74%) compared with the available FTHFS gene sequences in the database. The rest of the obtained clones corresponded to a partial FTHFS gene with no restriction site for the enzyme.
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used in T-RFLP analysis. In the clone library constructed from DNA samples from the experimental reactor on day 442 and 642, 97% of the clones retrieved represented the partial FTHFS gene sequence appearing as the highly abundant 220 bp T-RF. This sequence had 77% identity to an FTHFS gene of an uncultured environmental landfill bacterium (GenBank accession number AF459461; Fig. 4). The phylogenetic placement of the dominant sequences obtained from sludge samples from the reactors is presented in Fig. 4.

qPCR analysis

The results from qPCR analysis of the acetogenic population in the reactors showed relatively stable abundance in the control reactor, whereas minor fluctuations in the experimental reactor were observed. The average FTHFS gene abundance was about $10^{-10}$–$10^{8}$ gene copies mL$^{-1}$, which can be compared to the total bacterial abundance of $10^{15}$–$10^{16}$ rRNA gene copies mL$^{-1}$, retrieved in previous investigation (28) of the control and experimental reactor. The quantification of the FTHFS gene, corresponding to 220 bp T-RF, demonstrated high abundance in the experimental reactor on days 442 and 642. No or low presence of this gene was observed in the other reactor samples. The average log gene abundances obtained in the qPCR analyses from the triplicate reactor samples are presented in Table 1.

Discussion

In this study, the putative acetogenic population succession
in two anaerobic reactors was profiled during an operating period of more than 600 d. The experimental reactor stressed by high concentrations of ammonia displayed an altered T-RFLP profile, with two distinct shifts of the most abundant acetogenic population members. The change in the T-RFLP profile was further confirmed by quantitative analysis of the partial FTHFS gene, corresponding to the dominant T-RF (220 bp) in the experimental reactor on day 442 and 642 (Table 1). In contrast, the control reactor had a relatively stable structure throughout the experimental period, which supported the hypothesis that the changes observed in the experimental reactor occurred due to increased ammonia concentration. The NMS ordination further demonstrated the significant impact of increasing ammonia levels on the acetogenic population structure. Here, almost all samples from the control reactor clustered together, an indication of the stability of the population in the process during the complete operation. In addition, the T-RFLP profile of the experimental reactor on day 70 associated with the population profiles of the control reactor, demonstrating that the two processes started with a relatively similar structure of the acetogenic population; however, the T-RFLP profile of the experimental reactor after the initial sampling point was separated from this cluster and a strong correlation with the NH$_4^+$-N concentrations was observed on days 442 and 642. This finding was in accordance with the T-RFLP profile, which indicated the occurrence of a distinct acetogenic population on day 442 and 642 compared with the other sampling occasions.

Molecular approaches using the FTHFSf and FTHFSr primer pair as specific probes for acetogens have proven to be a useful tool for profiling these functional bacteria in several diverse environmental habitats. Regardless of the phylogenetic diversity of the group members, these primers have successfully targeted an extensive range of acetogens; however, the specificity of the degenerated FTHFSf and FTHFSr primer pair used to quantify the total acetogenic population in the present study targeted both C. ultunense, S. schinkii, and T. acetatoxydans seemed to be restricted and thus not all acetogens involved in syntrophic acetate oxidation were targeted in the T-RFLP profile and the clone libraries.

Interestingly, the qPCR analysis showed that ammonia had no impact on the quantity of the total acetogenic population, since the abundance was relatively stable and at a similar level in both the control and the experimental reactor. The slightly higher variation in abundance in the experimental reactor compared with the control reactor throughout the operating period could be explained by the observed shift in the acetogenic population structure. The quantitative changes in SAOB and the methanogenic population in the control and experimental reactor were investigated in a previous study in SAOB and the methanogenic population in the control and operating period could be explained by the observed shift in the experimental reactor occurred due to increased ammonia concentration. Here, almost all samples from the control reactor clustered together, an indication of the stability of the population in the process during the complete operation. In addition, the T-RFLP profile of the experimental reactor on day 70 associated with the population profiles of the control reactor, demonstrating that the two processes started with a relatively similar structure of the acetogenic population; however, the T-RFLP profile of the experimental reactor after the initial sampling point was separated from this cluster and a strong correlation with the NH$_4^+$-N concentrations was observed on days 442 and 642. This finding was in accordance with the T-RFLP profile, which indicated the occurrence of a distinct acetogenic population on day 442 and 642 compared with the other sampling occasions.

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