Growth Characteristics and Thermodynamics of Syntrophic Acetate Oxidizers

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ABSTRACT: Syntrophic acetate oxidation (SAO) plays a pivotal role in biogas production processes when aceticlastic methanogens are inhibited. Despite the importance of SAO, the metabolic interactions and syntrophic growth of the organisms involved are still poorly understood. Therefore we studied growth parameters and interactions within constructed defined co-cultures comprising the methanogen *Methanoculleus bourgensis* and one, or several, of the syntrophic acetate oxidizers *Syntrophaceticus schinkii*, [*Clostridium*] *ultunense*, *Tepidanaerobacter acetatoxydans*, and a novel, uncharacterized bacterium. Cultivation experiments in a design-of-experiment approach revealed positive effects on methane production rate of increased ammonium levels (up to 0.2 M), temperature (up to 45°C) and acetate concentrations (0.15-0.30 M). Molecular analyses and thermodynamic calculations demonstrated close interlinkages between the microorganisms, with available energy of -10 kJ/mol for acetate oxidation and -20 kJ/mol for hydrogenotrophic methanogenesis. The estimated generation time varied between 14-24 days for the bacteria and 13-29 days for the methanogen and the acetate minimum threshold level was 0.40-0.45 mM. The rate of methanogenesis depended on the SAO bacteria present in the culture. These data are beneficial for interpretation of SAO prevalence and competiveness against aceticlastic methanogens in anaerobic environments.

INTRODUCTION

Anaerobic digestion is an efficient waste management technique to convert organic material to biogas, a renewable fuel that can replace fossil fuel in heating/cooling, electrical power generation and transportation.[1] Several organic wastes from agriculture, households, and food manufacturing industries contain proteins, and thus have high biogas potential. The digestate generated from these wastes after anaerobic degradation is also a nutrient-rich and valuable biofertilizer.[2] However, high protein content can be a major drawback in biogas systems, due to formation of excess ammonia during protein degradation. Ammonia is toxic to many microorganisms and thus often gives rise to process instability and decreased biogas yields.[3] Increasing acetate level is often the first chemical signal of microbial ammonia inhibition, an effect related to the reduced activity of ammonia-sensitive aceticlastic methanogens.[4] Instead, ammonia-tolerant microorganisms performing syntrophic acetate oxidation (SAO) in association with hydrogenotrophic methanogens may take over.[4, 5] SAO involves a mutualistic interaction between syntrophic acetate-oxidizing bacteria and hydrogen (H_2)-oxidizing methanogens. The syntrophic bacteria oxidize acetate to H_2 and carbon dioxide (CO_2) or formate, which are subsequently converted to methane (CH_4) by H_2-utilizing methanogens. The bacteria rely on the methanogenic activity, since acetate oxidation rapidly becomes endergonic when H_2 accumulates.[6] Nevertheless, the H_2 (or formate) level still needs to be sufficient to favor the hydrogenotrophic methanogens and is thus restrained within a low and narrow range.[7] Only a few bacteria capable of SAO have been isolated and characterized to date. These include the mesophiles [*Clostridium*] *ultunense*[8] and *Syntrophaceticus schinkii*,[9] the thermotolerant *Tepidanaerobacter acetatoxydans*,[10] and the thermophiles *Thermacetogenium phaeum*[11] and *Pseudothermotoga lettingae*.[12, 13] These bacteria have the special physiological feature that they can utilize various substrates in pure culture, in addition to oxidizing acetate in SAO.[14] Co-culturing and genomic studies of some of these isolates have revealed and confirmed potential

physiological and metabolic capabilities of their syntrophic cooperation.[15-20] However, many key aspects within this area remain unexplored. Thus, further insights into syntrophic growth and metabolism are critical in order to reveal the behavior of these organisms in response to ecological parameters and their ability to compete for acetate under various conditions.

The aim of the present study was to identify the thermodynamic constraints on SAO under mesophilic conditions and to examine the impact of environmental parameters on the growth rate of defined SAO co-cultures. The impact of temperature, pH, ammonium, and initial acetate concentration on methane production and acetate degradation rate was established in batch cultures comprising *Methanoculleus bourgensis* and one, or several, of the syntrophic acetate oxidizers *S. schinkii*, [*C.*] *ultunense*, and *T. acetatoxydans* and a novel, yet to be characterized, bacterium. To allow calculation of thermodynamic constraints, the communities were maintained and sub-cultured in medium with sodium acetate as the sole energy source and H_2 partial pressure, pH, temperature, CO_2, methane formation, and acetate degradation were monitored throughout the growth experiments. The 16S rRNA gene abundance of the main species was quantified to obtain information on the interplay between the organisms in the co-cultures. The intention was to gain fundamental insights into the syntrophic mechanism of acetate oxidation, which will be useful in developing strategies to improve high-ammonia biogas processes.

MATERIALS AND METHODS

Organisms and Media. The syntrophic acetate-oxidizing bacteria [*C.*] *ultunense* strain Esp JCM 16670, *S. schinkii* strain Sp3 JCM 16669,[9] and *T. acetatoxydans* strain T1 DSM 21804[10] and the hydrogenotrophic methanogen *M. bourgensis* strain MAB1[21] used in this study were isolated at the Department of Microbiology, Swedish University of Agricultural Sciences. Recent phylogenetic analysis has affiliated [*C.*] *ultunense* to the family Tissierellaceae and not Clostridiaceae[17]. Therefore, the family[MOU1] name of this species awaits amendment. The

family name for [*C.*] *ultunense* is thus given in brackets. For co-cultivation, *S. schinkii* and *M. bourgensis* were inoculated (culture co_s) and another defined culture was established by inoculating all three SAO bacteria and *M. bourgensis* (culture co_{mix}). However, 16S rRNA gene Illumina sequencing revealed presence of an additional uncharacterized species, strain AMB_1 (*Keratinibaculum paraultunense* as closest relative with 96% sequence identity) in the methanogenic culture and it was thus also included in the co_s and co_{mix} cultures.

All cultures were grown in bicarbonate-buffered basal media with yeast extract (0.2 g/L) containing sodium acetate and ammonium chloride (NH_4Cl), prepared as described in Westerholm *et al*.[9] The initial pH of the media was 7.3. For incubation at pH 8.1, changes in medium composition and minor pH adjustments were performed as described in Westerholm *et al*.[10] The cultures were inoculated with co-cultures that had depleted acetate (100 mM) to below detection and were incubated in darkness, without shaking, at set temperatures. Temperature was monitored continuously during growth.

Analytical Procedures and Molecular Analyses. Methane and CO_2 in the gas phase were quantified by gas chromatography and concentration of volatile fatty acids (VFA, including acetate, propionate, butyrate, isobutyrate, valeriate, isovaleriate, capronate and isocapronate) in the liquid phase was determined using high-performance liquid chromatography as described previously.[9] In order to determine the final VFA levels, analyses using gas chromatography (6890 Series, Hewlett Packard, USA) were conducted as described previously.[22] The partial pressure of H_2 (p_{H2}) was analyzed by direct injection of 1 mL headspace gas (by syringe) from the cultures into a gas chromatograph with a reducing compound (HgO bed) photometer (Peak Performer Reduced Gas Analyzer PP1, Peak Laboratories, CA, USA). After being separated from the matrix gas through a system of two packed columns (a zeolite MS13X 60/80 column with length 16.5" and a Unibead silica 1S 60/80 column with length 81") using N_2 carrier gas, H_2 was indirectly detected and quantified by the mercury vapor liberated from the heated HgO

bed with an ultraviolet (UV, 254 nm) absorption photometer. A dilution of 20 000 ppm H_2 standard (Aga, Sweden) was used for calibration.

Extraction of total genomic DNA, construction of standard curves, and quantitative polymerase chain reaction (qPCR) analysis were conducted as described elsewhere.[23] 16S rRNA genes of [*C.*] *ultunense*, *S. schinkii*, *T. acetatoxydans*, and *M. bourgensis* were targeted using primer pairs Cult, THAC, Tp, and MAB developed by Westerholm *et al*.[23, 24] For targeting the novel strain AMB_1 in the co_{mix} culture, the primers AMB_1_1028F (5´-AACGAGCGCAACCCCTATTT-3´) and AMB_1_1227R (5´-CTGGGATCGGCTTTTTGGGA-3´) were designed using Geneious v6.1. To evaluate their specificity, these new primers were tested with genomic DNA from pure culture of all strains present in the co_{mix} culture. The qPCR protocol for analysis of AMB_1 consisted of 7 min at 95°C, 40 cycles of 95°C for 30 s, annealing at 60°C for 1 min and 72°C for 30 s, and finally temperature melt curve analysis. Construction of 16S rRNA gene amplicon libraries, Illumina MiSeq sequencing, and data analyses were carried out as described previously[25], with the exceptions that fastp v. 0.19.5[26] was used for quality control and read filtering, the software package Divisive Amplicon Denoising Algorithm 2 (DADA2)[27] version 1.6.0 was used for further processing, and the forward and reverse reads were truncated at positions 250 and 200, respectively. Assignment of taxonomy was performed with the DADA2 taxonomy classification, using the Silva training set v. 132. The data were organized into a single data object (*phyloseq* package[28]) and the graphic was produced using the plot_bar function (*ggplot2* package[29]) in R Studio software.[30] Reads were assigned at species level using the Blast algorithm[31] provided by the National Center for Biotechnology Information (NCBI http://www.ncbi.nlm.nih.gov). Raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under the study accession number PRJNA514445.

Cultivation Experiments. Two different cultivation experiments were conducted in this study, focusing on I) analysis of thermodynamics and II) determination of growth parameters for optimized methane production rates and calculation of doubling times.

Experiment I) Cultivation and thermodynamic calculations. Triplicate anaerobic batches of each culture (co_s and co_{mix}) were prepared using 200 mM acetate, 0.2 M NH_4Cl, and initial pH ($pH_{initial}$) of 7.3. Incubation was at 37°C, giving a free ammonia level of 0.09 g NH_3-N/L. The cultures were grown in 0.5 L glass flasks[JD2][MOU3], containing 0.25 L media and were inoculated with 12.5 mL of co-culture that had depleted acetate to below detection. Triplicate cultures incubated in medium without acetate served as negative controls. Determination of gas composition (H_2, CH_4, and CO_2) and analyses of liquid samples (pH, acetate and 16S rRNA gene abundance) were conducted throughout. Liquid samples of 1 mL for DNA extraction were collected during growth and stored at -20°C until further analysis. For the thermodynamic calculations, the reactions evaluated were: $CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$ (reaction 1; $\Delta G° = 55.0$ kJ), $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ (reaction 2; $\Delta G° = -130.8$ kJ), and $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ (reaction 3; $\Delta G° = -75.8$ kJ). Temperature corrections for 37°C versus 25°C (the standard temperature) were made using the Gibbs-Helmholz equation, with H_2, CO_2, and CH_4 in the gas phase (standard conditions: partial pressure at 1 atm), and acetate in the aqueous phase (standard concentration at 1 M).[32] G_f and H_f values were taken from Hanselmann.[33] Gibbs free energy (ΔG) values pertaining to actual concentrations and partial pressures were calculated using the Nernst equation, as described previously.[32] All calculation procedures are described in detail in Dolfing.[34]

Kinetic parameters. The generation time was calculated from the qPCR results using the logarithmic growth equation $N_t = N_0 \times e^{\mu t}$, where N_t is the number of cells at time t, N_0 is the number of cells at time zero, and μ is the growth rate constant. The value of μ (assumed constant) was calculated from the slope of the 16S rRNA gene increase during the period of

exponential growth. The generation time (g) was calculated as $g = (\ln 2)/\mu$, an expression derived from the logarithmic growth equation. The acetate threshold level was determined in these cultures after 100-200 days of cultivation.

Experiment II) Determination of methane production rates at different pH, temperature, ammonia and acetate levels and calculation of doubling times. The co_{mix} culture experiment was intended to generate data relevant for efforts to improve operating strategies in biogas digesters, particularly regarding the impact on methane production rate of temperature, ammonium level, and initial acetate concentration. The experiment was set up using the design-of-experiment approach (Modde software v. 11, Umetrics AB, Umeå, Sweden) and was run in two sets, at $pH_{initial}$ 7.3 and at $pH_{initial}$ 8.1. The range of the factors for both pH levels was set at 0.05-0.34 M NH_4Cl, 30-46°C, and 0.05-0.4 M sodium acetate (Table S1.1), with the intention of mimicking typical biogas digester conditions. The cultures were grown in 0.25 L glass flasks, containing 0.08 L media and were inoculated with 4 mL of co-culture that had depleted acetate to below detection. Methane yield was monitored one to two times a week until gas production ceased, and the logarithmic increase in methane concentration in the individual batches was recorded as the response factor. Response surface methodology (RSM), which evaluates the data with multiple linear regression, and a central composite face-centered design with three levels (low, middle, and high) were used in the Modde software to conduct the full factorial design. Duplicate samples at each set point were analyzed and three replicates of the central point were included in the design, to give a final experimental matrix of 60 batch experiments, in total representing 15 different set-up conditions, at each pH level (Table S1.1). Methane production and acetate degradation were measured throughout. The doubling times ($t_{d,CH4}$) were estimated using the specific methane production rate (μ_{CH4}) in the equation $t_{d,CH4} = \ln 2/\mu_{CH4}$, where μ_{CH4} was calculated from the slope of logarithmic methane increase during exponential methane production.

RESULTS AND DISCUSSION

To better understand the kinetics and ecophysiology of the organisms involved in syntrophic acetate oxidation, we created two syntrophic acetate-oxidizing communities, co_s and co_{mix}. Both communities relied on *M. bourgensis* as the hydrogen scavenger. This methanogen is an efficient hydrogen consumer in mesophilic SAO cultures[8-10] and is prevalent in SAO-dominated biogas systems.[35, 36] The syntrophic acetate oxidizer in co_s was *S. schinkii*, while the syntrophic co_{mix} community consisted of *S. schinkii*, [*C.*] *ultunense* and *T. acetatoxydans*. However, 16S rRNA gene Illumina sequencing revealed presence of an uncharacterized species, strain AMB_1 in the *M. bourgensis* culture and it was thus also included in the co_s and co_{mix} cultures.

Acetate consumption and methane production were equimolar in SAO cultures. Analysis of acetate and methane concentrations in the co_s and co_{mix} cultures during growth revealed a 1:1 ratio between consumption of acetate and production of methane (Figures 1a and 2a), which is in agreement with previous findings for mesophilic and thermophilic SAO cultures. [11, 20] This demonstrates the metabolic interdependence and tight coupling between the bacteria and the methanogen.

Hydrogen partial pressures and Gibbs free energy changes stabilize in SAO cultures. Production and consumption of four moles of hydrogen per mole of acetate oxidized, and per mole of methane produced, means that the amount of energy available from both reactions is very sensitive to the hydrogen partial pressure. Thus p_{H2} can be expected to stay within a narrow range, as both reactions need to be exergonic if the organisms involved are to obtain energy for growth from these reactions. We found this expectation to be fulfilled in both cultures. Except for early p_{H2} peaks of 10 Pa in co_s after 15 days and 7 Pa after 8 days in co_{mix}, p_{H2} essentially remained consistently between 3.1 and 5.3 Pa (average 4.2 Pa) over the course of the growth experiments in both co_s and co_{mix} (Fig 1a and 1b). pH increased from 7.3 to 7.9-8.0 and CO_2

varied between 12-29% in all cultures during growth (Table S1.2). Thermodynamic calculations demonstrated that both acetate oxidation and hydrogenotrophic methanogenesis were exergonic at all times and that the energetics in co_s and co_{mix} were consistent. In both cultures, the ΔG values started out at levels of about -30 to -40 kJ/reaction for acetate oxidation and hydrogenotrophic methanogenesis, respectively, and stabilized at -10 kJ/mol for acetate oxidation and -20 kJ/mol for hydrogenotrophic methanogenesis (Figures 3 and S1.1). Thus the methanogens obtained more energy from this partnership than their acetate-oxidizing partners. This is different to a previously analyzed methanogenic SAO tri-culture including *M. bourgensis* and [*C.*] *ultunense*, in which equal sharing was assumed (*i.e.* 17 kJ/mol each).[20] The hydrogen levels in that study [1.6-6.8 Pa] were similar to the steady state levels observed here, and substantially lower than those typically observed in thermophilic SAO-cultures (10-60 Pa).[19, 37] This was expected, since temperature increases the window of opportunity and allows for higher hydrogen levels.[32]

Idiosyncratic growth of the SAO bacteria and the methanogen. Given that the hydrogen partial pressure stayed within a narrow range during our experiments, one would expect methane production to result in growth of the methanogen throughout. We found that this was not the case. In both communities, 16S rRNA gene copies of the methanogen ceased halfway through the experiment (Figures 1b and 2b). Nevertheless, after growth had ceased methane production continued at a steady pace, demonstrating that the methanogenic population was still highly active. During the period of exponential growth, *i.e.*, about day 70 to 100 in co_s and day 8 to 30 in co_{mix}, the methanogen increased from 10^{6-7} to 10^8 gene copies/mL, giving generation times of 20 days in co_s and 9 days in co_{mix} (Table 1).

Growth of the SAO bacteria was even more idiosyncratic. In co_s, the 16S rRNA gene copy numbers of *S. schinkii* increased steadily between days 50 and 80 to reach 10^8 gene copies/mL (Figure 1b). After this, growth of *S. schinkii* continued, but at a lower rate, until day 100, when

growth of *S. schinkii* stopped and copy numbers tended to decrease, even though there was still plenty of acetate (100 mM) present, and acetate continued to be degraded until it was fully depleted at day 160 (Figure 1a). A similar pattern was seen for co_{mix}, where exponential growth of *S. schinkii* at its initial 'maximum' rate ceased at 10^8 gene copies/mL (~ day 30) (Figure 2b, Table SI.3), a point where there was still a lot of acetate present. Just as in co_s, growth of *S. schinkii* continued after this, but at a lower rate (Figures 2b). [*C.*] *ultunense* and strain AMB_1 grew faster than *S. schinkii*, but growth of these organisms stopped earlier than for *S. schinkii*. Thus *S. schinkii* also became the dominant microorganism in co_{mix} (Figures 2b and 4). Another interesting observation is that *S. schinkii* reached similar abundance in both co_s and co_{mix}, and thus the fact that it had to share the available substrate with other syntrophic acetate-oxidizing bacteria appeared not to affect its growth. Analyses of cultures without acetate suggested that [*C.*] *ultunense*, and in particular strain AMB_1, grew on yeast extract (Table SI.3). This implies that, even in cultures with acetate, the initial rapid increase in [*C.*] *ultunense* and strain AMB_1 during the first week of incubation was due to growth on yeast extract. Detection of traces of propionate and butyrate in co_{mix} at the end of the experiment supports this hypothesis. However, after the initial rapid increase, [*C.*] *ultunense* and AMB_1 continued to increase, but at a slower pace. At days 30-40, [*C.*] *ultunense* abundance reached higher levels in cultures with acetate (1-2 × 10^8 gene copies/mL) than in cultures without acetate (4 × 10^7 gene copies/mL), illustrating its ability to oxidize acetate. For AMB_1, there was no difference between cultures with and without acetate (10^8 gene copies/mL in all cultures), so the ability of this bacterial species to oxidize acetate remains to be investigated. Still, the fact that strain AMB_1 did not show any growth in the co_s culture suggests that its growth on yeast and/or acetate was regulated by the presence of [*C.*] *ultunense* or *T. acetatoxydans.* The slow growth and low gene abundance of *T. acetatoxydans* (Figure 2b), particularly considering that this species has two 16S rRNA copy numbers per genome whereas *M. bourgensis* sp. MAB1, *S. schinkii* and [*C.*]

ultunense have one[38-40], indicate a restricted metabolic contribution of *T. acetatoxydans* in the co_{mix} culture.

The Illumina sequencing supported the qPCR results and showed dominance of *S. schinkii* in both cultures (Figure 4). In addition, strain AMB_1 was only detected in the Illumina sequencing in samples taken at the first days in the co_s culture and likely did not to grow, as it was not detected after 50 days of incubation. This was further strengthened using the AMB-targeting primers in qPCR analysis, which indicated no presence of this species above the detection limit of 10^0 gene copies/mL in any of the samples. Moreover, in the Illumina analysis, sequences classified into the bacterial genus *Chungangia* were detected in co_s at days 5-48. *Chungangia* is strict aerobic bacteria[41] that is unlikely to grow in these anaerobic cultures. These sequences were thus considered to represent DNA contamination of extraction and sequencing reagents appearing in the analysis, due to the extremely low microbial biomass in the initial days of cultivation. Similarly to the qPCR results, the Illumina analysis also showed higher relative abundance of SAO bacteria than of the methanogen, even considering differences in 16S rRNA copy numbers per genome (Figure 4). This was rather unexpected, as the energy available from acetate oxidation was lower than that from methanogenesis. The reason for this is not immediately clear from the present results, but it can be speculated that the methanogen may have to invest more energy in assimilation (e.g. the synthesis of amino acids), whereas the SAO microorganisms may take more advantage of the yeast extract in the medium or use secondary metabolites from the methanogen. Another possibility could be that the methanogen simply dissipates more of the free energy and has a lower energy gain than the SAO microorganisms.

One interesting dissimilarity between the co_{mix} and co_s cultures was the number of days required for initiation of methane production after inoculation (8 days for co_{mix}, 83 days for co_s). This may be taken to suggest that one or more of the other organisms in the co_{mix} community (*viz*. *T. acetatoxydans* or [*C.*] *ultunense*) provided a factor that somehow lowered the threshold for

initiation of SAO. It also appeared to expedite growth of *S. schinkii* and *M. bourgensis*, which grew faster in co_{mix} than in co_s (Table 1). These differences between co_{mix} and co_s are not only intriguing, but also relevant, because they show that the more complex co_{mix} community instigated more efficient acetate degradation than the simpler co_s community. This indicates that information gleaned from studying a simple community cannot be directly extrapolated to the 'real world'. Mesophilic anaerobic digesters in which the SAO route dominates are complex and show more resemblance to the co_{mix} community than to the co_s community. There are generally a number of syntrophic acetate oxidizers present in SAO-dominated biogas systems, but *S. schinkii* is often the most abundant of the known syntrophic bacteria,[23, 35, 42]. This was also the case in the co_{mix} culture. Overall, our results indicate that cooperation between a consortium benefits the growth of syntrophic acetate oxidizers, confirming suggestions in previous research that syntrophy in the 'real world' is more complex than previously thought.[43]

Effects of environmental conditions on the methane production rate. Growth experiments and modeling of synthetic communities are useful to generate predictions of how different parameters control methane production in more complex systems. The main aim of the present experiment was to gain insights into the ecophysiology of syntrophic acetate oxidizers under prevalent biogas process conditions and the experimental set-up was therefore designed to replicate this environment. The parameters tested were temperature, pH, ammonia and acetate levels. The experiment was performed on co_{mix} due to its higher resemblance to the diverse SAO consortia prevailing in biogas systems.[5] The model obtained from methane production rates at varying growth conditions was shown to describe the variation in the data with high accuracy (Table SI.4). Under the conditions examined, temperature proved to have a strong impact on methane formation, with the highest production rates obtained at around 42-46°C at both $pH_{initial}$ 7.3 and 8.1 (Figure 3[JD4], 5, 6 and SI.2). This temperature range resembles the heterotrophic growth pattern of *T. acetatoxydans*,[10] whereas [*C.*] *ultunense* had its highest

growth rate around 37°C.[8] The effect of temperature on the growth rate of *S. schinkii* and strain AMB_1 has not been determined in pure culture. However, the upper temperature limit of *C. ultunense* strain Esp and *S. schinkii* during heterotrophic growth (40-45°C)[9] and the presence of relatively low numbers of heat shock proteins in their genomes[17, 44] raise many questions regarding the interplay of the different species in co_{mix} at higher temperatures. Whether [*C.*] *ultunense* and *S. schinkii* benefit from SAO conditions and/or other strains in the co-culture and remain active at 42-46°C, and whether *T. acetatoxydans* and/or strain AMB_1 is the main acetate consumer(s) in this setting, remain to be established. For practical application, this co_{mix} optimum range lies in between the mesophilic (35-38°C) and thermophilic (50-60°C) temperature intervals commonly applied in commercial biogas production.[45] Previous studies performed in our laboratory have assessed this temperature interval for its suitability for biogas production under high ammonia conditions, with the aim of supporting SAO activity.[35, 46] One such study revealed positive effects on methane production, enhanced degradation of amino acids, and increased abundance of *T. acetatoxydans* in degradation of thin stillage at 44°C instead of 38°C.[46] However, in processes fed household waste supplemented with protein, a similar temperature increase (from 37°C to 42°C) had less effect on methane yield and on the abundance of known SAO bacteria.[35]

An important aspect when considering the impact of temperature is its effect on ammonia level. Increased temperature shifts the equilibrium between ammonium (NH_4^+) and ammonia (NH_3) towards the latter, which is mainly responsible for inhibition of the microbial community.[47] In our experiments, the higher temperature resulted in ammonia levels of 0.4 and 1.8 g NH_3/L at $pH_{initial}$ 7.3 and 8.1, respectively. The continued methane formation at the higher ammonia level demonstrates extremely high ammonia tolerance of the syntrophs involved, which confirms previous findings for pure cultures of syntrophic bacteria.[8-10, 21] The absence of genes for ammonium uptake systems and encoding of potassium uptake proteins may be potential

underlying mechanisms.[15, 17, 40, 44, 48] Our cultivation experiments even showed a positive effect of higher ammonia levels on methane production rate, which is likely to be an highly important selective factor for establishing SAO in high-ammonia biogas systems.

The thermodynamic benefit of high acetate concentrations[6] was reflected in enhanced rates of methane production by co_{mix} at higher acetate levels. Maximum methane production rates were obtained at around 0.2-0.3 M (Figures 5, 6 and SI.2). Interestingly, the upper level of acetate seems to be slightly inhibitory at 7.3 but not at 8.1 (Figures 5 and 6), which might indicate a pH dependent inhibitory effect by the undissociated form of acetate on the methane production rate. Genome-based analyses will be helpful in obtaining further information about the impact of acetate on the SAO community, but the indication that *T. acetatoxydans* possesses a passive rather than active acetate uptake system[15] could be an explanation for higher growth of the co_{mix} culture at the higher acetate levels. Note that the optimum acetate concentration (0.2-0.40 M, 12-24 g/L) predicted in the present study for the co_{mix} culture is an extremely high level for commercial biogas production, but can still be found in ammonia-inhibited systems.[4, 49]

Competition with aceticlastic methanogens at high and low acetate levels in biogas processes. The results obtained here were used to estimate kinetic parameters describing the growth of SAO organisms and to provide a basis for comparison of our data with previous studies of aceticlastic methanogens. Using qPCR results for the period of exponential growth, generation times between 3-12 days were estimated for all the syntrophic microorganisms involved except strain AMB_1 (Table 1). This exceeds the generation time reported for *Methanosarcina* sp. (0.5-2 days), but is in a similar range as *Methanosaeta* (3.5-12 days sp.).[50] The higher growth rate of *Methanosarcina* and the high acetate affinity of *Methanosaeta* (further discussed below) indicate that SAOs have low competitiveness against aceticlastic methanogens in non-stressed environments. However, the generation times of these aceticlastic methanogens were obtained when grown at one set of parameters (35°C, low ammonia, pH 7.2

ect) and parameters such as acetate, pH, ammonia level and temperature will must likely effect their competitveness. It is well known that the lower ammonia tolerance of aceticlastic methanogens often causes SAO dominance in high-ammonia environments.[5] Still, the most rapid doubling times obtained in the present study (Table S1.4 [JD5]) also indicate SAO competiveness relative to aceticlastic methanogens in systems operating with low and moderate ammonia levels, if other conditions favor SAO. This would explain the SAO dominance reported at high temperature and/or high acetate in the absence of high ammonia as a selective factor.[51-55]

The minimum threshold for acetate utilization was 0.40-0.45 mM in the co_s culture and 0.7 mM in the co_{mix} culture (Table SI.6), which is comparable to the acetate affinity of *Methanosarcina* (0.2-2.5 mM).[56, 57] However, the minimum acetate level for *Methanosarcina* was determined in low-ammonia conditions and may be different when it is grown under higher ammonia levels, as used in the present study. Our data therefore indicate that SAO microorganisms can compete for acetate even when acetate is present at low levels, as confirmed by the dominance of SAO reported in biogas digesters operating at low acetate levels and high ammonia concentrations.[5, 58] The result of the present study also shows that the minimum threshold for acetate utilization of SAO microorganisms considerably exceeds the level of 7-70 µM reported for *Methanoseata* which is the other methanogenic group competing for acetate.[59] Consequently, SAO microorganisms will not be able to compete with *Methanosaeta*, in absence of inhibitory compounds and when acetate is the limiting parameter. However, *Methanosaeta* sp. have low tolerance to specific inhibitors and are seldom detected in high-ammonia biogas processes.[5]

SUPPORTING INFORMATION

The supporting information accompanying this manuscript contains information relating to:

- methods involving of factors of the experimental design used in experiment II
- results of performance of the experimental design, average 16S rRNA gene copy numbers of all strains when incubated with and without acetate, estimated doubling time (during growth at different temperatures, acetate concentrations, ammonia levels, and $pH_{initial}$), detailed values of minimum threshold for acetate utilization, change in Gibbs free energy over time and impact of acetate concentration and temperature on methane production rates (at different ammonia levels) by the co_{mix} culture at $pH_{initial}$ 7.3.

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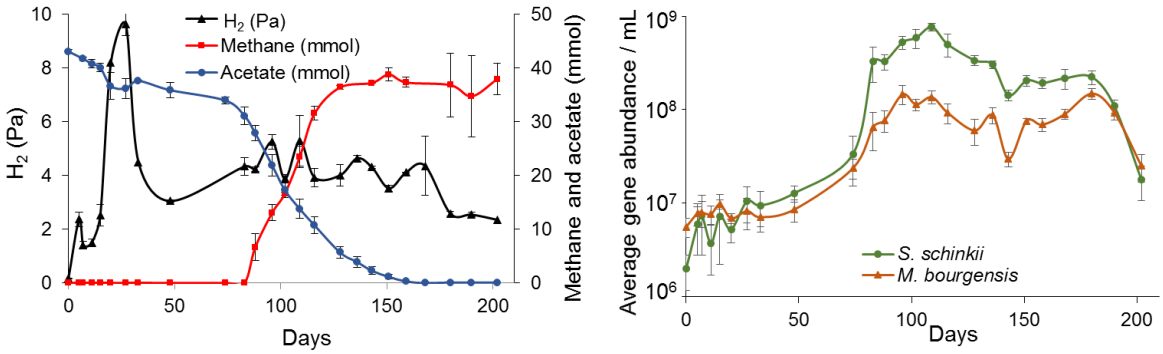
582 **Table 1.** Generation time estimated from the qPCR results for triplicate cultures of co_s and co_{mix}
583 when incubated at 37°C in medium containing 200 mM acetate, 0.2 M NH_4Cl, and an initial
584 pH of 7.3. The equation g = (ln2)/μ was used for calculation, where μ (assumed constant) was
585 estimated from the slope of the logarithmic increase in 16S rRNA genes of the respective
586 species

culture	*Syntrophaceticus schinkii*	[*Clostridium*] *ultunense*	*Tepidanaerobacter acetatoxydans*	strain AMB_1	*Methanoculleus bourgensis*
co_s	19.7 ± 1.5[1]	np	np	np	19.8 ± 1.4[2]
co_{mix}	9.5 ± 0.5[3]	4.5 ± 0.2[4]	11.7 ± 0.5[4]	3.1 ± 0.1[4]	8.5 ± 0.4[3]

587 np = not present

588 [1] Time frame used for calculation: days 48-83; [2] Time frame used for calculation: days 74-96; [3] Time frame used
589 for calculation: days 8-28; [4] Time frame used for calculation: days 0-14 (the qPCR data used for the calculations
590 are shown in Figures 1b and 2b)

591 a b



592

593 **Figure 1.** (a) Acetate consumption, methane formation, and hydrogen partial pressure and (b)
594 the 16S rRNA gene abundances (determined through qPCR analyses) of *Syntrophaceticus*
595 *schinkii* and *Methanoculleus bourgensis* during 200 days of co-cultivation of the co_s culture.
596 qPCR analyses using primers designed to target strain AMB_1 showed no detection of this
597 species in co_s. The co-cultures contained 0.2 M NH_4Cl and an initial acetate concentration of
598 200 mM (equal to 40 mmol per bottle), and were incubated at 37°C. Each sample point
599 represents an average of triplicate biological replicates.

600

a b

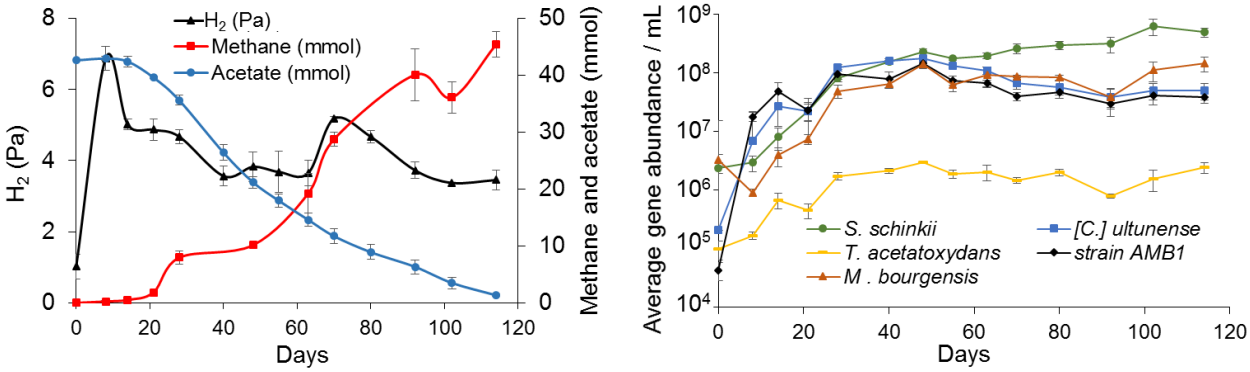
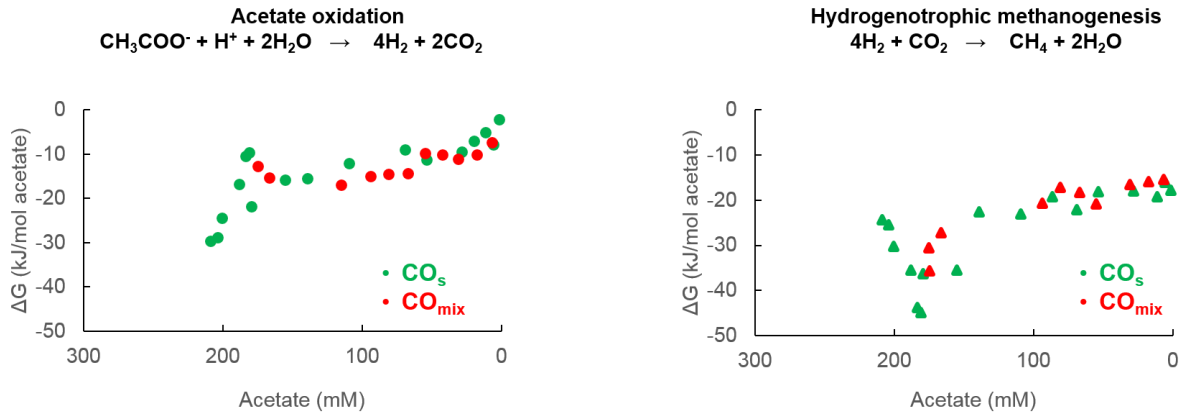


Figure 2. (a) Acetate consumption, methane formation, and hydrogen partial pressure and (b) 16S rRNA gene copies (determined through qPCR analyses) of *Syntrophaceticus schinkii*, [*Clostridium*] *ultunense*, *Tepidanaerobacter acetatoxydans*, strain AMB_1, and *Methanoculleus bourgensis* during 120 days of co-cultivation of the co_{mix} culture. The co-cultures contained 0.2 M NH_4Cl and an initial acetate concentration of 200 mM (equal to 40 mmol per bottle), and were incubated at 37°C. Each sample point represents an average of triplicate biological replicates.



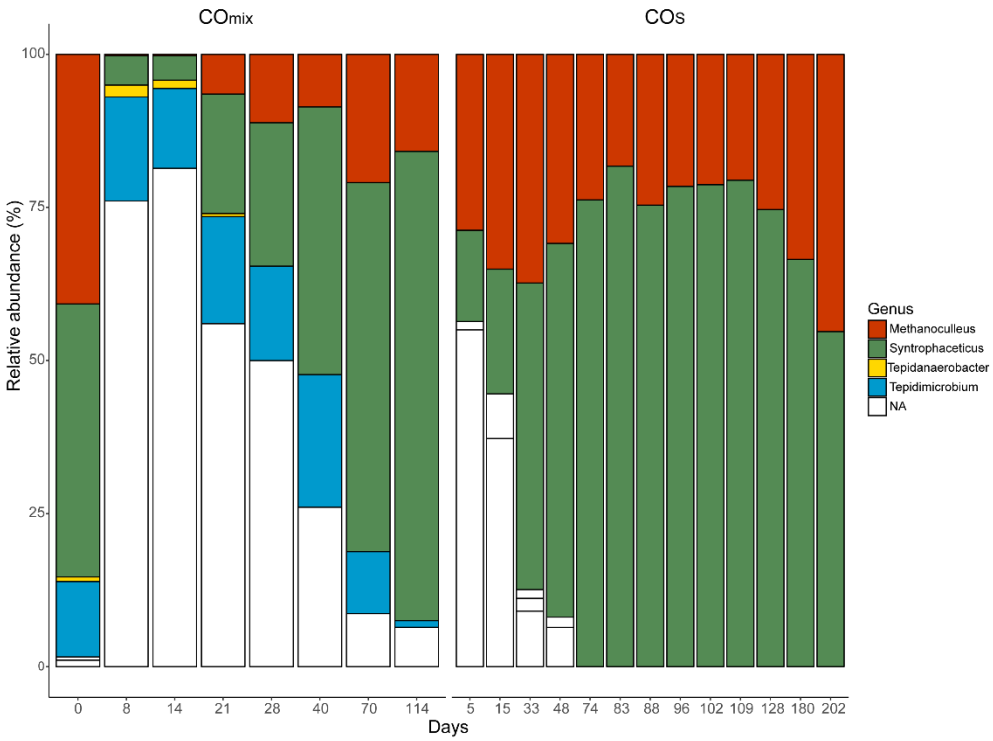
611

612 **Figure 3.** Change in Gibbs free energy (Δ*G*) for acetate oxidation (left panel) and

613 hydrogenotrophic methanogenesis (right panel) in the co_s culture (green symbols) and in the

614 co_{mix} culture (red symbols) during acetate degradation.

615



616

617 **Figure 4.** Relative abundance at genus level (based on total sequences) in the co_{mix} culture
618 (*Syntrophaceticus schinkii*, [*Clostridium*] *ultunense*, *Tepidanaerobacter acetatoxydans*, strain
619 AMB_1, and *Methanoculleus bourgensis*) and the co_s culture (*S. schinkii*, strain AMB_1, and *M.*
620 *bourgensis*) during degradation of 40 mmol (equal to 200 mM per bottle) acetate as shown in
621 Figures 2a and 3a. The results are mean values of biological triplicate batches. Blast searches
622 of the representative sequences revealed that Tepidimicrobium-classified reads had 97-99%
623 sequence similarity to [*C.*] *ultunense* and that the majority of the unclassified reads (NA in the
624 diagram) had 100% sequence similarity to the novel strain AMB_1.

625

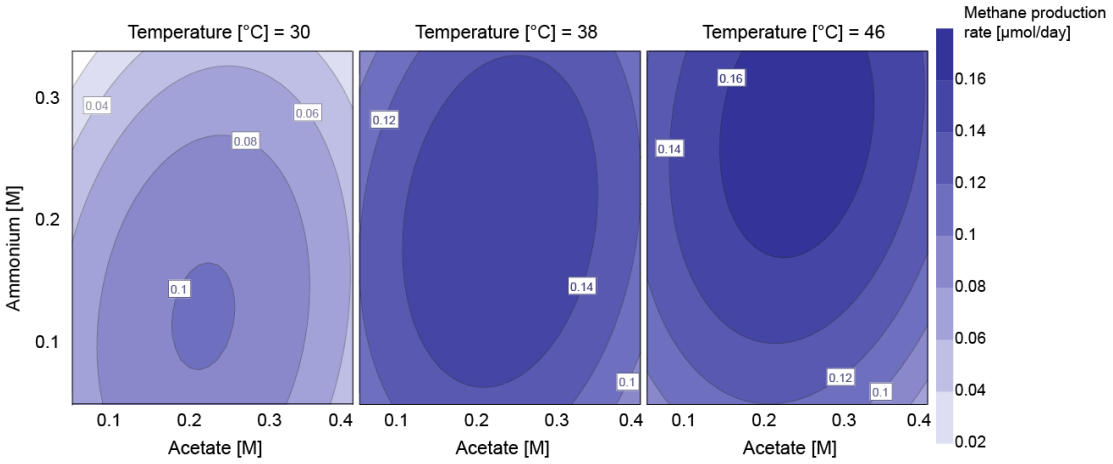


Figure 5. Impact of acetate concentration and temperature on methane production rates by the co_{mix} culture (*Syntrophaceticus schinkii*, *[Clostridium] ultunense*, *Tepidanaerobacter acetatoxydans*, strain AMB_1, and *Methanoculleus bourgensis*) at 0.05, 0.225, and 0.4 M acetate concentrations and $pH_{initial}$ 7.3. The parameters investigated were within the ranges 0.05-0.34 M NH_4Cl, 30-46°C, and 0.05-0.4 M sodium acetate.

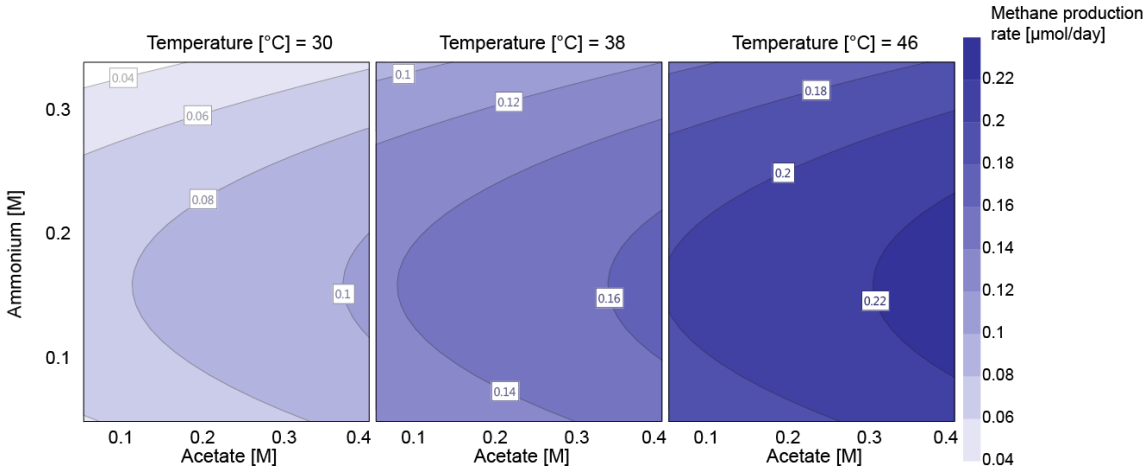


Figure 6. Co-cultivation at $pH_{initial}$ 8.1 and the impact of acetate and ammonia on methane production rates by the co_{mix} culture (*Syntrophaceticus schinkii*, *[Clostridium] ultunense*, *Tepidanaerobacter acetatoxydans*, strain AMB_1, and *Methanoculleus bourgensis*), when incubated at 30°C, 38°C, and 46°C. The parameters investigated were within the ranges 0.05-0.34 M NH_4Cl, 30-46°C, and 0.05-0.4 M sodium acetate.

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