Biogas Production through the Syntrophic Acetate-Oxidising Pathway

Characterisation and Detection of Syntrophic Acetate-Oxidising Bacteria

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Cover: Coloured versions of a scanning electron microscopy image of *Tepidanaerobacter acetatoxydans* (photo: M. Westerholm)

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Abstract

Biogas produced from wastes, residues and energy crops has promising potential to reduce greenhouse gas emissions and to secure future energy supply. Methane is the energy-rich component of biogas, and is formed as the end product during degradation of organic material without oxygen (anaerobic). Acetate is an important intermediate in anaerobic degradation and can be converted to methane through two pathways: aceticlastic methanogenesis and syntrophic acetate oxidation (SAO). SAO is a two-step reaction, consisting of acetate oxidation to hydrogen and carbon dioxide by syntrophic acetate-oxidising bacteria (SAOB), followed by conversion of these products to methane by hydrogenotrophic methanogens. Ammonia and acetate concentration, hydraulic retention time, temperature and methanogenic population structure are operational parameters considered to influence the acetate conversion pathway.

This thesis sought to increase understanding of SAO by examining syntrophic acetate oxidisers in pure culture, co-culture and methanogenic reactors. Two novel species of SAOB, *Syntrophaceticus schinkii* and *Tepidanaerobacter acetatoxydans*, were isolated and their phenotypic and phylogenetic traits were characterised. Quantitative molecular approaches were developed and applied to determine structural dynamics in the methane-producing population in a mesophilic biogas reactor during an ammonia-induced shift from aceticlastic to syntrophic acetate degradation. The abundance of SAOB increased, with a simultaneous decrease in aceticlastic methanogens. The majority of known SAOB are considered acetogens, and gradually increased ammonia concentration was shown to cause distinct shifts in the putative acetogenic population structure in mesophilic biogas reactors. However, the acetogenic bacterial abundance remained relatively stable. Bioaugmentation of syntrophic acetate-oxidising cultures did not improve process performance or support establishment of SAO as the dominant acetate degradation pathway.

In conclusion, SAOB are enduring and important components of the methane-producing community in mesophilic biogas reactors with high prevailing ammonia concentrations.

Keywords: syntrophic acetate oxidation, *Syntrophaceticus schinkii*, *Tepidanaerobacter acetatoxydans*, microbial population structure, biogas production, ammonia

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Westerholm, M., Roos, S. & Schnürer, A. (2010). *Syntrophaceticus schinkii* gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidising bacterium isolated from a mesophilic anaerobic filter. *FEMS Microbiology Letters* 309, 100-104.

II Westerholm, M., Roos, S. & Schnürer, A. (2011). *Tepidanaerobacter acetatoxydans* sp. nov., an anaerobic, syntrophic acetate-oxidising bacterium isolated from two ammonium-enriched mesophilic methanogenic processes. *Systematic and Applied Microbiology* 34, 260-266.

III Westerholm, M., Dolfing, J., Sherry, A., Gray, N.D., Head I.M. & Schnürer, A. (2011). Quantification of syntrophic acetate-oxidising microbial communities in biogas processes. *Environmental Microbiology Reports* 3, 500-505.

IV Westerholm, M., Müller, B., Arthurson, V. & Schnürer, A. (2011) Changes in the acetogenic population in a mesophilic anaerobic digester in response to increasing ammonia concentration. *Microbes and Environments* 26, 347-353.

V Westerholm, M., Levén, L. & Schnürer, A. Bioaugmentation of syntrophic acetate-oxidising culture in biogas reactors exposed to increasing levels of ammonia (manuscript).

The contribution of the author to the papers included in this thesis was as follows:

I Took part in planning the study and performed the majority of the laboratory work. Main writer of the manuscript.

II Participated in planning the study and performed all the laboratory work. Main writer of the manuscript.

III Involved in planning the study and analysing the results. Performed the majority of the laboratory work. Main writer of the manuscript.

IV Participated in planning the study and analysing the results. Performed the majority of the laboratory work, apart from construction of some of the clone libraries and the statistical analysis. Main writer of the manuscript.

V Participated in planning the study and analysing the results. Performed the molecular work and took part in monitoring the reactors. Main writer of the manuscript.

In addition to papers I-V, the author contributed to the following paper within the timeframe of the thesis work:

Westerholm, M., Hansson, M. & Schnürer, A. (2012) Improved biogas production from whole stillage by co-digestion with cattle manure. *Bioresource Technology* 114, 314-319.

Abbreviations

CSTR	Continuously stirred tank reactor
DNA	Deoxyribonucleic acid
FISH	Fluorescence *in situ* hybridisation
FTHFS	Formyltetrahydrofolate synthetase
HRT	Hydraulic retention time
OLR	Organic loading rate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate-oxidising bacteria
SIP	Stable carbon isotopic probing
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acids

1 Introduction

Intensifying demands to mitigate anthropogenic greenhouse gas emissions and to supply sustainably produced energy have in recent years increased attention in the production of biogas through anaerobic degradation of organic material. Methane, which represents the main component in biogas, can be used for replacement of fossil fuels in heat and electricity generation and as vehicle fuel. Furthermore, anaerobic digestion is a suitable method for waste and wastewater treatment, and the residue may be used as bio-fertiliser.

Overall, anaerobic degradation is a multi-step process and depends on numerous microbial populations, which operate in close interaction with each other. Within this process, acetate is an important source of methane (Kaspar & Wuhrmann, 1978; Hobson & Shaw, 1974) and degradation primarily proceeds through two pathways: acetate cleavage by aceticlastic methanogens and syntrophic acetate oxidation (SAO). SAO encompasses acetate oxidation to hydrogen and carbon dioxide by syntrophic acetate-oxidising bacteria (SAOB), and subsequent conversion of hydrogen and carbon dioxide to methane by hydrogen-utilising methanogens (Zinder & Koch, 1984). Ammonia and acetate concentration, dilution rate and methanogenic population structure are factors considered to influence the development of SAO (Hao *et al.*, 2011; Schnürer & Nordberg, 2008; Karakashev *et al.*, 2006; Shigematsu *et al.*, 2004; Ahring *et al.*, 1993; Petersen & Ahring, 1991). The significance of SAO has recently been emphasised in several studies of methanogenic systems (**V**; Hao *et al.*, 2011; Sasaki *et al.*, 2011a; Shimada *et al.*, 2011). Studies to date on the operation and optimisation of biogas reactors have tended to focus on maintaining the activity of the aceticlastic methanogens (Karakashev *et al.*, 2006), with the role of SAO neglected. Re-evaluation and optimisation of biogas reactors operating through SAO could be beneficial, in order to support growth and activity of the dominant microorganisms. Consequently, further

research within this area is important to increase the currently limited understanding of the nature of SAOB.

1.1 Aims of the thesis

The main aim of this thesis was to study syntrophic acetate oxidation, the microorganisms responsible, their growth conditions and their presence and appearance dynamics in biogas reactors with prevailing mesophilic conditions.

Cultivation of novel SAOB – Extended understanding of the microbes responsible for SAO in mesophilic conditions required isolation and characterisation of novel SAOB. By cultivation of the SAOB in pure culture, and co-culturing with a hydrogenotrophic methanogen, we sought to fully determine growth conditions, optimal parameters for growth and substrate pattern of the isolates. Chemotaxonomic and phylogenetic analyses were also included in the study in order to establish possible metabolic pathways, the taxonomic position and the relatedness of strains within the novel species, as well as closely related species (**I**, **II**).

Molecular investigations – To enable *in situ* investigations of microorganisms involved in methanogenesis of acetate, a molecular-based approach targeting SAOB had to be developed. The objective was to use the genetic information (16S rRNA gene) obtained (**I**, **II**) for development of a quantitative method specific for SAOB. This method would then be used to establish the abundance dynamics of high-ammonia biogas reactors (**III**). The majority of the SAOB identified to date have been classified as acetogens. Thus the aim in the next study was to use an established method for profiling and quantifying the acetogenic population in mesophilic high ammonia biogas reactors. The specific objective was to evaluate the influence of increasing ammonia concentration on the structure and incidence of the acetogenic population, and extend the current understanding of the SAO population in the reactors. An additional objective was to examine the possible presence of important and as yet unrecognised SAOB (**IV**).

Biogas reactors – Little is known of the ecological nature of SAOB in methanogenic reactors. We therefore required to link operational parameters and changes in the dominant methanogenic pathway with alterations in SAOB and methanogenic population abundance (**III**, **IV**). High ammonia concentrations have previously been identified as a promoter of SAO and the influence of this parameter on the SAOB present was therefore analysed.

Furthermore, by determining the dynamics of the hydrogenotrophic partner methanogen and the main competitors for acetate, the aceticlastic methanogens, we sought to obtain valuable information on the interaction between the methanogens and SAOB. The aim of the final study was to extend our previous findings and apply them to biogas systems. The main objective was to assess possible enrichment of microorganisms responsible for SAO through the addition of SAO cultures to biogas reactors exposed to increasing ammonia levels. By correlating the abundance of SAOB and methanogens to the prevailing operational characteristics and performance of the reactors, we investigated whether this was a procedure to circumvent unstable periods, often associated with a shift in the dominant methanogenic pathway, and to improve process stability during inhibition of ammonia (**V**).

Future generations may well have occasion to ask themselves, "What were our parents thinking? Why didn't they wake up when they had a chance?" We have to hear that question from them, now.

Al Gore (An Inconvenient Truth, 2006)

2 Biogas production

Biogas is formed during degradation of biomass without access to oxygen and is primarily composed of methane (CH_4), carbon dioxide (CO_2) and low levels of hydrogen sulphide (H_2S) and ammonia (NH_3). Methane is the energy-rich component of the gas and can be used for replacement of fossil fuel in electricity and heat production, in production of chemicals and materials, and as vehicle fuel (Weiland, 2010), which contributes to reduced greenhouse gas emissions.

Low sludge production and high cost-effectiveness compared with aerobic digestion make anaerobic digestion a suitable method for waste and wastewater treatment (Liu & Whitman, 2008; Talbot *et al.*, 2008; Steyer *et al.*, 2006; Batstone *et al.*, 2002; Ghosh & Pohland, 1974). A wide range of organic waste types can be used as substrate for the production of biogas from anaerobic degradation, such as animal manure, agricultural residues and by-products, sewage sludge, source-separated household wastes and organic industrial waste (Angelidaki *et al.*, 2011; Ahring, 2003). In addition to lowering greenhouse gas emissions through replacement of fossil fuel with biogas, the indirect environmental benefits associated with the anaerobic digestion of wastes are of great importance, especially due to the strong impact of methane on global warming. Such benefits include a reduction in the spontaneous emissions of ammonia and methane that otherwise occur during composting or storage of untreated animal manure (Börjesson & Berglund, 2007; Börjesson & Mattiasson, 2007).

Biogas residue can be applied as fertiliser on agricultural land, which contributes to recycling of nutrients and reduces the use of additional mineral nitrogen fertilisers. Anaerobic treatment converts organically bound nitrogen into ammonium, which makes it more available to plants. The use of biogas residue has also been reported to improve soil microbiological properties and to reduce the odour and the loss of nitrogen by ammonia emissions, due to

faster penetration into the soil (Odlare *et al.*, 2011; Weiland, 2010; Arthurson, 2009; Debosz *et al.*, 2002; Marinari *et al.*, 2000).

2.1 Biogas production in Europe

Lately primary energy production from biogas has enjoyed particularly strong growth and in 2010 more than 9200 biogas plants were operating in Europe (European Biogas Association, 2011), providing about 30.3 TWh biogas-derived energy in total. Germany is the leading European biogas producer and alone accounted for about 60% of the European Union's biogas-sourced primary energy production in 2010. An additional 1195 biogas plants were constructed in Germany during 2010, bringing the total amount to 7100 operating plants (EurObserv'ER, 2011). Hungary and the Czech Republic are other countries that had a high number of biogas plants installed in 2010. Germany is also a pioneer in biogas production per capita (543 kWh/capita), followed by Austria (176 kWh/capita) and Sweden (145 kWh/capita) (European Biogas Association, 2011). In Sweden there are currently 229 biogas plants in operation, producing about 1.4 TWh biogas-derived energy annually (Energimyndigheten, 2011). However, the theoretical biogas potential in Sweden has been estimated to be about 17 TWh, which of 14 TWh could be derived from the agricultural sector (Nordberg & Edström, 2005). Different crops and wastes from the agricultural sector are likely to become important substrates for Swedish biogas production in the future (Börjesson & Berglund, 2007). In 2010, the majority of Swedish biogas was produced at sewage treatment plants (44%), followed by co-digestion plants (25%), landfill (22%), industry (8%), and farm-scale biogas plants (1%) (Energimyndigheten, 2011).

While a large part of the biogas produced in Germany is derived from crops and liquid manure (Weiland, 2003), the common substrates in Swedish co-digestion plants are slaughterhouse waste, waste from food and feed industries, source-separated food waste and manure. About 75% of the biogas produced at co-digestion plants in Sweden is derived from materials with a high content of proteins, such as slaughterhouse waste, swine/poultry manure and distillation waste (by-products from ethanol production) (Nordberg, 2006). Highly proteinaceous materials represent energy-rich substrates for biogas production and the gas produced has a high ratio of methane (Weiland, 2010).

2.2 Substrate composition and operation parameters

For efficient degradation of organic material in a biogas process, the feedstock needs to have a balanced nutrient content in order to support the growth of the

microbial biota. Carbon (C), nitrogen (N), phosphorus (P) and other essential nutrients, vitamins and trace elements are required. One important factor is the ratio of carbon to nitrogen (C/N). Low C/N ratio poses a risk of accumulation of volatile fatty acids (VFA) and process disturbance due to ammonia inhibition, whereas a process with a high C/N ratio may experience N deficiency. The optimal ratio for production of biogas varies with different substrates and operational conditions, but often ranges between 10 and 30 (Schnürer & Jarvis, 2010; Weiland, 2010; Yadvika *et al.*, 2004).

At biogas plants a suitable composition of nutrients in the reactor sludge is often achieved through co-digestion of different substrates. The positive synergisms that may occur through the supply of missing nutrients by the co-substrate commonly improve the methane yield compared with that achieved during degradation of the individual substrates (Westerholm *et al.*, 2012; Alvarez & Lidén, 2008; Parawira *et al.*, 2008; Nordberg & Edström, 2005; Yadvika *et al.*, 2004; Ahring, 2003; van Lier *et al.*, 2001; Mata-Alvarez *et al.*, 2000).

Biogas plant operation commonly proceeds at mesophilic (30-40 °C) or thermophilic (50-60 °C) conditions (van Lier *et al.*, 2001). Increased process temperature generally favours the metabolic activity of the microorganisms (Zábranská *et al.*, 2000; Duran & Speece, 1997) and a higher sanitisation effect is achieved (Bagge *et al.*, 2005; Sahlström, 2003; Zábranská *et al.*, 2000; Olsen & Larsen, 1987). Reduced viscosity and increased diffusion rates are other advantages of the thermophilic process (Holst *et al.*, 1997). On the other hand, mesophilic processes require less energy input for heating (Gellert & Winter, 1997) and are less affected by inhibitory compounds, such as ammonia (Sánchez *et al.*, 2000; Angelidaki & Ahring, 1994; Hashimoto, 1983). Furthermore, microbial diversity and degradation efficiency of a number of different phenols have been reported to be lower at thermophilic process temperature than at mesophilic conditions (Levén *et al.*, 2011; Levén *et al.*, 2007).

For anaerobic degradation of agricultural residues, animal manure, and industrial and municipal wastes, continuously stirred tank reactors (CSTR) are the most common configuration. Due to the continuous loss of active microbial biomass, CSTR are preferably operated with a hydraulic retention time (HRT) exceeding the doubling time of the slowest growing microorganisms. Shorter retention time is likely to cause washout of the microbial population (Angelidaki *et al.*, 2011; Weiland, 2010). HRT is the average time the waste or sludge remains in the reactor. The HRT of CSTR is typically in the range 15-30 days under mesophilic conditions and 10-20 days under thermophilic conditions (Angelidaki *et al.*, 2011). The HRT depends on the organic loading

rate (OLR) and the reactor volume. OLR is the term commonly used for the amount of material that is added to the process per unit of time.

High HRT and low OLR allow extensive contact time between the microorganisms and the substrate, which often results in improved degree of digestion and enhanced methane yield from a given substrate. The degree of digestion is defined as the percentage of the organic material degraded and converted to biogas during a specific period of time and varies with the substrate. The degree of digestion can vary from 100% (for mainly water-soluble matter) down to 30% (for highly particulate matter). Anaerobic degradation of manure, for instance, commonly achieves a degree of digestion between 40-65% (Angelidaki *et al.*, 2011).

2.2.1 Reactor disturbance

A wide variety of compounds, such as ammonia, sulphide and heavy metals, are inhibitory for anaerobic microorganisms (Chen et al., 2008). Methanogenic reactors accommodate the activity of various strongly interrelated microbial populations, and nutritional shortage or inhibition causing imbalance in one trophic level may affect the entire community and reactor performance (Fernández et al., 1999). Furthermore, certain operational parameters such as high influent concentrations and short HRT pose an increased risk of reactor failure (Steyer et al., 2006).

Concentration of toxic or inhibitory compounds, HRT, temperature, pH and the microbial community are factors affecting the extent of the inhibition and the process response (Schnürer & Jarvis, 2010). However, process instability is commonly reflected by reduced biogas production and/or methane content, fluctuations in pH and alkalinity and accumulation of VFA (total or individual). Acetate and propionate in particular tend to accumulate during reactor disturbance (**V**; Westerholm *et al.*, 2012; Weiland, 2010; Boe *et al.*, 2008; Steyer *et al.*, 2006). The presence of high VFA concentrations subsequently causes further acidification and microbial stress (Chen *et al.*, 2008).

Complete breakdown can be avoided by increasing the HRT, decreasing the OLR or reducing the concentration of the inhibitory compound by dilution or adjustment of feedstock composition (Chen *et al.*, 2008). Immobilising the microorganisms with different types of inert material, sludge settling or acclimatisation of the microbial community may be other ways to circumvent loss of cell biomass and operational problems (Chen *et al.*, 2008; Whitman *et al.*, 2006). Acclimatisation attained by exposing the microbes to slowly augmented levels has also been shown to increase the tolerance, probably

through internal changes in the predominant species or a shift in population (Chen *et al.*, 2008).

2.3 The microbiology of the biogas process

The anaerobic digestion for biogas production can be divided into four main stages: hydrolysis, fermentation, anaerobic oxidation and methanogenesis (Figure 1).

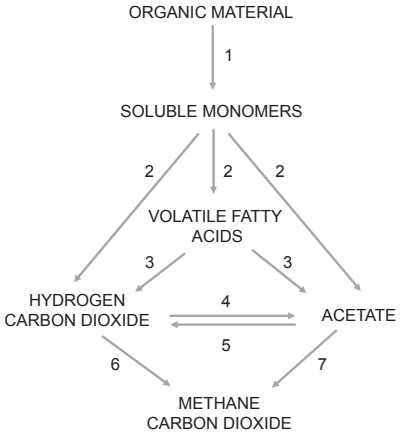

Figure 1. Anaerobic degradation of complex organic material to methane and carbon dioxide: 1. Hydrolysis. 2. Fermentation. 3. Anaerobic oxidation. 4. Hydrogen oxidation. 5. Syntrophic acetate oxidation (SAO). 6. Hydrogenotrophic methanogenesis. 7. Aceticlastic methanogenesis. Intermediary products are compounds such as alcohols, fatty acids, lactate *etc*. Modified after Zinder (1984).

Hydrolysis involves enzyme-mediated conversion of complex organic material (carbohydrates, proteins and lipids) to simpler compounds. Carbohydrates of insoluble or complex soluble form are initially hydrolysed by exoenzymes secreted by hydrolytic bacteria. The smaller products and soluble sugars enter the cells of facultative anaerobes and strict anaerobes, and are subsequently degraded by endoenzymes. The proteins are hydrolysed by exoenzymes (proteases or peptidases) to amino acids, which are subsequently transported into the bacterial cell and converted to organic acids (Ramsay & Pullammanappallil, 2001). Lipids are hydrolysed to glycerol, galactose and long-chain and medium-chain fatty acids by exoenzymes, named lipases, excreted by fermentative bacteria (Mackie *et al.*, 1991).

In the following step, a large variety of fermentative bacteria and hydrogen-producing acetogenic bacteria (acetogens) perform several different fermentative processes or anaerobic oxidation reactions to degrade the soluble

compounds produced through hydrolysis or added to the reactor. At this stage carbon dioxide, acetate, formate, hydrogen gas, alcohols, short-chain fatty acids and ammonia are produced. The short-chain fatty acids (other than acetate) are subsequently converted to acetate, hydrogen gas and carbon dioxide (Angelidaki *et al.*, 2011). This step is endergonic under standard conditions and can only be performed during product removal by methanogens, in a so-called syntrophic association (Schink, 1997).

Methanogens performing the final step in the anaerobic conversion of biomass to methane are members of the domain *Archaea*, which synthesise methane as the major product of their energy metabolism (Whitman *et al.*, 2006). The main groups of methanogens distinguished in the biogas process are hydrogenotrophic and aceticlastic. Aceticlastic methanogens cleave acetate, whereas hydrogenotrophic methanogens use hydrogen, or formate, as an electron donor in the conversion of carbon dioxide to methane (Demirel & Scherer, 2008). The methanogenic reactions generate a exceptionally small quantity of energy and the amount of ATP produced of one mole methane is one or less (Whitman *et al.*, 2006). The reduction of carbon dioxide by hydrogen is the most favourable methanogenic reaction, while the aceticlastic methanogens gain the lowest amount of energy. Consequently, the natural pressure of selection results in a more diverse hydrogenotrophic population than in the acetate-utilising methanogens (Garcia *et al.*, 2000). The hydrogen-utilising methanogens are distributed in all six phylogenetic orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales, Methanosarcinales* and *Methanocellales* (Angelidaki *et al.*, 2011). Aceticlastic methanogens belong to the genera *Methanosarcina* or *Methanosaeta*, within the order *Methanosarcinales* (Demirel & Scherer, 2008). Members of *Methanosaeta* use only acetate for growth, whereas *Methanosarcina* congregates species able to use acetate, methanol and methylamines and some species are also able to use hydrogen to reduce carbon dioxide or methanol to methane (Whitman *et al.*, 2006).

2.3.1 Structure and diversity in biogas reactors

The structure and activity of the microbial population involved in anaerobic sludge often depend on the original inoculum, and differences in operational and environmental conditions (Demirel & Scherer, 2008; Karakashev *et al.*, 2005; Guyot *et al.*, 1993). However, some general directions can be distinguished. In mesophilic anaerobic reactors the methanogens commonly observed are members of the orders *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales* and *Methanosarcinales*. Thermophilic conditions usually favour hydrogenotrophic methanogens (belonging to the *Methanobacteriales*),

although *Methanosarcina* and *Methanosaeta* species may occur (Demirel & Scherer, 2008; Liu & Whitman, 2008). The operational temperature is also considered to influence the microbial diversity. Higher bacterial and methanogenic diversity, both in species richness and representation of different phyla, has been reported in mesophilic reactors compared with the communities prevailing in thermophilic conditions (Levén *et al.*, 2007; Karakashev *et al.*, 2005; Sekiguchi *et al.*, 1998). The composition of the acetogenic population has also been shown to depend on the operational temperature and, in accordance to the bacterial and methanogenic population, higher diversity was observed in mesophilic than in thermophilic conditions (Ryan *et al.*, 2008) In addition, ammonia may be considered a strong impact factor on bacterial (Figure 2) and acetogenic population structure (Figures 2 and 3 in **III**).

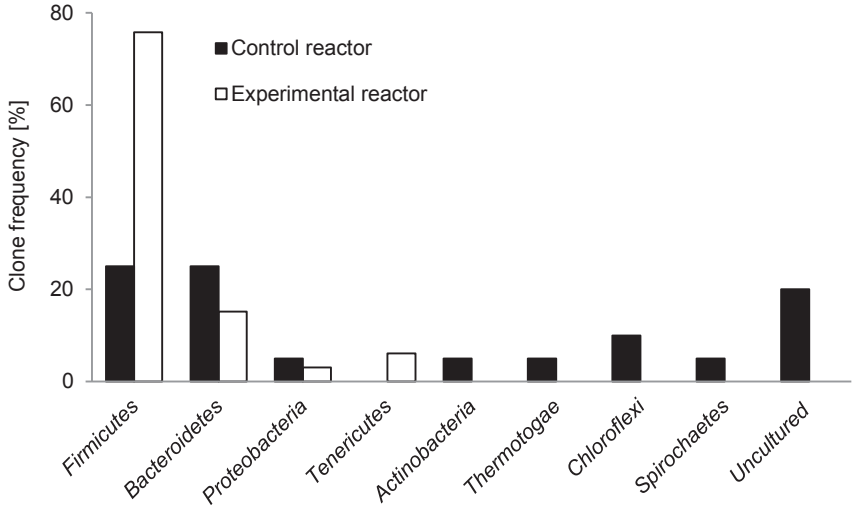

Figure 2. Phylogenetic affiliation of bacterial 16S rRNA sequences recovered from one control reactor (filled bars) with an ammonium-nitrogen concentration of 0.90 g NH_4^+-N L^{-1} and one experimental reactor (open bars) operating at 6.9 g NH_4^+-N L^{-1}. Both reactors operated under mesophilic conditions. Data were obtained from construction of clone libraries and clones were grouped into categories based on their phylum level affiliation. Distinctly higher diversity was revealed in the reactor with low ammonia compared with the reactor operating at high concentration.

Considering methanogens, the concentrations of ammonia and acetate have been demonstrated to impact on the population structure. Biogas reactors operating under stable conditions with a low content of acetate, NH_3 and VFA

are often characterised by *Methanosaetaceae* species, whereas *Methanosarcinaceae* or hydrogenotrophic *Methanomicrobiales* (particularly *Methanoculleus* species) normally dominate in unstable reactors with high ammonia and acetate concentrations (Nettmann *et al.*, 2010; Karakashev *et al.*, 2005; McMahon *et al.*, 2001).

2.3.2 Methane production pathways

In biological anaerobic digestion for biogas production, methane is mainly synthesised from hydrogen and carbon dioxide, or from acetate. Acetate is considered to be the most important intermediate (Kaspar & Wuhrmann, 1978; Hobson & Shaw, 1974) and the methanisation of acetate can proceed through two pathways. The most commonly described is the aceticlastic pathway, where acetate is cleaved directly to methyl and carboxyl groups by the aceticlastic methanogens. The methyl group is converted to methane, whereas the carboxyl group is oxidised to carbon dioxide (Whitman *et al.*, 2006). Acetate-oxidising bacteria in syntrophic interaction with hydrogen-consuming methanogens represent the second mechanism, SAO (Zinder & Koch, 1984), which is further described in section 3.

3 Syntrophic acetate oxidation (SAO)

Syntrophic acetate oxidation was originally proposed by Barker (1936) and later confirmed by Zinder & Koch (1984). SAO involves two reactions: oxidation of acetate to hydrogen and carbon dioxide by SAOB (reaction *i*), and the subsequent conversion of hydrogen and carbon dioxide to methane by hydrogenotrophic methanogens (reaction *ii*):

i. $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$

ii. $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_20$

Since the cooperating partners are able to metabolise both formate and hydrogen (Whitman *et al.*, 2006; Schink, 1997), formate has also been reported as a likely electron carrier in SAO (Hattori *et al.*, 2001).

SAOB and the methanogen mutually depend on each other to perform the metabolic activity. Acetate oxidation (reaction *i*) can only proceed if the hydrogen/formate level is kept low by the methanogen (reaction *ii*) (Schink, 2002; Schink, 1997; Stams, 1994). Thermodynamically, the oxidation of acetate to carbon dioxide and hydrogen (reaction *i*) is an unfavourable reaction in standard conditions ($\Delta G°' = +104.6$ kJ mol^{-1}). However, this reaction can proceed if hydrogenotrophic methanogenesis (reaction *ii*) ($\Delta G°' = -135.6$ kJ mol^{-1}) consumes the hydrogen (Hattori, 2008; Stams, 1994). Accordingly, the removal of hydrogen by the methanogen makes the acetate oxidation exorgenic and by combining the two reactions the free energy change ends up at $\Delta G°' = -31.0$ kJ mol^{-1}. The cooperating organisms thereby divide a diminutive amount of energy by their performance (Schink, 2002). The available energy for SAO has been calculated to be 17 kJ mol^{-1} for each organism in a tri-culture containing *Clostridium ultunense*, strain TRX1 and *Methanoculleus* MAB1 (under the following conditions: 37 °C, 25 mM acetate, 100 mM HCO_3^- and 31 kPa methane) (Schnürer *et al.*, 1997). This value indicates that only fractions of

ATP equivalents are gained per reaction run (the synthesis of ATP demands about $\Delta G^{\circ\prime}$=60-70 kJ mol^{-1}) (Schink, 1997).

Product formation by SAOB becomes more favourable if the hydrogen (or formate) concentration is kept low, but level must be sufficient to favour the hydrogen-consuming methanogenesis. These contrary requirements restrain the hydrogen and formate concentration within a low and narrow range (Stams, 1994). In thermophilic conditions (55-60 °C), the partial pressure of hydrogen measured in syntrophic co-cultures is in the range 10-50 Pa (Hattori *et al.*, 2001; Lee & Zinder, 1988a), where the low level indicates the threshold for hydrogen utilisation by the methanogen. In mesophilic conditions (37 °C), the hydrogen concentration can be as low as 1.6-6.8 Pa (Schnürer *et al.*, 1997), emphasising the importance of efficient hydrogen removal by the methanogen under mesophilic conditions.

High relative contribution of SAO to conversion of acetate to methane has been reported in several investigations of biogas reactors operating under diverse conditions (**V**; Sun *et al.*, 2012; Hao *et al.*, 2011; Sasaki *et al.*, 2011a; Shimada *et al.*, 2011; Laukenmann *et al.*, 2010; Schnürer & Nordberg, 2008; Karakashev *et al.*, 2006; Shigematsu *et al.*, 2004; Schnürer *et al.*, 1999). Furthermore, SAO has been suggested to occur in diverse natural environments, such as lake sediments (Nüsslein *et al.*, 2001), acidic peat (Horn *et al.*, 2003), oil reservoirs (Gray *et al.*, 2011; Jones *et al.*, 2008), soil (Chauhan & Ogram, 2006) and rice field soil (Rui *et al.*, 2011; Liu & Conrad, 2010).

3.1 Syntrophic acetate-oxidising bacteria (SAOB)

Only a restricted number of bacterial species known to perform syntrophic acetate oxidation in cooperation with a hydrogenotrophic methanogen have been characterised to date. Initially a thermophilic strain designated Reversibacter was described (Lee & Zinder, 1988b), but this bacterium was lost before its phylogenetic position was established. Since then, five SAOB have been isolated and characterised, the thermophiles *Thermacetogenium phaeum* (Hattori *et al.*, 2000) and *Thermotoga lettingae* (Balk *et al.*, 2002), the thermotolerant *Tepidanaerobacter acetatoxydans* (**II**) and the mesophiles *C. ultunense* (Schnürer *et al.*, 1996) and *Syntrophaceticus schinkii* (**I**). However, indications of the existence of as yet unrecognised SAOB have been reported (*e.g.* **IV**). *C. ultunense*, *T. phaeum*, *S. schinkii* and *T. acetatoxydans* are all affiliated with the phylum *Firmicutes*, whereas *T. lettingae* belongs to the phylum *Thermotogae*. Isotope tracer measurements and molecular analyses have indicated distribution of SAO phenotypes in other bacterial phyla. For instance, members of *Geobacter*, *Anaeromyxobacter* (anoxic rice field soil;

Hori *et al.*, 2007), *Smithella* (methanogenic crude oil-degrading enrichment cultures; Gray *et al.*, 2011), *Betaproteobacteria* and *Nitrospira* (lake sediments; Schwarz *et al.*, 2007) have been suggested to be responsible for acetate oxidation in syntrophic partnership with hydrogentrophic methanogens.

In addition, syntrophic bacteria able to oxidise acetate with the support of non-methanogenic hydrogen-utilising partner organisms have been characterised. For instance, the alkali bacterium *Candidatus* 'Contubernalis alkalaceticum' has been shown to oxidise acetate in co-cultivation with an hydrogenotrophic, alkaliphilic sulphate reducer, with the production of sulphide (Zhilina *et al.*, 2005). Another example is *Geobacter sulfurreducens*, which oxidises acetate in syntrophic association with nitrate- or sulphate-reducing hydrogentrophic bacteria. The hydrogen partial pressure recorded in the co-culture of *G. sulfurreducens* and the nitrate-reducing *Wolinella succinogenes* is considerably lower (0.02-0.04 Pa) than levels reported to occur during SAO with hydrogenotrophic methanogens. Consequently, the free energy for the bacterium is also higher and calculated to about -53 kJ mol^{-1} under the conditions investigated (Cord-Ruwisch *et al.*, 1998). Whether the two bacteria *Candidatus* 'C. alkalaceticum' and *G. sulfurreducens* are able to oxidise acetate in cooperation with methanogens has not yet been established and these bacteria are thus not included in further discussions in this thesis. Furthermore, the alkaliphilic feature of *Candidatus* 'C. alkalaceticum' (growth at pH 10) indicates that this syntroph is probably not a common component in biogas-producing consortia.

3.1.1 Growth conditions in pure culture

In pure culture, the above mentioned isolates (*i.e.* strain Reversibacter, *T. phaeum*, *T lettingae*, *T. acetatoxydans*, *C. ultunense*, *S. schinkii*) have the ability to use organic acids, alcohols and amino acids, and produce acetate as the main fermentation product (Balk *et al.*, 2002; Hattori *et al.*, 2000; Schnürer *et al.*, 1996; Lee & Zinder, 1988b). This substrate pattern suggests involvement in different fermentation and anaerobic oxidation reactions under non-SAO conditions, instead of contributing to acetate oxidation (**II**). The number of substrates used by *C. ultunense*, *T. phaeum* and *S. schinkii* for growth in pure culture is restricted (**I**; Hattori *et al.*, 2000; Schnürer *et al.*, 1996), whereas *T. lettingae* and *T. acetatoxydans* have been shown to use a broad spectrum of substrates (Table 1 in **II**; Balk *et al.*, 2002). *T. phaeum* and strain Reversibacter have been shown to be able to perform reverse acetate oxidation in pure culture, *i.e.* obtain energy by the reduction of carbon dioxide by hydrogen to acetate. This feature has not been demonstrated for *S. schinkii*, *T. acetatoxydans* (**I**, **II**), *C. ultunense* or *T. lettingae*. However, *C. ultunense* can

convert carbon dioxide and hydrogen to acetate in dense resting cell cultures, and *T. phaeum* and *T. lettingae* are able to oxidise acetate without a partner organism when an alternative electron acceptor is present (*i.e*. sulphate, thiosulphate, elemental sulphur, Fe(III) or anthraquinone-2,6-disulphonate). Representative characteristics of known SAOB are summarised in Table 1.

C. ultunense, *S. schinkii* and *T. acetatoxydans* possess a relatively high ammonium tolerance (0.6-1.0 M NH_4Cl) (**I**, **II**; Schnürer *et al.*, 1996). This feature probably confers the bacteria with a competitive advantage in ammonia-stressed systems. *S. schinkii* achieves extremely low cell density under laboratory conditions, even at optimal temperature and pH. However, addition of extra ammonia (>30 mM) to the culture media results in increased growth (**I**). Ammonia concentration ranges for growth are not available for *T. phaeum* and *T lettingae*, but the relatively high salt tolerance of the strains indicates that they may be able to grow at increased ammonia concentrations. *T. phaeum* and *T. lettingae* grow at concentrations up to 0.8 M and 0.5 M NaCl, respectively, and *T. lettingae* even displays an optimal growth rate at 0.2 M NaCl. The situation is similar for *C. ultunense*, which can grow at NaCl concentrations up to 0.6 M.

Temperature optimum and ranges for growth for the mesophilic, thermotolerant and thermophilic SAOB are specified in Table 1. The thermotolerant competency of *T. acetatoxydans* with optimum growth rate at 44-45 °C (Figure 3 in **II**) is somewhat surprising, as the strain originates from a reactor operating under mesophilic conditions (**II**). Growth of *S. schinkii* in pure culture was only confirmed at temperatures between 25 and 40 °C. The optimal temperature for growth of *S. schinkii* could not be determined due to the extremely low cell density achieved, even after several months of cultivation. However, it should be pointed out that these investigations were performed in defined growth media, and consequently the characteristics of *S. schinkii* may not adequately reflect growth properties in the conditions prevailing in anaerobic reactors. For instance, the increased cell density of *S. schinkii* at higher ammonia concentrations indicates that some components, essential for optimal growth of the strain, may not be included in the growth media. Reactor sludge is often quite complex and may thereby more satisfactorily provide the requirements for growth.

Table 1. *Characteristics of known SAOB according to Lee & Zinder (1988b), Schnürer et al. (1996), Hattori et al. (2000), Balk et al. (2002), Papers* ***I, II****.*

Characteristic	Reversi-bacter	*C. ultunense* BS^T	*T. phaeum* PB^T	*T. lettingae* TMO^T	*S. schinkii* $Sp3^T$	*T. acetat-oxydans* $Re1^T$
Origin:	Anae-robic reactor	Anaerobic reactor	Anaerobic reactor	Anaerobic reactor	Upflow anaerobic filter	Cont. stirred tank reactor
Feed to the reactor	solid waste	swine manure	ww kraft-pulp plant	methanol	ww fishmeal	alfalfa silage
Operational temp (C°)	55	37	60	65	37	37
Cell shape	rod	rod	rod	rod	rod	rod
Cell size (μm)	0.4-0.6 ×2-3	0.5-0.7 ×0.5-7	0.4-0.7 ×2-12.6	0.5-1 ×2-3	0.5-0.7 ×2-5	0.3-0.5 ×1.5-15
Motility	ND	+	ND	ND	-	+
Spore formation	-	+	+	-	+	+
Gram type	positive	positive	positive	negative	Gram-variable	positive
Temperature range (°C)	50-65	15-50	40-65	50-70	25-40	20-55
Optimum temperature	60	37	58	65	ND	44-45
NH_4Cl (M) range	ND	0.6	ND	ND	0.6	1.0
Supplement required	Yeast	Yeast	None	None	Yeast	None
G+C content (mol%)	47	32	53.5	39.2	ND	38.4
Acetogenesis from H_2/CO_2	+	+	+	-	-	-

ND - not determined, ww - wastewater

3.1.2 Growth conditions in co-culture

Work to elucidate the kinetic parameters of SAO is still at its infancy, but the syntrophs are generally considered to be slow growers. The low growth rate is probably a consequence of the low amount of free energy available for the syntrophic consortia (Hattori, 2008; Schnürer *et al.*, 1999). *T. phaeum* has been reported to consume acetate at a rate of 21.5 μmol (min g dry cell wt)$^{-1}$ in syntrophic interaction with a hydrogen-utilising methanogen. In comparison, the two mesophilic aceticlastic methanogens *Methanoseata soehngenii* and *Methanoseata concilii*, frequently identified in anaerobic digestion systems,

have been shown to reach maximal acetate consumption rates of 16 and 38 μmol (min g dry cell wt)$^{-1}$, respectively (Hao *et al.*, 2011).

However, growth of the mesophilic SAOB is even slower. Thermophilic syntrophic co-cultures of strain Reversibacter or *T. phaeum* coupled with partner methanogens grow on acetate with doubling times of a few days (1.5 days and 3 days, respectively) (Hattori *et al.*, 2000; Lee & Zinder, 1988b), whereas the mesophilic *C. ultunense* and the hydrogen-utilising methanogen *Methanoculleus* sp. MAB1 degrade acetate with a doubling time of 28 days (Schnürer *et al.*, 1994). The generation time of *S. schinkii* and *Methanoculleus* sp. MAB1 was calculated to be about 69-78 days (unpublished results). This can be compared with the generation time observed in cultivation of different aceticlastic methanogens, 8-36 h for *Methanosarcina* sp. and 1-9 days for *Methanoseata* sp. (Demirel & Scherer, 2008). However, by calculating the methane formation rate in the exponential phase of a syntrophic acetate-degrading culture containing the SAOB *C. ultunense*, *T. acetatoxydans* and *S. schinkii* and *Methanoculleus* sp. MAB1 (hereafter designated SAO-culture), a doubling time of only 9 days was found (Figure 3).

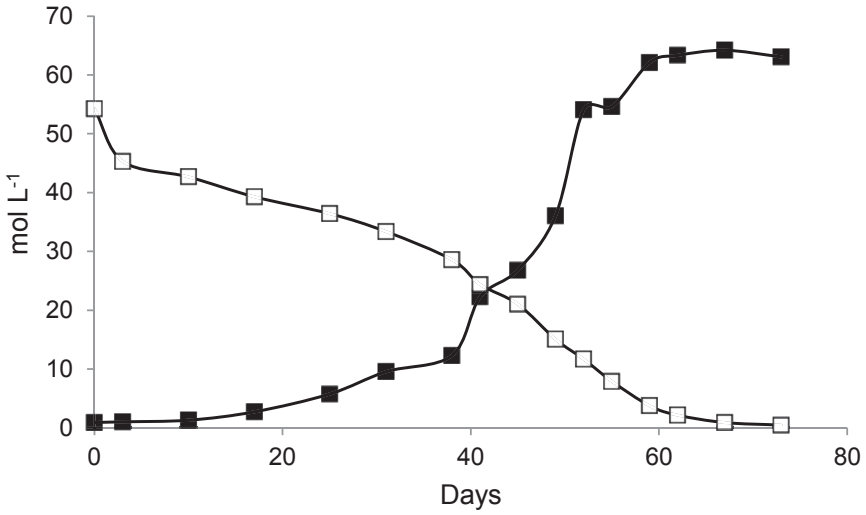

Figure 3. Syntrophic degradation of acetate □ and production of methane ■ by SAO-culture, including the SAOB *C. ultunense*, *S. schinkii* and *T. acetatoxydans* and the hydrogenotrophic *Methanoculleus* sp. MAB1, at 37°C and 2 M NH_4Cl.

Information about the doubling time of *T. acetatoxydans* in syntrophic cultivation with a hydrogen-utilising methanogen is not available at the moment (**II**). Consequently, only speculations can be made regarding the cause of the observed decrease in doubling time of the SAO-culture compared with

the co-cultures. Either presence of *T. acetatoxydans* is the origin of the relatively high rate of methane formation, or cooperation is carried out between the strains. Alternatively, the three different strains possess maximum acetate degradation rate in different acetate concentrations, resulting in increased overall degradation rate in the batch experiment.

The use of alternative substrates (except for acetate) for growth by the syntrophic cultures has not been examined adequately to date. However, *T. lettingae* has primarily been characterised as a syntrophic methanol-degrading bacterial strain in association with a hydrogenotrophic methanogen, but has also been shown to syntrophically oxidise acetate. The growth rate of the co-culture is considerably enhanced when methanol is used as substrate, compared with the growth rate observed during degradation of acetate. In addition to acetate, strain Reversibacter and *T. phaeum* are able to oxidise ethanol to methane in the presence of hydrogen-utilising methanogens (Hattori, 2008). Syntrophic degradation of ethanol or methanol by our SAO culture has not been established. However, degradation of fatty acids by the SAO culture was tested and revealed that butyrate was used as substrate, but no growth was observed when propionate was added as substrate (unpublished results).

3.1.3 Presence in biogas reactors

The importance of SAO and presence of SAOB have recently been stressed in several bacterial population studies of both laboratory and full-scale reactors. Furthermore, gene sequences affiliated to the thermophilic *T. phaeum* and *T. lettingae* have been detected in reactors operating at both high (Rademacher *et al.*, 2012; Sun *et al.*, 2012; Sasaki *et al.*, 2011a) and moderate (Shimada *et al.*, 2011; Schlüter *et al.*, 2008) temperature conditions. Sequences correlated to the mesophilic and thermotolerant SAOB (*C. ultunense*, *S. schinkii* and *T. acetatoxydans*) have also been retrieved from both thermophilic (Sun *et al.*, 2012; Weiss *et al.*, 2008) and mesophilic reactors (**III**, **V**; Karlsson *et al.*, 2012; Sun *et al.*, 2012; Shimada *et al.*, 2011). The presence of the thermophilic *T. lettingae* and *T. phaeum* in reactors operating under mesophilic conditions and of the mesophilic *C. ultunense* and *S. schinkii* in thermophilic systems is surprising. The reason may be deficit in availability of components essential for optimal growth in pure cultivation, as discussed previously. Alternatively, hitherto uncharacterised species closely related to these bacteria possess more diverse features than the SAOB isolated to date.

A noteworthy finding in this thesis is that despite the difference in origin and physiological traits of the mesophilic and thermotolerant SAOB (Table 1, **I**, **II**), all three isolates (*C. ultunense*, *S. schinkii* and *T. acetatoxydans*) have

been detected in reactors operating under diverse conditions regarding ammonia concentration, temperature, HRT and substrate feed (**III**, **V**).

During construction of bacterial 16S rRNA gene libraries of thermophilic municipal biogas plants, 1.7-6.7%, and 0.8-1.7% of the sequences analysed, respectively, were allocated to the genome of *C. ultunense* and *T. acetatoxydans* with high alliance (97% maximum identity). For *S. schinkii* the correlation was as high as 17.2-19.3% (Weiss et al., 2008). However, the levels obtained with quantitative analyses were considerably lower. In biogas reactors occupying SAO-mediated acetate degradation the relations of the specific SAOB gene abundance to the total bacterial gene abundance were 0-2.3, 0-0.01 and 0.4-1.0% for *C. ultunense*, *T. acetatoxydans* and *S. schinkii*, respectively (**III**; Sun *et al.*, 2012).

Interrelated levels of gene abundance determined in quantitative studies of SAOB can be distinguished. The abundance of genes affiliated to *S. schinkii* was found to range from about 10^9 to 10^{11} per mL reactor sludge (**III**, **V**; Karlsson *et al.*, 2012; Sun *et al.*, 2012), whereas abundance of *C. ultunense*-related genes was about 10^7 and *T. acetatoxydans* genes reached levels from 10^6 to 10^{10} per mL reactor sludge in mesophilic reactors dominated by SAO. The corresponding levels in reactors dominated by the aceticlastic pathway were 10^5-10^7, 0-10^5 and 0-10^3 for *S. schinkii, C. ultunense* and *T. acetatoxydans*, respectively (**III**, **V**; Sun *et al.*, 2012).

Thus these findings imply that the SAOB are enduring and important components of the biogas-producing consortia, abundant at interrelated levels determined by the prevalent acetate degradation pathway.

3.1.4 Partner methanogen

The available hydrogen-utilising partner is of most importance for successful acetate degradation through SAO. It is particularly important in mesophilic conditions, where the hydrogen released from acetate oxidation needs to be even more efficiently consumed in order to provide the required energy for the cooperating partner, compared with at higher temperatures (Schink, 1997). The partner methanogens used in investigations of syntrophic acetate oxidising cultures in thermophilic conditions have been: *Methanobacterium* sp. strain THF (Lee & Zinder, 1988b) and *Methanothermobacter thermoautotrophicus* strains ΔH and TM (Balk *et al.*, 2002; Hattori *et al.*, 2000). Phylogenic analyses of the methanogenic population in SAO-dominated thermophilic reactors have revealed high abundance of hydrogenotrophic *Methanobacteriales* (*e.g. Methanobacterium* and *Methanothermobacter*) and *Methanomicrobiales* (*e.g. Methanoculleus*), but the abundance of aceticlastic *Methanosarcina* species is also reported to be high (**V**; Hao *et al.*, 2011;

Shimada *et al.*, 2011). *Methanoculleus* was revealed to be the predominant group of methanogens in mesophilic biogas plants with syntrophic acetate oxidation (Schnürer *et al.*, 1999) and was therefore used (*Methanoculleus* sp. MAB1 and MAB2; Schnürer *et al.*, 1996) as partner methanogen in co-cultivation under mesophilic and thermotolerant conditions (**I**, **II**). Further indications of the importance of members of *Methanomicrobiales*, in particular *Methanoculleus* species, for SAO have been reported in mesophilic (Angenent *et al.*, 2002) and thermophilic conditions (Sasaki *et al.*, 2011a). It is worth considering in this regard the optimal methane production rate at 44-45 ºC obtained in a growth study of *Methanoculleus* MAB1 (Figure 4). This indicates possibilities to optimise the rate of SAO by adjustment of temperature if this species operates as the partner methanogen.

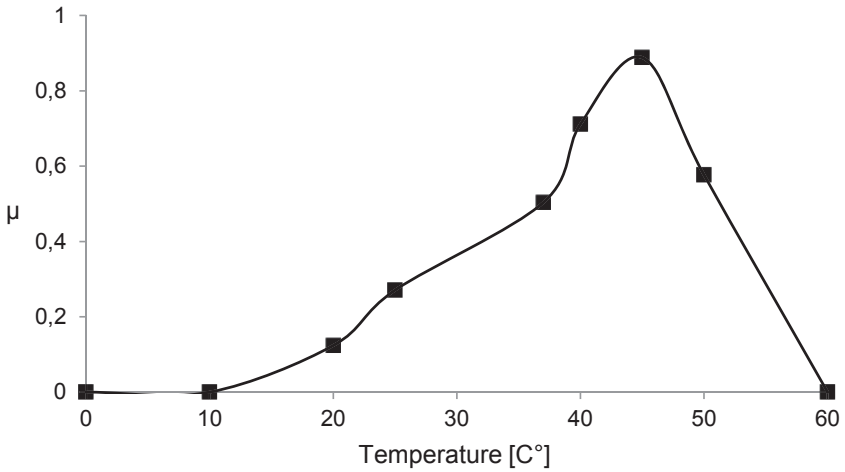

Figure 4. Methane production rate of *Methanoculleus* sp. MAB1 during growth at different temperatures.

Intriguingly, differential expression of methanogenesis genes was reported in an investigation of *M. thermoautotrophicus* strain TM during growth in pure culture compared with growth in co-culture with the SAOB *T. phaeum* in thermophilic conditions (Luo *et al.*, 2002). In pure culture, two genes encoding methyl coenzyme M reductase (MCRI and MCRII), representing the final enzyme in the methanogenesis pathway, were transcribed. However, in co-culture only one (MCR1) was transcribed by the methanogen. This result is in accordance with findings in previous pure culture investigations comparing the expression of genes under excess and limited supply of hydrogen (Morgan *et al.*, 1997; Bonacker *et al.*, 1992). Furthermore, initiation of co-culture of *C.*

ultunense, *T. phaeum*, *S. schinkii* and *T. acetatoxydans* with the hydrogenotrophic methanogen has proven to be troublesome and a time lag from three weeks (*T. phaeum*) (Hattori *et al.*, 2005) up to four months (*S. schinkii*) (**II**) has been reported. Even an attempt to obtain rapid conversion from pure growth on pyruvate or methanol to SAO by resting cells of *T. phaeum* with *M. thermoautotrophicus* grown with hydrogen/carbon dioxide has proven unsuccessful (Hattori *et al.*, 2005). Low energy yield has been suggested as a possible reason and the following factors are considered crucial for successful initiation of the co-cultivation: (i) combination of the SAO strain with the methanogen directly after isolation of the bacterium from acetate oxidising culture, (ii) high cell density of the methanogenic culture, (iii) low amount of hydrogen in the gas phase when adding the bacterium to the methanogenic culture and (iv) presence of carriers (**II**). However, the difference in gene expression of the methanogen observed by Luo *et al.* (2002) indicates that the genetics and metabolic activity of the partner methanogen are probably also of importance. They suggest significant physiological differences between methanogens that are exposed to high hydrogen partial pressure applied during pure culture and methanogens that are supplied with extremely low hydrogen levels, which occurs under syntrophic conditions. Consequently, another important factor for successful SAO co-culture might be to avoid extensive culture periods of the hydrogenotrophic methanogen at high hydrogen concentrations before combination with the bacterial strain.

A recent molecular study reported of indications that members belonging to the order *Methanosarcinales* may act as hydrogen-utilising methanogens in SAO (Karlsson *et al.*, 2012). The findings showed that over 90% of the methanogenic population analysed in biogas reactors with SAO as the main mechanism for methane formation consisted of *Methanosarcinales*. Furthermore, the relatively stable abundance of *Methanosarcinaceae* established in reactors experiencing a shift in the acetate methanisation pathway may also be indicative of involvement in SAO of species affiliated to this family (**V**). In contrast to *Methanosaetaceae*-related species, several members of the *Methanosarcinaceae* are mixotrophic and, in addition to acetate, are able to utilise methanol, methylamines and hydrogen and carbon dioxide for growth (Whitman *et al.*, 2006). Moreover, occurrence of hydrogen production and consumption during acetate metabolism to methane by *Methanosarcina* species (Lovley & Ferry, 1985) indicates an ability of these methanogens to mediate the entire process, both acetate oxidation and subsequent methanogenesis (Karakashev *et al.*, 2006).

3.1.5 Metabolic pathway

The majority of the SAOB (*C. ultunense, T. phaeum, T. acetatoxydans* and *S. schinkii*) are considered acetogens, which possess the ability to use the reductive acetyl-CoA (Wood) pathway for assimilation of carbon dioxide into cell carbon and for the conservation of energy (**IV**). The pathway includes several reactions, but overall combines two one-carbon units and produces one acetyl group. The acetyl-CoA pathway can progress under both autotrophic and heterotrophic conditions. That is, the carbon and energy for acetate and biomass formation can be derived from either inorganic (*e.g*. hydrogen/carbon dioxide) or organic (*e.g*. glucose) compounds (Ruhland *et al.*, 1955). During bacterial growth on sugars, the main function of the acetyl-CoA pathway is the reoxidation of electron carriers (NAD, ferredoxin, etc.). Carbon dioxide functions as the terminal electron acceptor and the presence of exogenous carbon dioxide is essential for growth of acetogens. However, other terminal electron acceptors, *e.g*. nitrate, halogenated compounds and aromatic acrylates, may be used by acetogens either in preference to, or concurrently with, carbon dioxide. The energy is conserved by substrate-level phosphorylation, membranous electron transport systems and ATPases (Drake *et al.*, 2006).

The capability to use the acetyl CoA-pathway distinguishes acetogens from organisms that produce acetate by other metabolic pathways. Acetogens may also produce compounds other than acetate if terminal electron acceptors other than carbon dioxide are used, since the acetyl-CoA pathway can be repressed (Drake *et al.*, 2006). Consequently, in order to assign an acetogen it is essential to confirm that the bacterium harbours the acetyl-CoA pathway. For *C. ultunense* and *T. phaeum* enzyme activity, tests indicate operation through the acetyl-CoA pathway (Hattori *et al.*, 2005; Schnürer *et al.*, 1997). However, for *T. acetoxydans* (**II**) and *S. schinkii* (unpublished results), detection and partial sequencing of the gene encoding the acetogen key enzyme formyltetrahydrofolate synthetase (FTHFS) indicate acetogenic features of the isolates. FTHFS is a highly conserved enzyme and targeting of this gene has recently been assessed to study acetogenic populations in anaerobic reactors (**IV**; Ryan *et al.*, 2008). No gene encoding acetyl-CoA synthase (also termed CO dehydrogenase), another important enzyme in the acetyl-CoA pathway, was encountered in the complete genomic sequence of *T. lettingae*, indicating that these bacteria possess an alternative metabolic mechanism for the oxidation of acetate (Hattori, 2008).

In syntrophic acetate oxidation, the SAOB are believed to operate the acetyl-CoA pathway reversibly. Thus, the syntrophic bacteria use the same biochemical reaction apparatus, and synthesise ATP, in both directions (Schink, 1997).

4 Detection of SAOB and the methanogenic pathway

The primary acetate conversion pathway and detection of the microorganisms involved can be determined through several different approaches. These implementations are of importance in order to increase the understanding of SAO, its ecological distribution and to substantiate the factors affecting the development of a certain degradation pathway. Tracer experiments are commonly used to establish the dominant pathway for acetate conversion in anaerobic biogas reactors, whereas T-RFLP, qPCR and culture-based studies have been performed for detection, quantification, isolation and characterisation of SAOB (**I**-**V**).

4.1 Culture-based studies

Enrichment, isolation and cultivation of syntrophic acetate oxidisers are time-consuming and reconstruction of co-cultures with a hydrogen-utilising methanogen requires a significant lag period (**II**; Schnürer *et al.*, 1996; Lee & Zinder, 1988b). Isolation of syntrophic acetate-oxidising co-cultures was therefore long considered to be extremely difficult or even impossible (Hattori, 2008). However, culture-based studies are of most importance in order to obtain detailed insights into their physiology, behaviour and interactions. Furthermore, information gained from culture-based studies may be essential for the development of molecular techniques. For instance, the isolation and characterisation of SAOB (**I**, **II**; Schnürer *et al.*, 1996) facilitated molecular studies of the mesophilic syntrophic acetate oxidisers *in situ* (**III**, **IV**, **V**).

Cultivation of anaerobic organisms demands implementation of special culture techniques and the methodology is based on the work of Hungate (1969). However, the isolation of SAOB is distinguished from the general anaerobic cultivation procedure due to the requirement to use a substrate in

pure culture, different from the compound (acetate) utilised during syntrophic growth, the mutual feature characteristic for this group of bacteria (**I**, **II**). Isolation is commonly performed with the agar shake or the anaerobic roll tube technique, but is advantageously preceded with enrichment of the source consortium on acetate. The intention with this is to enhance the number of abundant acetate-utilising microorganisms, which can then be further selected with serial dilution. Inclusion of ammonia at this stage may be an approach to inhibit aceticlastic methanogenesis. During isolation of the bacteria and growth in pure culture, several different compounds, known to be selective and support growth of acetogenic bacteria, are often tested. Subsequent establishment of a pure bacterial culture and confirmation of syntrophic acetate-oxidising capability in co-cultivation with a hydrogenotrophic methanogen are required in order to assign the isolated bacteria to the SAOB. This has been identified as an exceptionally troublesome and time-consuming step (**I**, **II**; Balk *et al.*, 2002; Hattori *et al.*, 2000; Schnürer *et al.*, 1996; Lee & Zinder, 1988b).

4.2 Selective inhibition

Methyl fluoride (CH_3F) specifically inhibits aceticlastic methanogenesis and can be used at certain concentrations to determine the relative contribution of acetate versus hydrogen/carbon dioxide to total methane production (Janssen & Frenzel, 1997). The selective inhibitor has especially been used in methanogenic environments, such as rice field systems (Penning & Conrad, 2006; Conrad & Klose, 1999), whereas the knowledge about applications in biogas reactors is limited (Hao *et al.*, 2011).

4.3 Tracer experiment

Labelled substrates (radiocarbon or stable isotopic probing) have been used in several studies to monitor the contribution of SAO to methane production in anaerobic reactors (**V**; Hao *et al.*, 2011; Sasaki *et al.*, 2011a; Schnürer & Nordberg, 2008; Karakashev *et al.*, 2006; Schnürer *et al.*, 1999; Petersen & Ahring, 1991). In the reactions involved in syntrophic acetate oxidation (see below), both the methyl and the carboxyl group of acetate are oxidised to carbon dioxide and hydrogen by SAOB, followed by reduction of one of the carbon dioxide molecules to methane by the methanogen. Aceticlastic degradation on the other hand, involves cleavage of acetate, where the methyl group is converted to methane and the carboxyl group forms carbon dioxide (Zinder & Koch, 1984).

Syntrophic acetate oxidation (SAO) reactions:

$$^{*}CH_3COO^- + 4H_2O \rightarrow H^{*}CO_3^- + HCO_3^- + 4H_2 + H^+$$

$$4H_2 + H^{*}CO_3^- (or\ HCO_3^-) + H^+ \rightarrow {}^{*}CH_4 (or\ CH_4) + 3H_2O$$

Aceticlastic cleavage (AC) reactions:

$$^{*}CH_3COO^- + 4H_2O \rightarrow HCO_3^- + {}^{*}CH_4 + 4H_2 + H^+$$

(Asterisks represent the carbon of methyl group in acetate.)

4.3.1 Radiocarbon

Tracer experiments with radiocarbon involve addition of ^{13}C or ^{14}C-labelled acetate in batch incubations for determination of the dominant pathway for acetate degradation (Schnürer & Nordberg, 2008; Karakashev et al., 2006; Schnürer et al., 1999). The aceticlastic methanogenesis of [2-^{14}C]-labelled acetate for instance, primarily produces $^{14}CH_4$, whereas SAO forms both $^{14}CO_2$ and $^{14}CH_4$ (see reactions above). Measurements of the labelled gaseous products then indicate the dominant pathway for methane formation, where a $^{14}CO_2/^{14}CH_4$ ratio exceeding 1 indicates dominance of SAO (Schnürer et al., 1999). However, the ratio does not reflect the quantitative contribution of each pathway, since dilution of the labelled bicarbonate may occur in unlabelled bicarbonate pool prevailing in the digester liquid.

4.3.2 Stable carbon isotopic probing (SIP)

Stable carbon isotopic probing (SIP) of methane ($\delta^{13}CH_4$) provides a more comprehensive insight into the carbon cycle in the methanogenic system compared with radiocarbon tracing or selective inhibition, and can principally be used to quantify the relative importance of the aceticlastic and hydrogenotrophic paths of methane production (Conrad, 2005). For estimation of the relative contribution of each pathway, determination of δCH_4, δCO_2, $\delta^{13}C$-acetate and the fractionation factors is required. SIP has commonly been combined with molecular analyses such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (Hao *et al.*, 2011), fluorescence *in situ* hybridisation (FISH) (Qu *et al.*, 2009) or construction of clone libraries (Sasaki *et al.*, 2011a) in order to link the dominant methane-producing pathway with the microbial community structure in anaerobic reactors.

Alternatively, nucleic acid-based SIP (DNA-SIP or RNA-SIP), which enables linking of a specific metabolic function to taxonomic identity, can be used. This approach is based on incorporation of ^{13}C-labelled atoms into the nucleic acid during growth on the labelled substrate. The ^{13}C-label increases the density of DNA or RNA and the labelled and unlabelled nucleic acids can

then be separated by density gradient centrifugation. The isolated DNA can be characterised taxonomically and functionally by molecular-based techniques, such as gene probing and sequencing. The microorganisms actively involved in a metabolic process, such as SAO, can subsequently be identified without the need for time-consuming performance of isolation and cultivation. Another positive feature of SIP is that it provides access to the genes involved in a specific function. However, dilution of the labelled substrate by unlabelled substrates produced by internal processes, cross-feeding during the enrichment phase and low growth rate of investigated microorganisms may appear as limitations during nucleic acid-based SIP application (Radajewski *et al.*, 2003; Radajewski *et al.*, 2000). The method has been used to detect organisms active in methane production from acetate in profundal sediment of a freshwater lake (Schwarz *et al.*, 2007) but, to my knowledge, not yet for characterisation of the acetate-degrading microbial population in biogas systems.

4.4 Molecular biological methods

Molecular-based assessments provide possibilities to study microbial communities in anaerobic reactors and the response to prevailing operational parameters. The genetic information obtained during the characterisation of *C. ultunense* (Schnürer *et al.*, 1996), *T. phaeum* (Hattori *et al.*, 2000), *S. schinkii* (**I**) and *T. acetatoxydans* (**II**) promoted the development of molecular approaches for quantification of SAOB *in situ* (**III)**. The molecular technique is based on targeting of the 16S rRNA gene encoding the major part of the small subunit of the ribosome. This gene is present in all prokaryotes in various copy numbers, and is commonly used in molecular-based analyses because of the high sequence conservation for precise alignment, but also the sufficient variability to distinguish differences at species level (Woese, 1987).

Alternatively, targeting of functional genes allows the study of microorganisms possessing a specific function or a specific functional group of organisms. A molecular biomarker for the gene encoding FTHFS has been used for investigation of the acetogenic population in biogas reactors operating at mesophilic (**IV**) and/or thermophilic conditions (Hori *et al.*, 2011; Ryan *et al.*, 2008), where some results indicate the occurrence of as yet unidentified SAOB. In the mesophilic reactor this was further investigated through performance of quantitative assessment of a FTHFS encoding gene, highly abundant in the SAO-dominated reactor, with relatively low identity to previously recovered gene sequences (**IV**). However, it should be taken into consideration that the FTHFS primer pair has a limited specificity for isolated SAOB (**IV**), and that bacteria other than acetogens, including *T. lettingae*, are

probably important for the performance of syntrophic acetate oxidation (Hattori, 2008). Furthermore, since the acetyl-CoA pathway is also used by non-acetogens, for instance purinolytic clostridia, the gene encoding FTHFS may be present in their genome and targeted in molecular assessment with this primer pair. Consequently, a more comprehensive survey of the SAOB population probably demands a biomarker targeting gene(s) distinguished for species able to mediate syntrophic acetate oxidation.

The molecular-based analyses of the SAO population that have been performed to date do not define the dominant pathway for methane formation. However, quantitative analyses of SAOB in biogas reactors indicate increased contribution of SAO for methanisation of acetate when the abundance of SAOB increases (**III**, **V**; Sun *et al.*, 2012). Molecular approaches used (*e.g.* **III**, **IV**, **V**), or having the potential to be used, for detection and quantification of SAOB in biogas reactors are described more comprehensively below.

4.4.1 Quantitative PCR

Quantitative polymerase chain reaction (qPCR) analysis has been extensively used to study the bacterial and archaeal population in biogas reactors (Blume *et al.*, 2010; Feng *et al.*, 2010; Munk *et al.*, 2010; Nettmann *et al.*, 2010; Yu *et al.*, 2005). qPCR provides measurements of gene abundance per unit sludge sample. The initial number of the original target molecules in the sample is specified by the curve rate of accumulated products in PCR. The amplicon synthesis can be monitored by probes labelled with a reporter dye. The probe binds to the target DNA between the two primer sites and liberates a fluorescence signal when released from the molecule during DNA synthesis. An alternative approach is to use a dsDNA-binding dye (*e.g.* SYBR Green), which emits fluorescence when it binds to dsDNA and the signal intensifies corresponding to the increase in amplified products (Sharma *et al.*, 2007). Species-specific primers targeting the 16S rRNA gene of several known SAOB have been developed (**III**), and can be used in qPCR for quantification of the bacterial gene abundance *in situ*. Conversion of gene abundance to cell densities requires knowledge of copy number per genome and/or genome size, which can be very variable (Klappenbach *et al.*, 2001). The complete genomic sequence of *T. acetatoxydans* was recently established (GenBank accession no. CP002728), and sequencing of the *C. ultunense* and *S. schinkii* genome is in progress (unpublished results), which will enable quantification of the bacteria abundance.

qPCR is an informative method for quantification of microbes *in situ*. However, an oligonucleotide is only as precise as the database from which it is derived. To date, only a few bacterial species known as SAOB have been

characterised and phylogenetically analysed. Furthermore, since amplified sequences in qPCR analysis are short, there is a risk that uncharacterised closely related strains belonging to other species might be targeted, resulting in overestimation of the gene abundance.

4.4.2 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is another approach applied to monitor changes in the most abundant microorganisms in response to operational variables of biogas reactors (**IV**; Ziganshin *et al.*, 2011; Feng *et al.*, 2010; Padmasiri *et al.*, 2007). In T-RFLP, PCR is conducted with primers labelled with a fluorescent dye, followed by digestion of the amplicons by restriction enzymes. Fluorescent-labelled terminal restriction fragments (T-RF) of different lengths (numbers of base pairs) are then produced due to the sequence polymorphism. These distinctive T-RFs are separated on a gel or capillary sequencer and detected by an automated sequencer. The relative intensity of the fluorescence is also registered, reflecting the relative abundance of the individual T-RF, which provides a profile of the microbial community composition. Individual T-RF can be identified and linked to a phylogenetic position by further analyses, *e.g*. by the performance of cloning and sequencing (**IV**). The survey of SAOB by T-RFLP with general primers may be restricted by the relatively low abundance of the bacteria in reactor sludge. For instance, in T-RFLP analyses using primers targeting the gene encoding 16S rRNA (unpublished results) or FTHFS (but only targeting *C. ultunense* and not *T. phaeum*, *S. schinkii* or *T. acetatoxydans*) none of the T-RFs obtained was affiliated to known SAOB (**IV**), even though presence of some of these bacteria has previously been determined in the reactors (**III**).

4.4.3 Cloning and sequencing

Several studies have identified SAOB in biogas reactors by assessment of cloning and sequencing (Sasaki *et al.*, 2011a; Shimada *et al.*, 2011; Weiss *et al.*, 2008). Cloning involves incorporation of PCR amplicons into plasmids, which are further used to transform host cells (*Escherichia coli*). The transformed cells are then plated, with the prospect of generating colonies from single cells. The clones can be analysed by colony PCR and restriction fragment length polymorphism (RFLP) using restriction enzymes. Clone families are distinguished by different restriction patterns, followed by sequencing of representatives from each group. As with T-RFLP analysis, the assessment of cloning for detection of SAOB in the biogas-producing microbial consortia may be limited by the relatively low abundance of the bacteria in reactor sludge. Genes affiliated to SAOB were not detected in our

studies during cloning with primers targeting the gene encoding 16S rRNA (unpublished results) or FTHFS (**IV**).

4.4.4 454 pyrosequencing

Contigs allocated to the genome sequence of SAOB have been obtained by 454 pyrosequencing of biogas-producing microbial communities (Rademacher *et al.*, 2012; Schlüter *et al.*, 2008). 454 pyrosequencing is an ultrafast sequencing approach that provides hundreds of thousands of single reads of nucleotide sequence data and is suitable for estimating biodiversity of complex microbial communities (Manichanh *et al.*, 2008). The read lengths obtained with the first generation of the technique were short (~100 to 200 bp) and not always sufficient for complete phylogenetic and functional characterisation of the biogas community (Wommack *et al.*, 2008). However, the read length has improved and with the latest generation of the 454 sequencing system more than 500 bp can be obtained, which is enough for an accurate phylogenetic characterisation (Logue *et al.*, 2011).

4.4.5 Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation (FISH) analysis has been widely used to visualise, identify and quantify bacterial and methanogenic members within biogas reactors (Krakat *et al.*, 2010; Nettmann *et al.*, 2010; Karakashev *et al.*, 2005). FISH is based on hybridisation of oligonucleotide probes to specific regions within the microbial ribosome (commonly RNAs). Since the organisms are examined without altering the cell structure morphology, abundance and spatial distribution can be documented (Amann *et al.*, 2001).

To the best of my knowledge, SAOB have not yet been identified in reactor sludge by assessment of the FISH approach. However, development of a SAOB-specific FISH method might reveal the preferred location of the bacteria in the reactors and their position relative to their partner methanogen.

Autofluorescence from the activated reactor sludge and from microorganisms (especially methanogens), and the relatively low abundance of the bacteria in the microbial community, might be limitations in development of a FISH approach for detection of SAOB. However, increased labelling of the probes or the use of indirect labelling techniques might circumvent these problems. Examples of indirect labelling are enzymatic signal amplification or linking the probe to a reporter molecule later detected by a fluorescent antibody (Moter & Göbel, 2000).

5 Factors regulating the dominant pathway

Syntrophic acetate oxidisers have been suggested to be less competitive for acetate than the aceticlastic methanogens (Rui *et al.*, 2011). Therefore SAO is considered to be the dominant pathway in systems operating under conditions that suppress growth of the aceticlastic methanogens, such as high ammonia (Schnürer & Nordberg, 2008; Schnürer *et al.*, 1999). Other factors considered to influence the acetate conversion pathway in anaerobic digestion systems are acetate concentration, HRT, temperature and the methanogenic population structure. However, the actual reason for change in prevalent pathway may not always be entirely obvious. Anaerobic reactors are generally characterised by complex microbial communities (Riviére *et al.*, 2009), and operational parameters, chemical components of the sludge and the microbial population structure and activity are often strongly correlated. However, in this section different factors proposed to regulate the dominant acetate-degrading pathway are further discussed.

5.1 Ammonia inhibition

Ammonia is readily released during anaerobic degradation of proteins and has inhibitory effects on the anaerobic microbial consortia, particularly on the methanogenic population (Sprott & Patel, 1986; Koster & Lettinga, 1984). Concentrations from 1.7 to 14 g L^{-1} of total ammonia nitrogen have been reported to restrain the methanogenic activity. The wide concentration range is probably caused by variations in substrates, inoculum, environmental conditions and acclimatisation periods (Chen *et al.*, 2008). It is primarily the dissociated form of ammonia (NH_3) that is considered to be responsible for inhibition, and there are different operational parameters that influence the equilibrium between the ammonium (NH_4^+) and ammonia (NH_3) in the reactor sludge (Hashimoto, 1986). Augmented temperature increases the ratio of NH_3,

but also contributes to reduced solubility of carbon dioxide, which increases the pH and thereby further shifts the equilibrium towards NH_3 (Gellert & Winter, 1997). The eventual ammonia concentration in the reactor is also influenced by substrate characteristics and the degree of decomposition (*i.e*. the proportion of the organic material converted to methane). At a low degree of decomposition a smaller proportion of the nitrogen in the reactor content is released as ammonia than at higher degrees of degradation (Schnürer & Jarvis, 2010).

Three mechanisms for ammonia toxicity are considered to influence growth and activity of methanogenic strains. One mechanism is based on the accumulation of ammonium inside the cell due to passive diffusion of uncharged ammonia across the cell membrane, equilibrating the intracellular and extracellular concentrations of ammonia. Inside the cell, ammonia is transformed to ammonium and a proton is subsequently taken up. This affects the intracellular pH and the uptake of protons also stimulates a K^+/H^+ antiporter and an influx of H^+, resulting in losses of cytoplasmic K^+ (Sprott *et al.*, 1984). A second possible mechanism involves direct inhibition by NH_4^+/NH_3 on the methane-synthesising enzymes. Ammonium is believed to interact with a Ca^+, Mg^{2+}-site, located on the external face of the cytoplasmic membrane, essential for the synthesis of methane by the methanogens (Sprott *et al.*, 1985). Increased maintenance energy requirement is a third mechanism proposed to cause ammonia inhibition (Cashion *et al.*, 1977).

Ammonia has been shown to be a selective agent for syntrophic acetate oxidation in moderate temperature conditions (**V**; Shimada *et al.*, 2011; Schnürer & Nordberg, 2008; Angenent *et al.*, 2002; Schnürer *et al.*, 1999). This may be due to the higher sensitivity of the aceticlastic methanogens to ammonia compared with the hydrogenotrophic methanogens (Steinhaus *et al.*, 2007; Angelidaki & Ahring, 1993b; Sprott & Patel, 1986; Zeeman *et al.*, 1985; Koster & Lettinga, 1984). Ammonia-induced inhibition of the aceticlastic methanogens has been suggested to encourage the relatively ammonia-tolerant SAOB (**I**, **II**) to be the dominant acetate degrader, together with the hydrogenotrophic methanogen (Karakashev *et al.*, 2006; Angenent *et al.*, 2002). This has been further indicated by molecular studies of biogas reactor sludge showing increased abundance of SAOB and hydrogenotrophic methanogens, and decreased abundance of the aceticlastic methanogens, coincidently with enhanced ammonia concentration and a shift from aceticlastic to syntrophic acetate degradation (**III**, **V)**. Accordingly, a study quantifying SAOB in 13 full-scale CSTR biogas reactors found correlations between high ammonia concentrations and presence of SAOB (Sun *et al.*, 2012). Here, genes assigned to *C. ultunense, S. schinkii* and *T. acetatoxydans*

were observed to a higher degree in ammonia-stressed biogas reactors compared with reactors operating at low ammonia concentrations. This concerned both mesophilic and thermophilic reactors. The analysis also indicated a correlation between absence of the aceticlastic methanogens of *Methanosaetaceae* and high concentrations of ammonia, which is in accordance with previous investigation (Nettmann *et al.*, 2010).

However, members of the *Methanosarcinaceae* and *Methanosaetaceae* have been abundantly detected in high ammonia reactors, indicating occurrence of ammonia-tolerant aceticlastic methanogens (**III**, **V**; Sun *et al.*, 2012; Calli *et al.*, 2005b).

The majority of the SAOB characterised to date have been identified as acetogens and ammonia has been shown to influence the population structure of this group of bacteria (**IV**). The propionate-degrading acetogenic bacteria in particular are considered to be susceptible to ammonia toxicity (Calli *et al.*, 2005a; Lokshina *et al.*, 2003; Poggi-Varaldo *et al.*, 1997).

Different possible approaches have been suggested in order to circumvent problems associated with high ammonia concentration in anaerobic digestion systems. Examples of such approaches include addition of materials with ion exchange capacity (Hansen *et al.*, 1999; Angelidaki & Ahring, 1993a), lowering the temperature from thermophilic to mesophilic conditions (Angelidaki & Ahring, 1994), pH regulation (Karlsson & Eljertsson, 2012), increasing the C/N ratio and diluting the biomass with water (Kayhanian, 1999). However, long retention time is often required, which restrain the economic feasibility of the process (Nielsen & Angelidaki, 2008). Acclimatisation is another factor that could increase the tolerance and retain viability of the microbes at ammonia concentrations far exceeding the initial inhibitory concentrations (Angelidaki & Ahring, 1993b; Zeeman *et al.*, 1985; Koster & Lettinga, 1984; Kroeker *et al.*, 1979).

5.2 Hydraulic retention time

The dilution rate and the hydraulic retention time have been suggested to influence the pathway for acetate conversion to methane in anaerobic reactors. In an anaerobic, mesophilic acetate-fed chemostat (Shigematsu *et al.*, 2004), SAO was reported as the primary pathway at low dilution rate (0.025 day^{-1}), whereas the aceticlastic pathway dominated at a higher rate (0.6 day^{-1}). This is in accordance with the results from a laboratory-scale CSTR study, identifying HRT as a crucial factor in avoiding washout of the microbes. Here, activity of both SAO and aceticlastic methanogenesis was indicated in reactors operating at relatively low ammonia concentrations (1.5 g NH_4^+-N L^{-1}), and at HRT and

OLR of 57 days (0.04 day^{-1}) and 0.8 gVS $(L\ day)^{-1}$, respectively. Aceticlastic methanogenesis became dominant, when HRT was decreased to 26 days (**V**).

Furthermore, doubling times obtained from cultivation studies of the mesophilic acetate-degrading co-cultures indicate HRT as a significant factor for maintenance of the syntrophs in the reactor. As mentioned previously, the HRT should exceed the microbial doubling time in order to avoid washout of the population (Angelidaki *et al.*, 2011; Weiland, 2010). Considering the difference between the doubling times of mesophilic acetate-degrading co-culture (28 days) (Schnürer *et al.*, 1994) and the SAO-culture (9 days; Figure 3), and between mesophilic (9-28 days) and thermophilic (1.5-3 days) co-cultures (Hattori *et al.*, 2000; Lee & Zinder, 1988b), the HRT needed for maintenance of syntrophic acetate oxidation in a continuous methanogenic reactor is probably difficult to predict. However, presence of *S. schinkii*-related species has been established in mesophilic reactors with HRT ranging from 8 (in upflow anaerobic filter) to 64 days (in CSTR) (**I**, **III**, **V**; unpublished results; Sun *et al.*, 2012; Schnürer *et al.*, 1999). Furthermore, reactor configuration, recycling of reactor sludge and presence of support material probably also influence the resulting HRT needed to maintain SAOB and their partner methanogen in the system.

5.3 Support material

The dissolved hydrogen concentration is maintained low by methanogens through close interaction with hydrogen producers. Consequently, the majority of hydrogen-dependent methane formation occurs in granular sludge, flocks or biofilms, which facilitate interspecies hydrogen transfer by reducing the distance between the hydrogen-producing bacteria and the hydrogen-consuming methanogens (Angelidaki *et al.*, 2011; Liu & Whitman, 2008; Stams, 1994). Furthermore, mixing has been reported to inhibit syntrophic oxidation of volatile fatty acids, as indicated by disruption of the spatial juxtaposition of the syntrophic bacteria and their methanogenic partner (McMahon *et al.*, 2001). Factors such as the ready formation of granules or adherence to bottle walls by the cells in syntrophic acetate-oxidising cultures (unpublished results), the origin of *S. schinkii* (anaerobic filter reactor) and the requirement of addition of carriers to growth media for successful initiation of acetate oxidation (**I**, **II**) indicate that incorporation of support material, or formation of granular sludge, in the reactors could promote the presence of SAOB and help to counteract losses of these slow-growing syntrophs. Increased microbial tolerance to high ammonia concentration through inclusion of carbon fibre textiles has been assessed in biogas reactors (Sasaki *et al.*,

2011b). It was found that the fibre textiles supported maintenance of a microbial population with higher tolerance to ammonia, possibly characterised by SAOB and hydrogenotrophic methanogens.

5.4 Acetate concentration

There is conflicting information about how the level of acetate influences the development of SAO. Under thermophilic conditions, SAO has been proposed as the predominant acetate degradation pathway in anaerobic reactors at both low (0.2-1 mM) (Hori *et al.*, 2006; Ahring, 1995; Ahring *et al.*, 1993; Petersen & Ahring, 1991) and high (100 mM) acetate concentrations (Hao *et al.*, 2011). The influence of acetate concentration has not been extensively examined in methanogenic systems prevailing within mesophilic reactors. However, one study using mesophilic long-term acetate-fed chemostats showed dominance of SAO at low acetate concentrations (0.2 mM), while higher concentrations (4 mM) favoured the aceticlastic pathway (Shigematsu *et al.*, 2004). However, the concentration of ammonia was probably low (not specified in the report), which probably influenced the constitution of the acetate degradation pathway at low and high acetate concentrations. Since high ammonia commonly is accompanied by high VFA concentrations, it is difficult to distinguish the cause and effect within this aspect.

Thermodynamically, SAO is favoured by high concentrations of acetate. Without the presence of competitors for acetate, high acetate concentrations have been shown to stimulate growth (the methane formation rate) of syntrophic acetate-oxidising cultures in growth studies under mesophilic conditions (Schnürer *et al.*, 1996; Blomgren *et al.*, 1990). This observation is consistent with growth experiments performed with the mesophilic SAO-culture, comprising *C. ultunense*, *T. acetatoxydans*, *S. schinkii* and the hydrogen-utilising partner *Methanoculleus* sp. MAB1. The results from this trial also indicate that a relatively high initial concentration of acetate (>25 mM) is required for initiation of syntrophic acetate degradation (unpublished data).

The minimum threshold level for acetate of the SAOB, *i.e*. the lowest concentration of acetate that can be metabolised in an active culture, has yet to be determined. Schnürer *et al.* (1996) observed ceased degradation of the syntrophic co-culture (*C. ultunense* and *Methanoculleus* sp. MAB1) at an acetate concentration of 30 mM. Similar result was obtained with co-culture of *T. acetatoxydans* and *Methanoculleus* sp. MAB2. However, acetate degradation proceeded below the detection limit of 2 mM in a co-culture of *S. schinkii* and *Methanoculleus* sp. MAB1, which also occurred in the culture

holding the three SAOB (*C. ultunense*, *S. schinkii* and *T. acetatoxydans*) and the partner methanogen (unpublished results). These findings indicate that the threshold concentration of acetate varies between the strains, resulting in a competitive interaction, or existence of some sort of metabolic cooperation, perhaps by production of essential intermediates otherwise absent in the cultivation media. An attempt was made to quantify the different SAOB during the syntrophic conversion of 50 mM acetate to methane in batch cultivation. However, no distinct variance in increased gene abundance between the bacteria was observed throughout the experiment (unpublished data). The cultivation in batch probably restricted the distinction between active and inactive strains, which a study in a continuous system would enable.

The concentration of acetate influences the diversity and the dominance of the aceticlastic methanogens. *Methanosarcina* generally exhibits higher growth rate but requires higher acetate concentration (McMahon *et al.*, 2001; Jetten *et al.*, 1990), whereas *Methanosaeta* species dominate at low acetate concentrations, due to their higher affinity for acetate (Blume *et al.*, 2010; Zheng & Raskin, 2000). Consequently, one possible explanation for dominance of the SAO pathway at low acetate concentrations in thermophilic methanogenic systems is the lower growth rate reported for some *Methanoseata* species compared with thermophilic syntrophic acetate-degrading cultures (Ahring, 1995).

5.5 Temperature

Syntrophic acetate oxidation becomes energetically favourable at elevated temperatures (Hattori, 2008; Schink & Stams, 2006; Schink, 1997), and SAO has been established as an important pathway in several thermophilic methanogenic reactors (Hao *et al.*, 2011; Krakat *et al.*, 2010; Ryan *et al.*, 2010; Hori *et al.*, 2006; Karakashev *et al.*, 2006; Shigematsu *et al.*, 2004; Petersen & Ahring, 1991; Zinder & Koch, 1984). As previously mentioned, the lower growth rate of certain *Methanoseata* species compared with syntrophic acetate oxidisers in thermophilic conditions might favour SAO (Ahring, 1995). However, indications of SAO have also been reported in subtropical lake sediments at temperatures as low as 15 °C (Nüsslein *et al.*, 2001).

The temperature ranges for growth of known SAOB are specified in Table 1. However, as mentioned previously, gene sequences allocated to the different SAOB have been recovered from processes operating at temperatures far from the optimum growth conditions determined during pure cultivation.

5.6 The methanogenic population structure

Syntrophic acetate oxidisation has been suggested to be the dominant methanogenic pathway in the absence of *Methanosaetaceae* (Karakashev *et al.*, 2006). Furthermore, statistical analysis of the methanogenic population in large-scale reactors has established negative correlations between presence of SAOB and members of the *Methanosarcinaceae* (Sun *et al.*, 2012). However, these studies considered the methanogenic population of the biogas plants at one sampling point, displaying the existing profile in the reactors dominated by the SAO pathway. Therefore, it might be inaccurate to identify the existing community structure as the actual factor of origin for the prevailing acetate-degrading pathway. Our quantification of SAOB and the methanogenic population in biogas reactors operating at gradually increased ammonia concentration showed a decrease in *Methanosaetaceae* before increased abundances of SAOB and introduction of the SAO pathway (Figure 1B in **III** and Figure 4 in **V**). However, in both these studies *Methanosaetaceae* was still present when SAO was the dominant pathway. Furthermore, high abundance of *Methanosaetaceae*-related species has been found in a laboratory-scale reactor, maintaining SAO as the predominant acetate-degrading pathway (Figure 2 in **III**), which contradicts the results by Karakashev *et al.* (2006).

5.7 Promoting SAO in biogas reactors

Aceticlastic activity is generally considered to be the dominant pathway for methane production in anaerobic digestion processes (Pavlostathis & Giraldo-Gomez, 1991; Zinder & Koch, 1984). Hence, operation and optimisation of biogas reactors is generally based on maintaining aceticlastic methanogens, which overlooks the importance of the SAO pathway. Thus, re-evaluation based on suitable conditions for syntrophic acetate oxidisers is essential in order to optimise SAO-dominated processes (Karakashev *et al.*, 2006).

As stated above, high levels of ammonia, long HRT, presence of support material, high/low acetate concentration and high temperature may enhance the relative contribution of SAO to acetate removal in biogas reactors. The methanogenic population structure has also been suggested to influence this aspect. Reactor studies have indicated that ammonia-induced dynamic transition of the methanogenic pathway from aceticlastic to SAO is associated with periods of unstable process performance, accumulation of VFA and increased risk of process failure (**V**; Schnürer & Nordberg, 2008). This highlights the importance of interpretation of syntrophic acetate oxidisers under particular conditions and their impact on process performance. Hypothetically, start-up, operation and recovery of a biogas process, planned to

operate at parameters commonly promoting SAO, might be optimised if adjusted to support growth of the SAOB and the hydrogenotrophic partner methanogen.

Dominance of SAO has been observed to involve a reduction in gas production rate and methane yield compared with aceticlastic-mediated acetate degradation (**V**; Schnürer & Nordberg, 2008). However, the shift might involve development of an ammonia-tolerant biogas-producing community and possible operation of the process even at levels reported as inhibitory for the anaerobic consortia. This is of interest due to the comparably high methane potential of the anaerobic degradation of protein-rich materials. Furthermore, these processes produce digestion residue with high amounts of plant-available ammonia, thus representing a valuable fertiliser. These positive aspects may then compensate for the reduction in gas production rate and methane yield observed with SAO.

Optimised growth of *T. acetatoxydans* (Figure 3 in **II**) and the highest methane production rate of *Methanoculleus* sp. MAB1 (used as partner methanogen in SAO, Figure 4) obtained at 44-45 °C might be indicative of optimal operation conditions for SAO-dominated processes at a temperature between the conditions normally used today, *i.e*. mesophilic (37 °C) and thermophilic (55 °C).

With the aim of promoting SAO and decreasing the adaptation period to high ammonia concentrations, bioaugmentation of the natural biogas-producing consortia with syntrophic acetate oxidisers was recently assayed in laboratory-scale biogas reactors. The bioaugmentation seemed to increase the presence of the SAOB *C. ultunense* and *T. acetatoxydans*, but did not have an impact on the abundance of *S. schinkii* (Figure 4 in **V**). No significant influence on process performance of bioaugmentation was observed under the conditions studied. Instead, the findings indicated that the presence or supply of SAO microorganisms is not a crucial factor for introduction of the SAO pathway. It is rather the operational conditions, such as high ammonia, that promote the dynamic transition to SAO (**V**).

6 Conclusions and Perspectives

Acetate is an important intermediate in methanogenic systems and degradation proceeds through two pathways: acetate cleavage by aceticlastic methanogenesis and SAO. The aceticlastic pathway is the most commonly described, but several recent investigations and the studies reported in this thesis describe the significant contribution of SAO to methanogenic systems. SAO involves acetate oxidation to hydrogen and carbon dioxide by SAOB, and subsequent conversion of hydrogen and carbon dioxide to methane by hydrogenotrophic methanogens.

The true extent of the phylogenetic and ecological distribution of bacteria able to degrade acetate in syntrophic relationship with a partner hydrogenotroph is currently unknown. Prior to this work, only one mesophilic and two thermophilic SAOB had been isolated. The isolation and characterisation of the novel SAOB *S. schinkii, T. acetatoxydans* and strain sp. Esp, closely related to *C. ultunense*, thereby contributes substantially to research into SAO. These isolates tolerate relatively high ammonia concentration, which probably provides the SAOB with a competitive advantage against other acetate users at elevated ammonia levels. *T. acetatoxydans* uses a broad spectrum of substrates, indicating involvement in fermentative and oxidative reactions under non-SAO conditions, whereas the growth patterns for *S. schinkii* and strain Esp are restricted. *T. acetatoxydans* has an optimal growth rate at 44-45 °C, which is also a feature of the partner *Methanoculleus* sp. MAB1.

The molecular technique developed in this thesis proved useful for capturing quantitative changes in SAOB abundance and revealed increased abundance of *C. ultunense*, *S. schinkii* and *T. acetatoxydans*, coincidently with an ammonia-induced shift from aceticlastic to syntrophic acetate degradation. Furthermore, examination of the dynamics of abundant hydrogenotrophic methanogens indicated a shift in population structure, whereas a decrease in

aceticlastic methanogenic abundance suggested restrained activity due to elevated ammonia levels.

Increased ammonia concentration influenced the putative acetogenic population structure and caused distinct shifts in the most abundant members. However, the abundance of the acetogenic population remained relatively stable. Furthermore, a functional gene possibly harboured by an essential unknown SAOB was identified.

Finally, presence of some SAOB was induced by supplementation of syntrophic acetate-oxidising culture, but bioaugmentation did not significantly influence process performance under the conditions investigated. High abundance of SAO microorganisms is probably not a crucial factor for mediation through this pathway. Instead, operation under certain environmental conditions, such as high ammonia, promotes the dynamic transition to SAO.

6.1 Future perspectives

Knowledge and understanding of the behaviour of syntrophic acetate oxidisers and their influence on process performance and stability are still in their infancy and further research is needed. Several interesting directions can be taken to further study this area.

The questions raised in paper **V** can be investigated to a greater extent. For example, operational parameters such as enhanced temperature, changed substrate composition or a more gradual increase in ammonia levels might improve the establishment and activity of the added SAO-culture. It would also be interesting to determine the impact of bioaugmentation on the remaining microbial community in anaerobic reactors. When the individual effects on the population of altered operational parameters have been identified in the reference reactors, any differences in population structure between the reactors would then indicate the influence of the added culture. T-RFLP analyses, combined with cloning and sequencing or 454 pyrosequencing, could be suitable assessments in this context. Moreover, the impact of inclusion of support material or formation of granular sludge, which allows attachment of microbes in the reactors and counteracts washout of slow-growing microorganisms, could be investigated in SAO-dominated processes. The support material could either be placed in the reactor directly or after an incubation period in acetate-degrading co-culture chambers, in order to let the organisms adhere to the support material. Another interesting area to investigate is the influence of increased sludge retention time, achieved through recirculation, on SAOB abundance. Survivability and placement of the

syntrophs can be analysed by performance of SAOB-specific qPCR and FISH. Identification of preferred locations in the reactor might enable development of methods or recommendations that can stimulate the maintenance of SAOB in a continuous reactor system.

Important insights about the beneficial or unfavourable roles of the syntrophic acetate oxidisers for profitable anaerobic digestion could be obtained by development of a rapid *in situ* detection system for organisms with SAO attributes. For example, a DNA microarray targeting SAOB and specific hydrogenotrophic methanogens found in anaerobic reactors could be designed and used to study the expression of genes under different environmental conditions. Large amounts of sludge samples could then be screened and linked to process parameters and performance.

More comprehensive research on syntrophic acetate-degrading cultures is required in order to establish optimal growth parameters, such as temperature, ammonia concentration and pH. Determination of growth rates of SAO cultures in continuous systems prevailing under different operational conditions in terms of *e.g.* temperature and acetate and ammonia concentrations is one possible approach to further extend the understanding and mutual interaction among the SAOB, and between the SAOB and the methanogens. In addition, the affinity of the different SAOB for acetate could be determined and compared against available data on the aceticlastic methanogens, allowing further prediction of the behaviour of the SAOB in methanogenic systems. This information could then be used to implement strategies for improved reactor operation under certain conditions, such as high ammonia concentrations.

Knowledge of the mutualistic interaction and mechanism of interspecies electron transfer between the SAOB and the methanogens is limited and should be extended. For example, *Methanoculleus* sp. MAB1 and sp. MAB2 generate methane from both hydrogen and formate, implying that both these compounds could be used for interspecies transfer.

Isotope tracer measurements and molecular-based analyses have suggested that SAO is a widely distributed phenotype in several different bacterial phyla. Future research will probably provide more isolates, which is of substantial importance in obtaining valuable insights into the nature of these syntrophs.

The SIP technique probably possesses high potential for identification of hitherto uncultivated SAOB and for development of SAOB-specific molecular techniques. The use of this culture-independent approach would probably produce significant insights into the SAO population in anaerobic reactors. The possibility to link microbial function to taxonomic identity is a major benefit of

this approach and identification of novel SAOB could possibly facilitate successful isolation and characterisation of additional syntrophic strains.

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