Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes

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Summary
Changes in communities of syntrophic acetate-oxidizing bacteria (SAOB) and methanogens caused by elevated ammonia levels were quantified in laboratory-scale methanogenic biogas reactors operating at moderate temperature (37°C) using quantitative polymerase chain reaction (qPCR). The experimental reactor was subjected to gradually increasing ammonia levels (0.8–6.9 g NH₄⁺N l⁻¹), whereas the level of ammonia in the control reactor was kept low (0.65–0.90 g NH₄⁺N l⁻¹). Acetate oxidation in the experimental reactor, indicated by increased production of ¹⁴CO₂ from acetate labelled in the methyl carbon, occurred when ammonia levels reached 5.5 and 6.9 g NH₄⁺N l⁻¹. Syntrophic acetate oxidizers targeted by newly designed qPCR primers were Thermacetogenium phaeum, Clostridium ultunense, Syntrophaceticus schinkii and Tepidanaerobacter acetatoxydans. The results showed a significant increase in abundance of all these bacteria except T. phaeum in the ammonia-stressed reactor, coincident with the shift to syntrophic acetate oxidation. As the abundance of the bacteria increased, a simultaneous decrease was observed in the abundance of aceticlastic methanogens from the families Methanosaetaceae and Methanosarcinaceae. qPCR analyses of sludge from two additional high ammonia processes, in which methane production from acetate proceeded through syntrophic acetate oxidation (reactor SB) or through aceticlastic degradation (reactor DVX), demonstrated that SAOB were significantly more abundant in the SB reactor than in the DVX reactor.

Introduction
Methane formation from acetate can proceed through two different mechanisms. The most commonly described involves aceticlastic methanogens that perform acetate cleavage for methane production. The second mechanism proceeds through syntrophic acetate oxidation (Zinder and Koch, 1984). This pathway entails fermentation of acetate to hydrogen and carbon dioxide by syntrophic acetate-oxidizing bacteria (SAOB). Hydrogen utilizing methanogens then reduce carbon dioxide to methane. High ammonia levels, formed during the anaerobic degradation of protein-rich material, have been shown to be one important factor regulating the shift from aceticlastic methanogenesis to syntrophic acetate oxidation in mesophilic biogas processes (Schnürer et al., 1999; Schnürer and Nordberg, 2008). The shift is probably a consequence of the inhibitive effect of ammonia on the activity of the aceticlastic methanogens (Koster and Lettinga, 1984; Sprott and Patel, 1986). The concentration of acetate, dilution rate and presence of the aceticlastic Methanosaetaceae are other factors suggested to have an impact on the development of syntrophic acetate oxidation (Petersen and Ahring, 1991; Ahring et al., 1993; Shigematsu et al., 2004; Karakashev et al., 2006).

So far a restricted number of SAOB have been isolated and characterized, namely the mesophilic bacteria Clostridium ultunense (Schnürer et al., 1996; 1997) and Syntrophaceticus schinkii (Westerholm et al., 2010), the thermotolerant Tepidanaerobacter acetatoxydans (Westerholm et al., 2011), and the thermophilic bacteria Thermacetogenium phaeum (Hattori et al., 2000; 2005) and Thermotoga lettingae (Balk et al., 2002). Initially, a thermodhilic bacterium (Lee and Zinder, 1988) named Reversibacter was described, but unfortunately this bacterium was lost before its phylogenetic position could be established.

Information about syntrophic acetate oxidation, the organisms involved, and their role in the methanogenic environment is currently limited. However, greater understanding of microbial dynamics in response to inhibitory compounds, such as ammonia, should facilitate further development and also optimization of the anaerobic treatment process. In the present study, primers targeting 16S rRNA gene sequences of known SAOB were designed. Quantitative real-time polymerase chain
reaction (qPCR) analyses were then performed in order to determine changes in SAOB and methanogenic communities caused by elevated ammonia concentrations. Two mesophilic biogas reactors were included in the analysis. In one (experimental) reactor a shift from aceticlastic acetate degradation to syntrophic acetate oxidation had been established previously, while in the second (control) reactor aceticlastic methanogenesis was the main pathway for methane formation (Schnürer and Nordberg, 2008). Two high ammonia processes, in which methane production from acetate proceeded through syntrophic acetate oxidation (reactor SB) or through aceticlastic degradation (reactor DVX), were also included in the investigation.

Results and discussion

Pathway for acetate degradation in the biogas reactors

In the control reactor, acetate degradation was primarily through aceticlastic methanogenesis throughout the operating period. In the experimental reactor, which was subjected to gradually increasing ammonia levels, a shift from aceticlastic acetate degradation to syntrophic acetate oxidation was established between 225 and 442 days of operation, when the ammonia level reached 5.5 and 6.9 g NH₄⁺-N l⁻¹ (Fig. S1). Labelling analysis with [2-¹⁴C]-acetate demonstrated occurrence of syntrophic acetate oxidation in reactor SB (Schnürer and Nordberg, 2008; Ek et al., 2010), while in reactor DVX the analysis indicated that aceticlastic methanogenesis was the main pathway for acetate degradation (Fig. S1). The dominance of aceticlastic methanogenesis in reactor DVX was somewhat unexpected, as the ammonia concentration in this reactor exceeded the levels previously shown to cause development of syntrophic acetate oxidation (Schnürer et al., 1999; Schnürer and Nordberg, 2008). Parameters other than ammonia [e.g. substrate change, increased loading rate and decreased hydraulic retention time (HRT)] apparently had an impact on the mechanism developed for methane formation in reactor DVX.

Real-time PCR quantification of SAOB and methanogens

All standard curves for the quantitative PCR analyses, constructed as described in Table S1, had a linear correlation coefficient (r²) ranging between 0.985 and 0.999, and the calculated qPCR efficiency of the reactions varied between 86.2% and 108%.

The qPCR analyses showed a distinct increase in C. ultunense, S. schinkii and T. acetatoxydans in the experimental reactor when the ammonia level increased above 3.3 g NH₄⁺-N l⁻¹ (Fig. 1A). The increase was confirmed by an additional assay with triplicate DNA samples from the experimental reactor on day 225 and day 442, which demonstrated a significant increase (un-paired t-test, P < 0.05) in C. ultunense from 4.1 ± 1.2 × 10⁵ to 2.3 ± 0.9 × 10⁷ gene abundance ml⁻¹, in S. schinkii from 6.3 ± 1.4 × 10⁶ to 6.8 ± 2.1 × 10⁸ gene abundance ml⁻¹, and in T. acetatoxydans from 4.7 ± 2.4 × 10⁵ to 5.7 ± 0.4 × 10⁹ gene abundance ml⁻¹. In parallel, a decrease in the abundance of acetate utilizing methanogens from the family Methanosarcinaceae occurred from day 225 onwards, when the ammonia concentration exceeded 3.3 g NH₄⁺-N l⁻¹. However, the abundance of the acetate utilizing Methanosaetaceae declined after only 70 days of operation (Fig. 1B). Hydrogenotrophic methanogens of the order Methanomicrobiales initially decreased in abundance between days 70 and 142, but subsequently increased to around their initial abundance by day 642. It is possible that certain members of the Methanomicrobiales declined initially due to ammonia inhibition or pH change and subsequently (> 142 days) ammonia-tolerant members of the Methanomicrobiales were favoured as the ammonia concentration increased. The observed decrease in ace-

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ticlastic methanogens and increase in hydrogenotrophic methanogens in response to increasing ammonia levels, most likely caused by a comparatively higher tolerance of Methanomicrobiales to ammonia (Koster and Lettinga, 1984; Sprott and Patel, 1986), have been reported at population level previously (Angenent et al., 1984; Sprott and Patel, 1986), have been reported at population level previously (Angenent et al., 1984; Sprott and Patel, 1986). However, the present study represents the first detailed analysis of changes in both the population of methanogens and SAOB in response to increasing ammonia concentration.

In the control reactor, methanogen and SAOB abundance remained stable throughout the 642 days of operation (Fig. 1). Total bacterial abundance in the control reactor and experimental reactor was stable (4.9 ± 1.8 × 10^{10} and 3.7 ± 2.2 × 10^{10} gene abundance ml^{-1} respectively) throughout the operating period. These results supported the presumption that the changes in the microbial communities in the experimental reactor were a consequence of increased ammonia concentration.

16S rRNA genes from T. phaeum were not detected in any of the reactors. This was not surprising, as the temperature range of this thermophilic bacterium is 40–65°C, with an optimum around 58°C. The conditions in the reactors, operating at 37°C, were therefore unfavourable for T. phaeum.

In previous studies, C. ultunense, S. schinkii and T. acetatoxydans proved capable of withstanding rather high levels of ammonium chloride (~ 8 g NH_4•N L^{-1}) at neutral pH (Schnürer et al., 1996; Westerholm et al., 2010; 2011), an ammonium level that has strong inhibitory effects on aceticlastic methanogens from the families Methanosaetaceae and Methanosetaeaceae (Sprott and Patel, 1986; Hajarnis and Ranade, 1993). The ammonia tolerance of these syntrophic acetate-oxidizers probably gives them a

### Table 1. Primer sets and PCR programs used in the investigation.

| Primerd | Target species or group | Sequence (5′→3′) | Position in target species | Tm (°C) | Amplicon size (bp) |
|---------|-------------------------|------------------|---------------------------|--------|-------------------|
| CulStf | Clostridium ultunense | CCT TCG GGT GGA ATG ATA AA | 56–76 | 57 | 127 |
| CulSr | TCA TGC GAT TGC TAA GTT TCA | 162–183 |
| THACfd | Syntrophaceticus schinkii | ATC AAC CCC ATC TGT GGC | 802–820 | 61 | 171 |
| THACrd | TGC CCG CCA GAC CAT AAA | 1182–1200 |
| Tpf | Tepidanaerobacter acetatoxydans | AGG TAG TAG AGA GCG GAA AC | 963–983 | 63 | 237 |
| Tpr | TGT GGC CCA GAC CAT AAA | 1182–1200 |
| Thfd | Thermacetogenium phaeum | GGG TGG TGT GAA GGC ATC | 795–813 | 68 | 175 |
| Thrd | AGG TCC GCA GAG ATG TCA AG | 970–990 |
| Tbf | Total bacteria | GTG ITG CAI GGI IGT CTA IGT | 1048–1068 | 61 | 323 |
| Tbr | AGC TCI TCC ICI CTT TCC | 1371–1391 |
| Mscdf | Methanosarcinaceae | GAA ACC GYG ATA AGG GGA | 380–397 | 60 | 408 |
| Mscrf | TAG CGA RCA TCG TTT ACG | 811–828 |
| MMBGd | Methanomicrobiales | ATC GRT ACG GGT TGT GGG | 282–299 | 66 | 506 |
| MMBrd | CAC CTA ACG CCR ATH GTT TAC | 812–832 |
| Mstfd | Methanosetaeaceae | TAA TCC TYG ARG GAC CAC CA | 702–721 | 61 | 164 |
| Mstrd | CCT ACG GCA CCR ACM AC | 812–832 |
| pAfd | Bacteria | AGA GTT TGA TCC TGG CTC AG | 8–28 | 55 | 1534 |
| pHfd | AAG GAG GTG ATC CAG CCG CA | 1542–1522 |
| Arch46fd | Archaea | YTA AGC CAT GCR AGT | 46–61 | 40 | 971 |
| Arch1017rd | | GCC CAT GCA CCG CTC | 1017–999 |

a. f, forward; r, reverse primer.
b. l, inosine.
c. 16S rRNA gene sequence.
d. Designed by Stefan Roos and Maria Westerholm, Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.
e. Designed by Dr Neil Gray, School of Civil Engineering and Geosciences; Newcastle University.
f. (Maeda et al., 2003).
g. (Yu et al., 2005).
h. (Edwards et al., 1989).
i. (Ovreás et al., 1997).
j. (Barns et al., 1994).
competitive advantage in ammonia-stressed systems. These bacteria, in association with ammonia-tolerant hydrogenotrophic methanogens, may consequently adopt the role of dominant acetate consumers in environments where ammonia restrains aceticlastic methanogenic activity.

In reactor SB, *C. ultunense*, *S. schinkii* and *T. acetatoxydans* were present at significantly (t-test, P < 0.05) greater abundance than in reactor DVX (Fig. 2). The total bacterial gene abundance in reactor SB (1.0 \times 10^{10} ml^{-1}) was also slightly higher than in reactor DVX (3.2 \times 10^{9} ml^{-1}). The comparatively low abundance of acetate oxidizers in reactor DVX agreed with the labelling analysis, demonstrating dominance of aceticlastic methanogenesis in this reactor. In contrast, there was no significant difference (t-test, P > 0.05) in mean gene abundance of *Methanosarcinaceae* or *Methanomicrobiales* between reactors DVX and SB, and the *Methanosaetaceae* abundance was even significantly lower in reactor DVX. The high abundance of *Methanosaetaceae* in reactor SB was unexpected and contradicted results reported by Karakashev and colleagues (2006), showing that acetate oxidation is the dominant pathway only in the absence of *Methanosa-
etaceae. The relatively high abundance of Methanosarcinaceae and Methanosetaeaceae in reactor DVX is also noteworthy, indicating occurrence of ammonia-tolerant aceticlastic methanogens in this reactor operating at a high ammonia concentration. However, the accumulation of VFA and the decline in pH demonstrated the instability of aceticlastic methanogenesis in the conditions under which reactor DVX was operated, thereby reflecting the importance of SAOB for the maintenance of process stability in methanogenic systems with high ammonia concentrations.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Degree of acetate oxidation measured as $^{14}\text{CO}_2/^{14}\text{CH}_4$ over the operational period of 642 days in the control and experimental reactor, described by Schnürer and Nordberg (2008). The concentration of ammonia-nitrogen (g NH$_4^+$-N l$^{-1}$) is stated above the bars. Samples withdrawn at a single sampling point from two high ammonia processes, designated SB and DVX were also included in this investigation. Samples for DNA extraction and [2-$^{14}$C]-acetate tracer analysis were withdrawn from the SB reactor after 12 years of operation and from DVX reactor after 270 days of operation. Degree of $^{14}\text{CO}_2/^{14}\text{CH}_4$ in DVX and SB are mean values of two measurements. All processes operated at moderate temperature (37°C).

**Table S1.** Construction of DNA standards for quantification.

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