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Stable isotope probing of acetate fed anaerobic batch incubations shows a partial resistance of acetoclastic methanogenesis catalyzed by *Methanosarcina* to sudden increase of ammonia level

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form
5 November 2014
Accepted 8 November 2014
Available online 18 November 2014

Keywords:
Ammonia inhibition
Acetotrophic methanogens
Acetate-utilizing bacteria
Metabolic pathway dynamics
Pyrosequencing
Stable isotope signature

ABSTRACT

Ammonia inhibition represents a major operational issue for anaerobic digestion. In order to refine our understanding of the terminal catabolic steps in thermophilic anaerobic digestion under ammonia stress, we studied batch thermophilic acetate fed experiments at low (0.26 g L^{-1}) and high (7.00 g L^{-1}) Total Ammonia Nitrogen concentrations (TAN). Although methane production started immediately for all incubations and resulted in methane yields close to stoichiometric expectations, a 62–72% decrease of methanogenic rate was observed throughout the incubation at 7.00 g L^{-1} of TAN compared to 0.26 g L^{-1}. Stable Isotope Probing analysis of active microbial communities in ^{13}C-acetate fed experiments coupled to automated ribosomal intergenic spacer analysis and 16S rDNA pyrotag sequencing confirmed that microbial communities were similar for both TAN conditions. At both TAN levels, the ^{13}C-labeled bacterial community was mainly affiliated to *Clostridia*-relatives, with OPB54 bacteria being the most abundant sequence in the heavy DNA 16S rDNA pyrotag library. Sequences closely related to *Methanosarcina thermophila* were also abundantly retrieved in the heavy DNA fractions, showing that this methanogen was still actively assimilating labeled carbon from acetate at free ammonia nitrogen concentrations up to 916 mg L^{-1}. Stable isotopic signature analysis of biogas, measured in unlabeled

Abbreviations: FAN, Free Ammonia Nitrogen; TAN, Total Ammonia Nitrogen concentration; SIP, Stable Isotope Probing; ARISA, Automated Ribosomal Intergenic Spacer Analysis; AM, Acetoclastic Methanogenesis; HM, Hydrogenotrophic Methanogenesis; SAO, Syntrophic Acetate Oxidation; SAOB, Syntrophic Acetate Oxidizing Bacteria; α_C, Apparent Fractionation Factor.

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http://dx.doi.org/10.1016/j.watres.2014.11.010

acetate fed experiments that were conducted in parallel, confirmed that acetoclastic methanogenic pathway was dominant at both ammonia concentrations. Our work demonstrates that, besides the syntrophic acetate oxidation pathway, acetoclastic methanogenesis catalyzed by *Methanosarcina* can also play a major role in methane production at high ammonia levels.

1. Introduction

Ammonia is a major toxicant during anaerobic treatment of nitrogen-rich organic wastes, such as slaughterhouse byproducts, animal manure and food processing waste. High concentration of ammonia may indeed lead to unstable performance and operational failure in full-scale anaerobic digesters, due to inhibition of methanogens (Westerholm et al., 2012). Under ammonia stress, acetotrophic methanogens are usually considered to be more susceptible than the hydrogenotrophic ones (Angelidaki and Ahring, 1993) and Acetoclastic Methanogenesis (AM) is more easily suppressed than Hydrogenotrophic Methanogenesis (HM); however, conflicting results have been reported as well (Fujishima et al., 2000; Wiegant and Zeeman, 1986). Due to the different effects on methanogenic populations and metabolic activities, ammonia stress has been found to be an important factor inducing variation of the microbial community composition and metabolic pathways (Schnürer and Nordberg, 2008; Westerholm et al., 2012; Fotidis et al., 2013a).

To date, two possible patterns for microorganisms responding to ammonia stress have been reported in methanogenic systems. It was recently observed that a growth of Syntrophic Acetate Oxidizing Bacteria (SAOB) and hydrogenotrophic methanogens, which catabolize acetate by tandem reactions of Syntrophic Acetate Oxidation (SAO) and HM, was initiated by increasing ammonia concentration above 3 g L^{-1} of Total Ammonia Nitrogen (TAN) (Schnürer and Nordberg, 2008; Westerholm et al., 2011a, 2012; Lü et al., 2013). However, three of the four studies simultaneously detected *Methanosarcinaceae* and *Methanosaetaceae* in large abundance by using the QPCR method (Westerholm et al., 2011a, 2012; Lü et al., 2013). Another pattern was also reported which consisted of dominance of acetotrophic methanogens. For instance, *Methanosarcinaceae* was observed as the sole dominant methanogen at 6 g L^{-1} of TAN (Calli et al., 2005) and 7 g L^{-1} of TAN (Fotidis et al., 2013a) by using Fluorescence In Situ Hybridization (FISH); *Methanosarcinaceae* and *Methanosaetaceae* were identified as the most abundant populations by QPCR, although hydrogenotrophs were also detected (Westerholm et al., 2011b; Lü et al., 2013) together with SAOB in relatively lower abundance (Westerholm et al., 2011b). *Methanosarcinaceae* appearing in this pattern is a versatile population, which is able to perform both AM and HM pathways, and was even indicated to possess SAO function under high stress conditions (De Vrieze et al., 2012; Fotidis et al., 2013a; Qu et al., 2009). Documentation of in-situ pathway dynamics is still lacking in current literature, which may increase the difficulty to identify the functional populations and their in-situ contributions.

Except for the acetotrophic methanogens and the well-recognized SAOB which are coupled with hydrogenotrophs, some other bacterial groups, which are functionally related to acetate degradation, have been frequently discovered in anaerobic ecological niches, such as members affiliated to the phylum Proteobacteria (Li et al., 2009), the phylum Nitrospira (Schwarz et al., 2007) and the *Syntrophus* sp. (Chauhan and Ogram, 2006). However, their behavior under high ammonia conditions has never been reported. These studies are suggesting, acetate degradation under ammonia stress might involve a competition between acetotrophic *Methanosaetaceae*/*Methanosarcinaceae*, acetate oxidizing syntrophs and possibly bacterial members other than the identified SAOB. Response of the microbial communities to ammonia stress should be further investigated, since a deeper insight into the populations of acetate consumers, their activity and pathway dynamics under ammonia stress, could facilitate the development of methods to counteract ammonia inhibition in anaerobic digestion, such as bioaugmentation with ammonia-tolerant microorganisms (De Vrieze et al., 2012; Fotidis et al., 2013a).

This study aimed to refine our understanding of the terminal catabolic steps in thermophilic anaerobic digestion under ammonia stress and of the microbial groups actually involved, by taking advantage of stable isotope-based analytical techniques. Batch acetate-fed experiments at low and high ammonia concentrations were conducted by using non-acclimatized inoculum. Pathway dynamics were evaluated by stable isotopic signature of biogas. DNA-based Stable Isotope Probing (SIP) coupled to 16S rRNA gene pyrosequencing was utilized in parallel experiments to analyze microbial communities actively involved in acetate assimilation. Automated Ribosomal Intergenic Spacer Analysis (ARISA) was applied to record their dynamics. This study is the first report investigating the influence of ammonia on microbial communities by combining stable isotopic signature of biogas and stable isotopic probing of DNA in thermophilic methanogenic reactors, which allows to directly link carbon in the substrate to carbon incorporated into the microorganisms.

2. Materials and methods

2.1. Experimental set-up

All experiments were conducted using freshly collected methanogenic sludge, which was previously cultivated in an