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Effects of nitrogen oxides and denitrification by *Pseudomonas stutzeri* on acetotrophic methanogenesis by *Methanosarcina mazei*

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Abstract

Nitrate and its reduced forms produced during denitrification, nitrite and nitrous oxide, were studied for their influence on methane production from acetate by *Methanosarcina mazei*. While 0.18 mM nitrite and 0.8% nitrous oxide in the gas phase completely suppressed methane production, 71.4 mM nitrate resulted in only 83.3% inhibition. Co-culture experiments showed that *M. mazei* growing with 15 mM nitrate produced methane from acetate until the denitrifying bacterium *Pseudomonas stutzeri* was inoculated and nitrate denitrification began. The presence of nitrous oxide in the gas phase after cessation of denitrification activity by *P. stutzeri* in co-cultures flasks prevented *M. mazei* resuming methane production. Nitrous oxide, instead of dinitrogen, was the end product of denitrification by *P. stutzeri* either in pure cultures or in co-cultures with *M. mazei*, probably because of the highly reduced culture conditions that were used. This study strongly suggests that acetate-dependent methane production by *M. mazei* was inhibited by reduced nitrogen forms produced during bacterial nitrate denitrification, rather than by competition for acetate between denitrifying and methanogenic bacteria. These results are consistent with previous studies with H_2/CO_2 methanogens.

Keywords: Acetate; Methanogenesis; Denitrification; Nitrogen oxide; *Methanosarcina mazei*; *Pseudomonas stutzeri*

1. Introduction

Anaerobic digestion is a well known process of carbon removal that leads to energy generation and low sludge production [1]. On the other hand, the biological nitrification-denitrification process is the most common and efficient method to remove nitrogen pollution [2]. A combined process involving a nitrifying reactor coupled with an anaerobic denitrifying-biogas producing digester could be an interesting way to treat carbon and nitrogen high strength wastewaters.

Previous work has shown that nitrogen and carbon could be efficiently removed from synthetic wastewaters using coupled anaerobic and aerobic filters [3] and that the nature of the nitrogen reduction product depended on the C/N ratio as well as the type of carbon source [4,5]. In preliminary studies on combined nitrogen and organic carbon removal from piggery wastewaters, the end product of nitrate and nitrite reduction by anaerobic sludges was found to be dinitrogen rather than ammonium [6]. This predominance of the denitrification pathway instead of

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nitrate dissimilation was explained by the low C/N-oxide ratio and the volatile fatty acid composition of the carbon source in piggery wastewaters. Nitrate and especially its denitrification metabolic intermediates are known to inhibit methanogenic activity in natural habitats and in methanogenic sludges [7–9]. To our knowledge, studies on the influence of nitrate and nitrite, nitric oxide, and nitrous oxide on methanogens have only been conducted with cultures using methanogenic substrates other than acetate, mostly H_2 and CO_2 [9,10]. Nevertheless, acetate is known to account for about 70% of the methane produced in anaerobic digesters [11] and no data are available on the effect of nitrate and its reduced forms (through denitrification) on the activity of acetate-utilizing methanogens.

The purpose of this study was to investigate the influence of the denitrification activity on the methanogenesis from acetate using two well known microorganisms: the acetoclastic methanogen *Methanosarcina mazei*, and the denitrifying bacterium *Pseudomonas stutzeri*. The effect of direct additions of nitrate, nitrite, and nitrous oxide on the methanogen culture medium was studied, and then, co-cultures of the two bacteria were investigated in the presence of nitrate.

2. Materials and methods

2.1. Organisms and culture conditions

M. mazei S-6 (DSM 2053) and *P. stutzeri* ZoBell (ATCC 14405) were grown under anaerobic conditions as previously reported [12,13] in serum bottles sealed with rubber stoppers (Bellco Glass, USA). Serum bottles of 60 ml containing 25 ml medium were used for the experiments with nitrogen oxide experiments and 120-ml serum bottles containing 75 ml medium were used for co-culture experiments. Cultures were incubated at 37°C with shaking (200 rpm).

The medium used contained (in g l^{-1}) 0.75 KH_2PO_4, 1.45 K_2HPO_4, 0.5 NH_4Cl, 0.1 $MgCl_2 \cdot 6H_2O$, 0.04 $CaCl_2 \cdot 2H_2O$, 0.5 yeast extract, 0.5 trypticase, 0.5 L-cysteine·HCl, 10 ml of trace mineral solution and 1 ml of 0.1% resazurin. The medium was boiled under argon, dispensed into serum bottles, and autoclaved (20 min, 120°C). After autoclaving, the following solutions were added: a sterile solution of 42.5 g l^{-1} $NaHCO_3$ (final concentration 0.85 g l^{-1}), the substrate solutions, sodium acetate or methanol (final concentration 45 mM) and solutions of KNO_3 and KNO_2, when needed. Filter sterilized N_2O was added to the gas phase the night before the experiment and atmospheric pressure was readjusted by withdrawing the same volume of added gas. The pH of the medium was 6.9–7.1.

Volumes of inoculum for both organisms were 1 ml. Inocula of *M. mazei* were 7-day cultures grown with 45 mM acetate and 4-day cultures grown with 45 mM methanol. Inocula of *P. stutzeri* were grown in 45 mM acetate and 15 mM KNO_3 for 3 days.

2.2. Analytical methods

Gas was analyzed by gas chromatography (Shimadzu GC-8A) with argon carrier using a katharometer detector. 1 ml of the gas phase was withdrawn under sterile conditions and injected into the chromatograph. Concentrations are given in μmol per milliliter of the gas phase.

Liquid samples (1 ml) were collected under sterile conditions, and analyzed. Analysis of acetate and methanol was by gas chromatography using a flame ionization detector (Chrompac CP 9000). Nitrate and nitrite were analyzed by ion chromatography using conductivity detection (Dionex-100). Separation of the anions was by an Ionpac AS12A analytical column using a carbonate/bicarbonate eluent and a cation suppresser system.

3. Results and discussion

3.1. Influence of nitrogen oxides on methanogenic activity

Methane production of *M. mazei* grown in acetate medium containing either nitrate, nitrite or nitrous oxide was monitored (Fig. 1 and Table 1). The highest nitrate concentration tested (71.4 mM) did not exert a complete inhibition of methanogenesis. Nitrite and nitrous oxide were stronger inhibitors since either 0.18 mM nitrite or 0.32 mM nitrous oxide in the gas phase almost completely suppressed methane

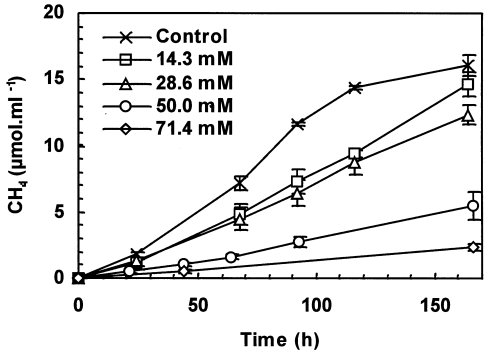

Fig. 1. Influence of nitrate on methane production of *M. mazei* grown in acetate medium (45 mM acetate). Methane production is shown as the results of two cultures for each nitrate concentrations (arithmetic mean ± range).

production (Table 1). Methanol-grown *M. mazei* cultures showed a pattern of sensitivity toward nitrate and nitrite which was similar to that of acetate-grown cultures (Table 1).

Similarly, Balderston and Payne [9] showed in studies on salt-marsh sediments and whole-cell suspensions of *Methanobacterium thermoautotrophicum* and *Methanobacterium formicium* that the reduced forms of nitrate, produced via denitrification, exerted a stronger inhibitory effect on methanogenesis from H_2 and CO_2 than nitrate. These authors suggested that cessation of methanogenesis during denitrification was a consequence of an inhibition of the methanogen metabolism rather than due to competition for substrates between methanogens and denitrifying bacteria. Our results strengthen this suggestion since acetate- and methanol-dependent methane production was also significantly inhibited by products of nitrate denitrification. The same suggestion has also been made by Chen and Lin [7] who observed that 7.1 mM nitrate but 10 times less nitrite was required to completely inhibit methane production from methanol in methanogenic sludges. Regarding Chen and Lin's results [7], striking differences of the strength of inhibition by nitrate of methanol-dependent methane production were found between the methanogenic sludges and our cultures of *M. mazei*. *M. mazei* was only 46% inhibited by 14.3 mM nitrate in methanol medium (Table 1) while nitrate at only 0.71 mM exerted 63% inhibition on the methanogenic sludge studied by these authors. This difference could be explained by the presence in the methanogenic sludge of denitrifying bacteria producing nitrite and/or nitrous oxide from nitrate, which is more inhibitory to methanogenic bacteria. However, this does not exclude that various methanogenic bacteria could be affected very differently by nitrogen oxides.

Indeed, Belay and co-workers [10] reported that the strength of growth inhibition by nitrate strongly depended on the methanogenic bacterium. Among five H_2/CO_2 utilizing methanogens tested, *Methanococcus thermolithotrophicus* could tolerate concentrations as high as 200 mM nitrate, whereas *Methanosarcina barkeri* was most affected with nearly complete inhibition at 20 mM nitrate. The methanogen we tested, *M. mazei*, could withstand concentrations at least four times higher than those reported for *M. barkeri* [10]. Although the substrates used to study the influence of nitrate on the two methanogens were different, it seems that nitrate tolerance can vary drastically among species of the same genus. This has also been observed with *M. thermoautotrophicum* and *M. formicium* using H_2 and CO_2 [9].

3.2. Influence of denitrification on methanogenic activity

Co-culture experiments were conducted in acetate

Table 1
Inhibition by nitrogen oxides of methane production by *M. mazei* grown with either 45 mM acetate or 45 mM methanol as substrate[a]

Compound	Concentration (mM)	Substrate	
		Acetate	Methanol
NO_3^-	14.3	35	46
	28.6	41	51
	50.0	65	nd[b]
	71.4	83	nd
NO_2^-	0.04	29	64
	0.18	97	96
N_2O	0.32[c]	95	nd
	0.99	100	nd

[a]Average values of the percentage of inhibition of methane production with respect to the control (nitrate free) in the late exponential phase. Each nitrogen oxide concentration was tested in duplicate.
[b]Not determined.
[c]Concentration in the gas phase under atmospheric pressure at 35°C.

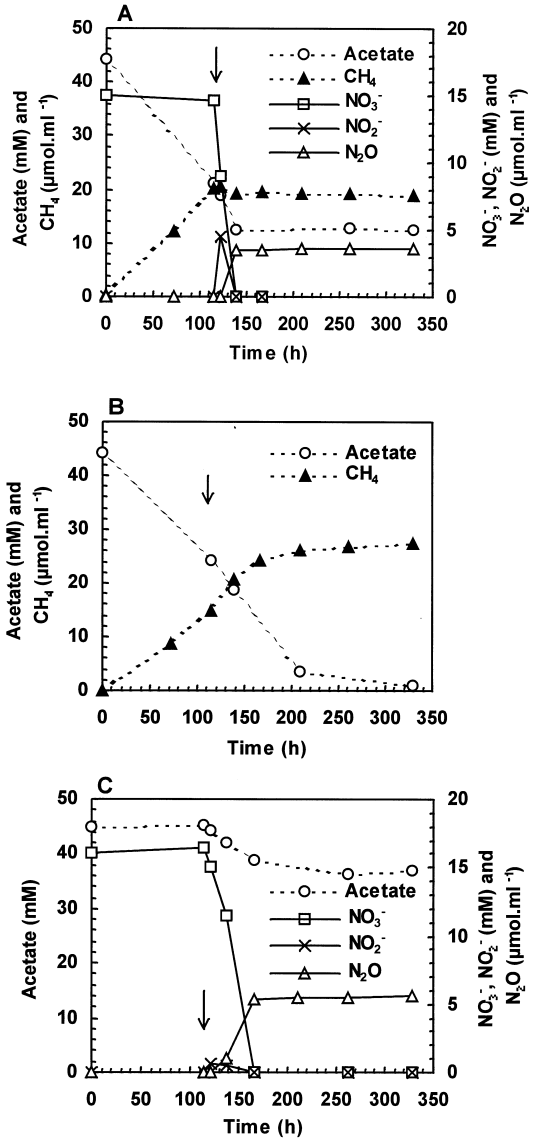

Fig. 2. Time courses of methanogenesis and/or denitrification in co-culture of *M. mazei* and *P. stutzeri* (A) and in control cultures of *M. mazei* (B) and *P. stutzeri* (C). Cultures were grown in acetate medium (45 mM acetate) with 15 mM nitrate. Arrows indicate times of inoculation of *P. stutzeri* in the co-culture and in the *P. stutzeri* control culture, and time of injection of filter-sterilized culture medium of *P. stutzeri* inoculum in the *M. mazei* control culture.

medium containing 15 mM nitrate by inoculating first *M. mazei* and then the denitrifying bacterium *P. stutzeri*, once methane production was initiated (~4–5 days). At the same time, separate cultures of *M. mazei* and *P. stutzeri* were used as controls. This experiment was replicated but data for only one experiment are presented in Fig. 2A–C. In co-culture (Fig. 2A), methane production by *M. mazei* stopped as soon as *P. stutzeri* was inoculated (t=116 h) and denitrification began. The *M. mazei* control culture, into which 1 ml of filter-sterilized culture medium of the *P. stutzeri* inoculum (i.e. cell free) was injected, was not affected (Fig. 2B). Therefore, methanogenesis in the co-culture was inhibited due to nitrate denitrification by *P. stutzeri* and not by some inhibitors that could be present or produced in the culture medium of the inoculum of *P. stutzeri*, or air contamination during inoculation.

The co-cultures accumulated nitrite to concentrations up to 4.43 mM during nitrate consumption (Fig. 2A) in contrast to the *P. stutzeri* control cultures which accumulated only 0.61 mM (Fig. 2C). Unexpectedly, in both co-culture and *P. stutzeri* control cultures, nitrous oxide, which appeared when the nitrite concentration was highest, could not all be reduced to dinitrogen by the denitrifying bacterium. The inability of *P. stutzeri* to reduce nitrous oxide was related neither to acetate limitation (residual acetate was present) nor to pH conditions which had risen to a maximum value of only 7.9 from 7.0 during the experiments. Nitrous oxide as end product of denitrification has been reported before with some strains of *P. fluorescens*, *P. aureofaciens*, *P. aeruginosa* and *P. chlorophalis* but not with *P. stutzeri* [14,15]. However, the transient accumulation of denitrification intermediates by *P. stutzeri* and other denitrifiers was reported to be dependent on the culture conditions [16–19]. The inability of *P. stutzeri* strain ZoBell to reduce nitrous oxide to dinitrogen in our study might reflect the culture conditions used which were designed to allow growth of the strict anaerobe *M. mazei*. Since minerals and nutrients usually needed for both methanogens and denitrifying bacteria were present, the main feature of the culture medium was its very low redox potential to enable growth of the methanogens ($Eh \leq -340$ mV using cysteine as reductant [20]). Most of the studies on denitrifying bacteria reported in the literature, in contrast, were carried out without reductant since nitrate reduction can occur at Eh values as high as +200 mV [21,22]. Methane production by *M. mazei* could not resume in co-cultures after the denitrifica-

tion activity of *P. stutzeri* ceased (Fig. 2A), presumably because of the presence in the gas phase of nitrous oxide (3.2 mM of gas phase, i.e. 8.1% of the gas phase) which was shown earlier to be a strong inhibitor of the methanogenic activity of *M. mazei* at much lower concentrations (Table 1).

3.3. General conclusions

Recent experiments in methanogenic reactors fed with an industrial wastewater agree with these results [23]. Methane production was observed during 45 h in the presence of 16.4 mM nitrate at a low oxido-reduction potential (ORP), confirming that nitrate is not an inhibitor of methanogens at this concentration. As soon as denitrification began, an increase of the ORP and nitrite production caused a failure of methanogenesis. It was then concluded that the growth of denitrifying organisms (or a phenomenon directly related to their growth) was responsible for the methane production failure rather than nitrate itself.

In terms of treating carbon and nitrogen high strength wastewaters (e.g. piggery wastewaters) by coupling nitrifying reactors with denitrifying-biogas producing digester, this study points to the critical influence that denitrification can have on methanogenesis and especially on the main methanogenic metabolic pathway, i.e. acetate transformation to methane. Consequently, technological alternatives need to be found in order to allow both methanogenesis and denitrification to take place in the same reactor. A sequencing batch reactor system, which permits methanogenesis to occur after completion of denitrification, has been studied in this laboratory [24]. Conversely, continuous feeding systems may be suited to the non-compatibility of denitrifiers and methanogens, as Lin and Chen [25] demonstrated this by using gel beads to immobilize acclimatized sludges growing on methanol which allowed both populations to grow in distinctive zones (termed 'habitat segregation'). The use of plug-flow reactors was shown to cause a similar segregation of microbial ecosystems [3].

Finally, regarding the diversity of sensitivity to nitrogen oxides between methanogenic bacteria, the methanogenic population used in such a process might be of importance and might be selected with respect to its tolerance to nitrogen oxides. It would therefore be interesting to extend the study on the effects of nitrate and its denitrification intermediates to a range of different methanogens.

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