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Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion

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ABSTRACT

In this study, the microbial response of anaerobic sludge digestion to the addition of two classic methanogenic inhibitors (chloroform, 2-bromoethanesulfonate) was investigated. Both the toxicants showed their effectiveness on CH_4 production, whereas the hydrogen responses and acetate accumulations were elicited to different extent. Terminal restriction fragment length polymorphism analyses in combination with clone library showed that both toxicants inhibited not only methanogenic activity but the structure of methanogenic communities. The acetoclastic *Methanosaetaceae* was more sensitive than hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales*. Interestingly, as reflected by the favorable thermodynamic condition and the increase of formyltetrahydrofolate synthetase (*fhs*) gene copy numbers, reductive homoacetogenesis from H_2/CO_2 was also stimulated by selective inhibition of methanogenesis with 2-bromoethanesulfonate (BES).

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1. Introduction

Methanogens are important participants in anaerobic waste treatment and bioconversion, converting one-carbon compounds and acetate to methane via pathways with a series of unusual coenzymes (Gerardi, 2003; Liu and Whitman, 2008). Accordingly, numerous chemical inhibitors have been tested and applied to study the ecological relationship between methanogens and other carbon mineralizing microbial groups, as well as the flow of carbon and electrons in both methanogenic cultures and environmental microbial communities (Conrad and Klose, 2000; Fey and Conrad, 2000; Metje and Frenzel, 2007). Moreover, since volatile fatty acids (VFA) and hydrogen produced at the acidogenesis step have higher added values, methanogenic inhibitors have been used for energy saving in various anaerobic processes (Anderson et al., 2006; Božic et al., 2009; Hu and Chen, 2007).

There have been reports of many chemical substances inhibiting methane formation of methanogenic archaea with different specificities and optimum inhibition concentrations (Scholten et al., 2000; Ungerfeld et al., 2004; Valdez-Vazquez and Poggi-Varaldo, 2009). In general, these compounds can be classified into two major groups: nonspecific and specific inhibitors. For example, chloroform ($CHCl_3$), as a classic example of nonspecific inhibitor for methanogens, is known to block the function of corrinoid enzymes and to inhibit methyl-coenzyme M reductase of methanogens (Chidthaisong and Conrad, 2000). It not only inhibits methanogenesis, but also inhibits partially acetate-dependent sulfate reduction and possibly H_2-dependent homoacetogenesis. By contrast, 2-bromoethanesulfonate (BES), as a structural analogue of coenzyme M, is used to specifically inhibit methanogenesis (Zinder et al., 1984). These two classic inhibitors were widely used in the past many years as metabolic probes for quantitative analysis of the contribution of methanogens in natural and engineered methanogenic systems, such as rice field soil (Fey and Conrad, 2000), freshwater sediment (Scholten et al., 2000), and anaerobic sludge digester (Hickey et al., 1987). In addition, a number of successful commercial applications have been also successfully established. These applications include: reducing energy loss and improving the efficiency of ruminal energetic transformations (Anderson et al., 2006; Božic et al., 2009), selection of mixed microbial consortia from waste biomass for hydrogen and acid production (Hu and Chen, 2007; Kaushik and Debabrata, 2004), increasing the coulombic efficiency of microbial fuel cell (Kim et al., 2005), etc.

Although the inhibition of methanogenesis by BES and $CHCl_3$ in anaerobic environments has been well established, most studies only focused on the practical applications. Little is known about

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their effect on the *in situ* quantity or diversity of targeted microbial community in natural environments to accomplish the decrease in methane output. It was reported that a low dose of BES (1.0 mM) is sufficient for inhibiting acetoclastic methanogenesis whereas a much higher dose (50 mM) is required to completely inhibit CO_2 reduction in a thermophilic anaerobic digester (Zinder et al., 1984). We thus hypothesized that there would be differences among the members of the methanogenic community in their sensitivity to the inhibitors.

In the absence of inorganic electron acceptors other than carbonate (e.g. nitrate, ferric iron, sulfate), methanogenic archaea and homoacetogenic bacteria are the main H_2 consumers. Homoacetogenic bacteria are another group of microorganism able to grow at the expense of hydrogen plus CO_2 as the sole energy source (Xu et al., 2009). In most cases, homoacetogens do not compete well with methanogens due to their less favorable thermodynamic characteristics, since the affinity of the methanogens for H_2 is 10–100 times higher than the affinity of the reductive homoacetogens (Liu and Whitman, 2008). Consequently, when methanogenesis was selective inhibited, we hypothesized that the accumulation of H_2 during the process of anaerobic sludge digestion might be favorable for homoacetogenesis. Interestingly, Siriwongrungson et al. (2007) recently also found that homoacetogenesis instead of methanogenesis likely became the sink of hydrogen to accumulate acetate during anaerobic degradation of butyrate under suppressed methanogenesis. Studies on rice roots gave similar results (Conrad and Klose, 2000). However, the knowledge on the population dynamics of homoacetogen coupled with the change of hydrogen partial pressure is lacking to date.

Therefore, with anaerobic sludge as a model system, the aims of the present study were (1) to compare BES and $CHCl_3$ for their effects on the formation of gases and fatty acids, (2) to determine the structure of the archaeal community under different inhibition types by a combination of clone library and terminal restriction fragment length polymorphism (T-RFLP) analysis, (3) to evaluate their effect on the dynamics of methanogenic groups by real-time PCR, and (4) to investigate the possibility of reductive homoacetogenesis process under conditions suppressing methanogenesis.

2. Methods

2.1. Source of anaerobic sludge and its incubation conditions

Anaerobic sludge was obtained from a mesophilic sludge digester in Chengbei Municipal Wastewater Treatment Plant (Wuxi, China). Only domestic wastewater was treated in this plant, which avoids accumulation of toxic substance inhibiting acidogenic bacteria and methanogens. In order to shorten the lag phase, mature anaerobic granular sludge collected from an efficient internal circulation reactor treating citric acid wastewater was used as seed (DSM Ltd., Netherlands). The characteristics of sludge are shown in Table 1, which were determined as previously described by Liu et al. (2008). Freshly collected mesophilic sludge (225 g sludge with 25 g seed per incubation) were placed into glass bottles (Hujing, China) filled with 500 ml trace element solution described elsewhere (Oh et al., 2003). The bottles were closed with latex stoppers and pumped with N_2. Methanogenesis were inhibited by addition of 50 mM 2-bromoethanesulfonate (BES) (97%; Biorgchem, China) and 0.05% (v/v) chloroform ($CHCl_3$) (99.5%; Advtechind, China). BES was added as aqueous solution of the sodium salt, and $CHCl_3$ was added directly as pure chemicals. Both the toxicant doses were selected based on literature values (Hu and Chen, 2007; Zinder et al., 1984) and preliminary experiments conducted in our laboratory. Each experiment was conducted so that the range of inhibition levels examined encompassed mildly to not only inhibit methane production (>IC_{95}), but also not to affect the performance of acidification. Treatments and controls without inhibitor were incubated in triplicate at 35 °C in the dark without shaking to avoid destruction of microbial consortia.

Table 1
Characteristics of sewage sludge and seed sludge.

Parameter	Unit	Sewage sludge	Seed sludge
Volatile solid	wt.%	54.16 ± 1.18	85.47 ± 1.21
Total proteins	g g^{-1} VS%	59.6 ± 1.47	65.32 ± 1.01
Total carbohydrate	g g^{-1} VS%	12.5 ± 0.81	20.84 ± 1.74
Total lipids	g g^{-1} VS%	1.2 ± 0.22	1.7 ± 0.21
SO_4^{2-} (mg/L)	mg/L	12.6 ± 0.14	8.4 ± 0.11
pH	–	7.28 ± 0.06	7.12 ± 0.08
Alkalinity ($CaCO_3$)	mg/L	2554.3 ± 58.91	ND[a]

[a] ND, not determined.

2.2. Measurement of gases and volatile fatty acids

The concentrations of VFAs were measured using filtrate samples through a 0.45 μm micron membrane. A gas chromatograph (GC-2010, Shimadzu, Japan) equipped with an auto injector (AOC-20i, Shimadzu) was used to determine the VFAs concentration. The detector was a flame ionization type and the column was a fused-silica capillary (PEG-20M, 30 m × 0.32 mm × 0.5 μm, China). Gas samples (0.5 ml) were taken with a gas-tight pressure lock syringe (Shimadzu, Japan), after the bottles were vigorously shaken for 30 s by hand to reach equilibration of the gas and liquid phase, and analyzed immediately by gas chromatography. The concentrations of CH_4, CO_2, and H_2 in the gas headspace were quantified via another channel of GC-2010 equipped with a packed column Porapak Q (50/80 mesh) and a thermal conductivity detector (Shimadzu, Japan).

2.3. DNA extraction

The procedure used for extraction of DNA was a modification of previously described protocols (Peng et al., 2008). Briefly, a sludge sample (1 ml) was mixed with 0.5 ml of sodium phosphate buffer (pH 8.0, 120 mM), 200 μl of sodium dodecyl sulfate (10%), and 1 g of glass beads (0.1 mm in diameter). After incubation (10 min, 65 °C) and two 30 s cycles of bead beating at 6.0 m s^{-1} (FastPrep bead-beater, Bio101/Savant, USA), the slurry was centrifuged (10 min, 13,000*g*). The DNA was then extracted from the supernatant using phenol–chloroform–isoamyl alcohol (25:24:1), and the extracts were precipitated with a 0.1 volume of sodium acetate (3 M, pH 5.3) and 2 volumes of ethanol as described elsewhere (Roh et al., 2006). Finally, the DNA pellet was purified with a commercial DNA fragment purification kit (TaKaRa, Japan) according to the manufacturer's instructions. DNA quality and size was checked by 0.8% (wt/v) agarose gel electrophoresis and spectrophotometer at 260 nm using a BioPhotometer (Eppendorf, Germany).

2.4. Archaeal 16S rRNA gene library

Clone library was constructed with the mixed extracted DNA from different sources (control/$CHCl_3$/BES incubations) to provide a better resolution for differentiating the individual T-RFs as phylogenetic lineages under different conditions. Two universal primers for archaea, Arc109F (5′-ACKGCTCAGTAACACGT-3′) and Arc934R (5′-GTGCTCCCCCGCCAATTCCT-3′) (Peng et al., 2008), were used to amplify the archaeal 16S rRNA gene. A PCT-200 DNA Engine (BioRad, USA) with a TaKaRa PCR Kit (TaKaRa, Japan) was used for the DNA amplification. The reaction mixture contained, in a total volume of 50 μl, 5× PCR buffers, 5.0 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.5 mM each primer, and 2 U of Ex-*Taq* DNA polymerase, and 1 μl

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