

# Methanol as the Primary Methanogenic and Acetogenic Precursor in the Cold Zoige Wetland at Tibetan Plateau

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**Abstract** Previous studies suggested that methanol and acetate were the likely methanogenic precursors in the cold Zoige wetland. In this study, the contribution of the two substances to methanogenesis and the conversion in Zoige wetland were analyzed. It was determined that methanol supported the highest CH<sub>4</sub> formation rate in the enrichments of the soil grown with *Eleocharis valleculosa*, and even higher at 15°C than at 30°C; while hydrogenotrophic methanogenesis was higher at 30°C. Both methanol- and acetate-using methanogens were counted at the highest (10<sup>7</sup> g<sup>-1</sup>) in the soil, whereas methanol-using acetogens (10<sup>8</sup> g<sup>-1</sup>) were ten times more abundant than either methanol- or acetate-using methanogens. Both methanol and acetate were detected in the methanogenesis-inhibited soil samples, so that both could be the primary methanogenic precursors in *E. valleculosa* soil. However, the levels of methanol and acetate accumulated in 2-bromoethane-sulfonate (BES)- and CHCl<sub>3</sub>-treated soils were in reverse, i.e., higher methanol in CHCl<sub>3</sub>- and higher acetate in BES-treated soil, so that methanol-derived methanogenesis could be underestimated due to the consumption by acetogens. Analysis of the soil 16S rRNA genes revealed *Acetobacterum bakii* and *Trichococcus pasteurii* to be the dominant methanol-using acetogens in the soil, and a strain

of *T. pasteurii* was isolated, which showed the high conversion of methanol to acetate at 15°C.

## Introduction

Natural wetlands in which abundant methanogenic archaea inhabit contribute a great portion of global methane emission [18]. In particular, the wetlands in cold areas, like permafrost and tundra can be the important contributors since about 75% earth's biosphere is under permanently low temperature (below 5°C) [2]. Methanogenesis pathways in a few cold wetlands have been studied since the 1990s [23, 24], those indicated that aceticlastic methanogenesis was prevalent at low temperature and could contribute at least 67% total methane production [4]; while hydrogenotrophic methanogenesis occurred mostly in the warmer environments and acidic peats [17]. Methylotrophic methanogenesis was believed to be limited in marine or other saline environments [20] and contributed less than 5% methane production in freshwater [16]. By using <sup>13</sup>C-isotop labeling experiment, Conrad and Claus [5] showed that methanol-derived methane was only marginal in anoxic rice field soil. However, studies on different types of boreal wetlands with acidic peat bogs in Finland revealed distinct methanogenesis pathways and different methanogen communities [8], most likely related to different vegetations. Our recent study on the composition and abundance of methanogen population in Zoige wetland also suggested that methyl compounds especially methanol might contribute a higher portion of CH<sub>4</sub> production than expected.

Zoige wetland at Tibetan plateau is a tundra wetland under an average annual temperature around -1.7°C to 3.3°C attributed to its high altitude. Distinct from the boreal tundra wetlands, Zoige wetland is located in the lower latitude

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(33°56'N, 102°52'E) region so that different vegetations such as *Eleocharis vallecucosa*, a plant with tuber in rich of pectin, thrive in addition to *Carex muliensis*. Annual methane emission in Zoige wetland was estimated from 0.56 to 1 Tg [6, 7, 11]. In our previous study, the most efficient psychrophilic methanogenesis was determined in the methanol enrichment of *E. vallecucosa* soil; furthermore, a predominant psychrophilic and methylotrophic methanogen was isolated [30]. To assess the contribution of methanol-derived methane in Zoige wetland, in the present study, we quantified the various methanogens and acetogens in the soil in which *E. vallecucosa* grew and determined methanogenic precursors through inhibition of methyl-coenzyme M reductase in order to assess the contribution of methanol-derived methane in Zoige wetland.

## Methods

### The Sampling Site

Zoige wetland (33°56'N, 102°52'E) is located at the eastern edge of the Qinghai-Tibetan Plateau, Southwest China at an altitude of 3,430 to 3,460 m. The soil temperatures were 6°C and 15°C from June to Sept. [27], and the pH of the soil slurry was 6.8–7.2. The mean annual rainfall was approximately 650 mm, and the water depth remained around 5 cm. Two predominant plant populations, *C. muliensis* and *E. vallecucosa* were scattered in the water, which covered about 95% of the whole site. Soils were collected from 10 to 30 cm in depth at the end of April, 2007 and filled into sterile serum bottles sealed with butyl rubber stoppers, transported in a box with ice within 24 h and then stored at 4°C.

### Media and Growth Experiments

The pre-reduced basal medium was prepared as described previously [28] but omitting rumen fluid and titanium solution. The basal medium contained cysteine-HCl, 0.05 g; tryptone, 0.25 g; yeast extract, 0.5 g; peptone, 0.25 g; and the following compositions at the indicated final concentrations (% v/v): 0.2 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> solution, 2; 0.2 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub> solution, 2; NaHCO<sub>3</sub> solution, 7; Na<sub>2</sub>S·9H<sub>2</sub>O solution, 2; mineral solution, 4; trace element solution, 0.8; vitamin solution, 0.8; 0.1% resazurin, 1.

Mineral solution comprised following chemicals (gram per liter): NH<sub>4</sub>Cl, 6 g; NaCl, 6 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 g; MgCl<sub>2</sub>·6H<sub>2</sub>O, 2 g; trace element solution contained the following compositions (gram per liter): FeCl<sub>2</sub>·4H<sub>2</sub>O, 2 g; ZnCl<sub>2</sub>, 0.05 g; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.05 g; CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.03 g; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.05; AlCl<sub>3</sub>, 0.05; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.2;

H<sub>3</sub>BO<sub>3</sub> saturated solution, 1 ml; HCl, 1 ml; vitamin solution contained the following compositions (gram per liter): biotin, 0.002 g; folic acid, 0.002 g; pyridoxine-HCl, 0.01 g; riboflavin, 0.005 g; thiamine-HCl, 0.005 g; niacin, 0.005 g; cyanocobalamin, 0.005 g; *p*-aminobenzoic acid, 0.005 g; pantothenic acid, 0.005 g. NaHCO<sub>3</sub> solution was prepared by dissolving 8.0 g in distilled water to make 100 ml. Na<sub>2</sub>S·9H<sub>2</sub>O solution was prepared by dissolving 24.2 g in distilled water to make 100 ml, filter-sterilizing and equilibrating with N<sub>2</sub> gas phase.

The medium was dispensed into screw-capped tubes sealed with butyl rubber stoppers and flushed with N<sub>2</sub> gas for routine cultivation unless indicated. Various methanogenic media were made by addition of the following substrates into the basal medium, 20 mmol L<sup>-1</sup> each of acetate, formate, methanol, trimethylamine, and 1.01 × 10<sup>5</sup> Pa H<sub>2</sub>/CO<sub>2</sub> (80:20). One gram of soil was inoculated into the media inside of an anaerobic box (Forma Anaerobic System 1029) and incubated at either 15°C or 30°C. Ampicillin solution (final concentration, 2 mg ml<sup>-1</sup>) was added to inhibit bacterial growth [14]. CH<sub>4</sub> production was measured for all the enrichments every 2 to 5 days during the incubation. The rate of methane production was calculated from the linear part of methane production curve against incubation days.

To measure the accumulation of methanogenic precursors, 2-bromoethane-sulfate (BES; final concentration, 2 mg ml<sup>-1</sup>) was added to inhibit methanogenesis [9, 19]. Chloroform (final concentration, 100 μmol L<sup>-1</sup>) was added to inhibit both methanogens and acetogens [15, 21].

### The Most Probable Number Method

Methanogens of different trophic groups in the soil were counted using most probable number (MPN) technique with three tubes for each dilution. One gram of soil was homogenized and used to prepare the tenfold serial dilution inside of an anaerobic box (Forma Anaerobic System 1029). The soil suspension was serially diluted prior to using as an inoculum and the pre-reduced basal medium was used for MPN determination. Five types of media containing each of acetate, formate, methanol, trimethylamine, and 1.01 × 10<sup>5</sup> Pa H<sub>2</sub>/CO<sub>2</sub> (80:20) for the five trophic types of methanogens, respectively, and by addition of ampicillin to inhibit bacteria. Quantification of methanol-trophic acetogens was performed by a serial dilution of the soil slurry into methanol-containing basal medium, and with BES (final concentration, 2 mg ml<sup>-1</sup>) to inhibit methanogens. Various methanogen populations were estimated after 60 days of incubation at 15°C by taking CH<sub>4</sub> production as the positive identification [26]. The counting of acetogens was based on acetate production at about 20-day incubation at 15°C.

## Analytical Techniques

Methane, acetate, and methanol were analyzed by a gas chromatograph GC-14B (Shimadzu) equipped with C18 column as described previously [31], and the C18 column (1 m by 2 mm) was packed with GDX401, a medium with polar copolymer of nitrogen heterocyclic ring monomer and divinylbenzene. Temperature parameters for the analyses of methane, acetate, and methanol were as follows, column 50°C, 230°C, and 150°C; injector 80°C, 250°C, and 170°C; and detector 100°C, 280°C, and 220°C, respectively. Methanol was also determined by using gas chromatograph GC-2010 (Shimadzu) equipped with capillary column GB5 (25 m by 0.25 mm), a non-polarity compound with (5% phenyl) polydimethylsiloxane. The temperature parameters were column 150°C, injector 170°C, and detector 230°C, respectively.

## DNA Extraction, Polymerase Chain Reaction Amplification, and Phylogenetic Analysis

DNA was extracted from the soil enrichments with methanol using a modified cetyltrimethylammonium bromide method as described previously [32]. Samples were suspended in TE buffer (10 mmol L<sup>-1</sup> Tris-HCl [pH 8.0], 1 mmol L<sup>-1</sup> ethylenediamine tetraacetate (EDTA)) with 7.5% chelex (Sigma), 50 mmol L<sup>-1</sup> EDTA (pH 7.0), 2% SDS, and 200 µg of proteinase K in 750 µl extract mixture. After the chelex layer was removed upon centrifugation, 100 µl of NaCl (5 mol L<sup>-1</sup>) and 80 µl of cetyltrimethylammonium bromide (10% w/v in 0.7 mol L<sup>-1</sup> NaCl) was added to the mixture and incubated at 65°C for 30 min. Then crude DNA was purified with DNA gel purification kit (Qiagen, America) and dissolved in TE buffer.

16S rRNA genes were polymerase chain reaction (PCR) amplified using bacterial-universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and prokaryote universal reverse primer 1492R (5'-TACG GYTACCTTGTTACGACTT-3'), and the DNA preparation was used as template. PCR mixture (25 µl) contained 2.5 µl *Taq* buffer, 1 µl of dNTP (5 mmol L<sup>-1</sup>, final concentration), 1 µl of each primer (0.2 µmol L<sup>-1</sup>, final concentration), 1 µl template DNA, 0.1 µl *Taq* DNA polymerase (5 U, TaKaRa, Japan), and 18.5 µl ddH<sub>2</sub>O. PCR amplification was performed at Bio-rad MyCycler, and the thermo-cycling parameters were as follows, initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s.

PCR products were purified as described previously [12]. The purified 16S rRNA gene fragments of about 1,465 bp were cloned into pUCm-T vector, and sequenced by SinoGenMax Co., Ltd. (Beijing, China). Chimera sequences

of 16S rRNA genes were identified by Chimera Check of Ribosomal Database Project II (release 8.1) [3]. The 16S rRNA sequences were submitted to GenBank to search for the similar sequences using BLAST algorithm. The best matching sequences were retrieved from the database and aligned and similarity analysis was performed by CLUSTAL X [25].

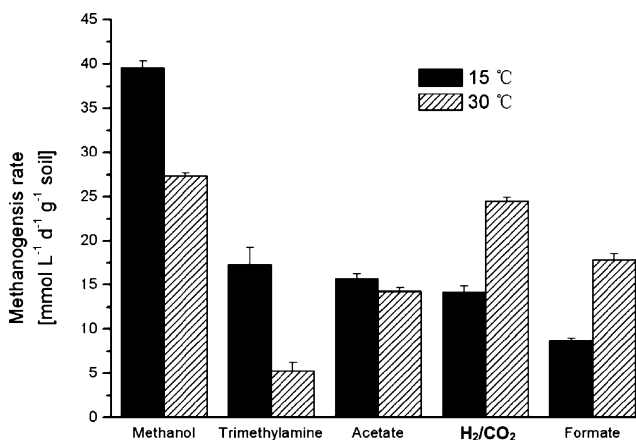
## Isolation and Physiology Analysis of Methanol-Using Acetogens

The acetogenic enrichment in methanol at 15°C was tenfold serially diluted in the same medium, and then Hungate rolling tube technique was performed. Colonies were picked for further purification. The purity of cultures was examined periodically by monitoring cell morphology under a normal bright-field microscope and colony homogeneity. Cell morphology was examined by using light microscope (Olympus BH-2 microscope). The acetate production rate from methanol was measured by cultivating the strain in methanol-contained freshwater mineral medium [10].

## Results

### The Rates of Methanogenesis in Various Methanogenic Enrichments of the Soil

To know the prevalent methanogenic pathways in Zoige wetland, 1 g of soil sampled from the rhizosphere of *E. valleculosa* was inoculated to the media containing each of the five types of methanogenic substrates, and incubated at either 15°C or 30°C. By following the methane yields during a 55-day incubation, methanogenesis rates of the soil enrichments with each substrate were determined. It turned out (Fig. 1) that methanogenesis occurred in all the



**Figure 1** Methanogenesis rates (mmol L<sup>-1</sup> d<sup>-1</sup> g<sup>-1</sup> soil) of the soil enrichments of Zoige wetland from various methanogenic substrates at 15°C or 30°C. Data are the means of three replicates, and standard deviations are shown

five enrichments and methanol supported the highest CH<sub>4</sub> formation rate at both temperatures, even higher at 15°C (39.58±0.76 mmol L<sup>-1</sup>d<sup>-1</sup>) than at 30°C (27.33±0.35 mmol L<sup>-1</sup>d<sup>-1</sup>). Trimethylamine also supported a higher methanogenesis rate at 15 °C (17.29±1.98 mmol L<sup>-1</sup>d<sup>-1</sup>) than at 30°C (5.25±0.97 mmol L<sup>-1</sup>d<sup>-1</sup>) though the rates were relatively lower than other substrates. These data implied that methylotrophic methanogenesis could be the cold active pathway. The rate of aceticlastic methanogenesis at 15°C was lower than methanol-trophic methanogenesis, and appreciably higher at 15°C than at 30°C. However, hydrogenotrophic methanogenesis rate was much higher at 30°C (24.50±0.45 mmol L<sup>-1</sup>d<sup>-1</sup>) than 15°C (14.17±0.72 mmol L<sup>-1</sup>d<sup>-1</sup>), confirming the mesophilic feature.

#### Quantification of Various Trophic Methanogens in the Soils

To determine the abundance of the cold active methanogens in the soil, MPN method was applied to quantify various trophic types of methanogens in the soil at 15°C; meanwhile, methanol-trophic acetogens were counted as well. The results (Table 1) showed that in the soil, the quantities of methanogens using H<sub>2</sub>/CO<sub>2</sub>, acetate and methanol were all numbered to 10<sup>7</sup> cells per gram of soil. While the methanogens using formate (10<sup>5</sup>) or trimethylamine (10<sup>4</sup>) were quite low in this soil. The cold active methanol-using acetogens (10<sup>8</sup> cell g<sup>-1</sup>) were ten times more than methanol-trophic methanogens.

#### Accumulation of Methanol and Acetate in the Methanogenesis-Inhibited Soil Sample

Both methanol and acetate could be the major methanogenic precursors in the *E. vallecuclosa* grown soil based on the experimental data above. To determine this hypothesis, accumulation of the two compounds in the soil samples were measured in which methanogenesis was inhibited either by BES or chloroform. Methane, methanol and acetate were monitored during the incubation at 15°C. While CH<sub>4</sub> was emitted in the non-inhibited soil sample, methanogenesis was completely suppressed either in BES- or chloroform-inhibited experiments (Fig. 2a). Meanwhile, maximal amounts of methanol at 7.14±1.86 mmol L<sup>-1</sup> and 19.23±2.63 mmol L<sup>-1</sup> were detected in the soil sample treated by BES and CHCl<sub>3</sub> at 35-day incubation, respectively; however,

the accumulated methanol was rapidly degraded in the two experimental vials though no CH<sub>4</sub> measured (Fig. 2b). Simultaneously, maximal acetate was detected in the inhibited soil samples (Fig. 2c) at 20.80±2.30 mmol L<sup>-1</sup> and 11.90±1.50 mmol L<sup>-1</sup> in BES- and CHCl<sub>3</sub>-treated experiments, respectively. Different from the accumulated methanol, acetate was gradually degraded. Methanol accumulation in CHCl<sub>3</sub>-treated soil was much higher than in BES-treated one; in contrast, more acetate was accumulated in BES treatment than in CHCl<sub>3</sub>-treated one, implying that part of accumulated methanol could be converted to acetate.

The accumulation of methanol and acetate in methanogenesis inhibited *C. muliensis* soil sample was quite different. Comparing with the soil sample without inhibitors, no CH<sub>4</sub> produced in BES- or CHCl<sub>3</sub>-treated soil (Fig. 2d), however, only 2.28 mmol L<sup>-1</sup> methanol was detected in CHCl<sub>3</sub>-treated soil and almost none in BES-treated one (Fig. 2e). While pronounced acetate accumulation was detected in BES-treated soil (17.32 mmol L<sup>-1</sup>) on the 47th day, in the CHCl<sub>3</sub>-treated one, acetate level was constantly low (about 3.59±0.68 mmol L<sup>-1</sup>; Fig. 2f), even lower than in blank experiment (13.65 to 6.21 mmol L<sup>-1</sup> during incubation).

#### Methanol Metabolism Channeled to Acetogenesis in the Soil Sample

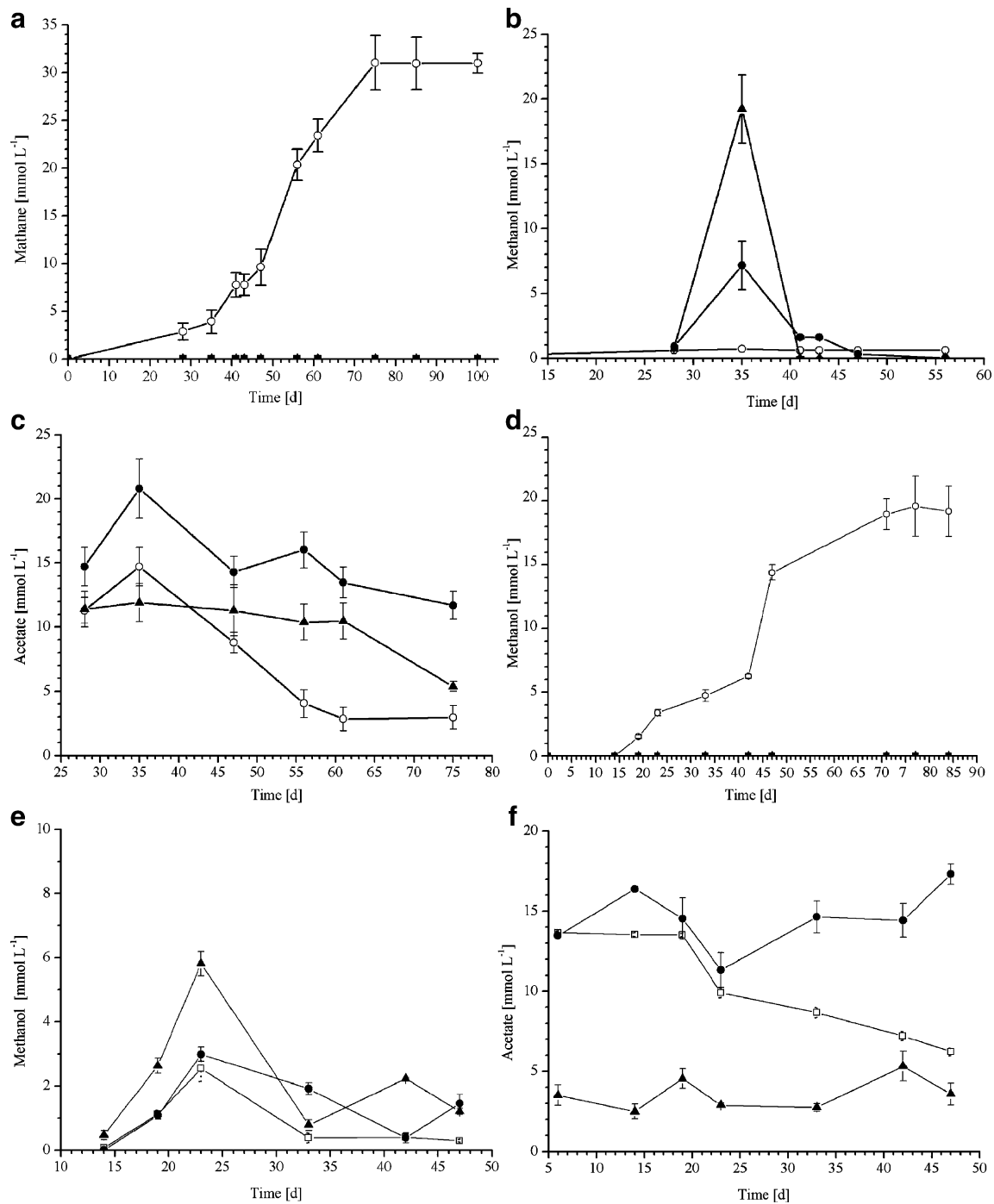
Since a conversion of methanol to acetate was speculated and abundant methanol-trophic acetogens were detected in the soil, in this experiment methanol-derived acetogenesis was determined in the soil sample with BES to inhibit methanogens. By inoculating the soil into methanol (20 mmol L<sup>-1</sup>) and monitoring the levels of methanol and acetate during incubation at 15°C, efficient methanol conversion to acetate (0.46 mmol L<sup>-1</sup>d<sup>-1</sup>) with a lag phase of less than 5 days was shown (Fig. 3).

#### Dominant Methanol-Trophic Bacterial Community in the Soil

To get insight of the dominant methanol-using bacteria community structure, the highest diluted MPN cultures with methanol and BES were used for analysis. Total DNA was extracted from the 20-day incubated enrichment at 15°C, and the phylogenetic diversity of bacterial 16S rRNA genes

**Table 1** Quantification (g<sup>-1</sup> soil) of methanogens and methanol-using acetogens in the soil by MPN method at 15°C

Methanogens		Acetogens			
H <sub>2</sub> /CO <sub>2</sub>	Formate	Acetate	Methanol	Trimethylamine	Methanol
1.1×10 <sup>7</sup>	4.5±0.2×10 <sup>5</sup>	3.0±0.1×10 <sup>7</sup>	4.5±0.5×10 <sup>7</sup>	1.5±0.2×10 <sup>4</sup>	4.5±0.3×10 <sup>8</sup>

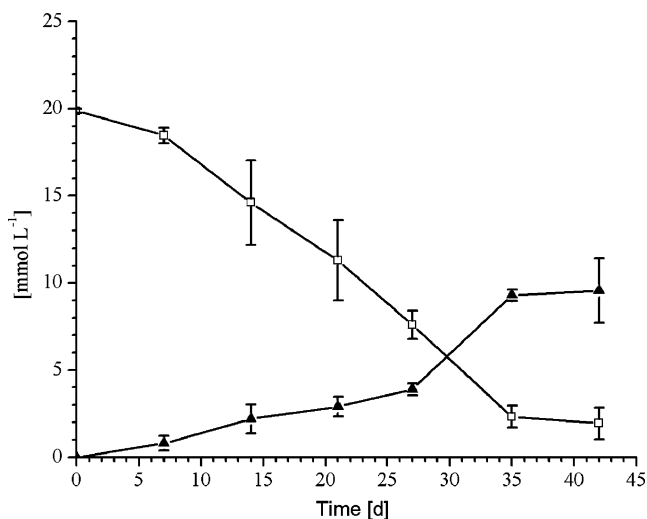


**Figure 2** Methane production (**a** and **d**) and accumulation of methanol (**b** and **e**) and acetate (**c** and **f**) in the methanogenesis-inhibited soil slurries of *E. valleculosa* (**a**, **b**, and **c**) and *C. muliensis* (**d**, **e**, and **f**) with 2-bromoethanesulfonate (2 mg ml<sup>-1</sup>) or chloroform (100 μmol L<sup>-1</sup>)

during a 60-day incubation at 15°C. Data are the means of three replicates and standard deviations are shown. *Empty circle* blank. *Filled triangle* CHCl<sub>3</sub> inhibited. *Filled circle* 2-bromoethanesulfonate (BES) inhibited

was analyzed. Sixteen fragments of 16S rRNA genes in total were retrieved and the sequence homology analysis showed that the majority was from two bacteria, of 12 sequences (GenBank accession nos. FJ794701–FJ794704, FJ794706–FJ794708, FJ794710–FJ794713, and FJ794715)

displaying 99–100% similarities with that of a psychrophilic acetogen *Acetobacterium bakii* (DSM 8239); the remaining four sequences (GenBank accession nos. FJ794700, FJ794705, J794709, and FJ794714) showed 99–100% similarities with *Trichococcus pasteurii* (KoTa2).



**Figure 3** Acetate production from methanol (20 mmol L<sup>-1</sup>) at 15°C in the soil enrichment. Data are the means of three replicates, and standard deviations are shown. Empty square methanol. Filled triangle acetate

#### Characterization of *T. pasteurii* Strain Isolated from Methanol Enrichment

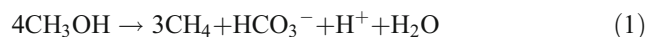
A strain (J-9) was isolated from the highest diluted MPN cultures of methanol enrichment by continued dilution and incubated at 15°C anaerobically. Cells were Gram-staining positive and consisted of cocci in chains. Strain J-9 produced acetate from methanol at 15°C with the maximal rate of  $0.96 \pm 0.08$  mmol L<sup>-1</sup> d<sup>-1</sup>, but quite lower rate at 30°C ( $0.33 \pm 0.02$  mmol L<sup>-1</sup> d<sup>-1</sup>), and no lactate formed from methanol. The 16S rRNA gene sequence (GenBank No. GQ406052) analysis indicated strain J-9 as the member of *T. pasteurii*.

#### Discussion

In Zoige wetland, two predominant plant populations, *C. muliensis* and *E. vallecuculosa* covering about 95% of the whole site grow in the water. While *C. muliensis* is prevalent in most cold wetlands, *E. vallecuculosa* seems just prevalent in the wetlands at low latitude. Our preliminary study on methanogenesis pathways suggested that methanol and acetate could serve as the important methanogenic precursors in *E. vallecuculosa* grown soil. In this study, both methanogenesis rates and quantification of various trophic methanogens in the soils showed that methanol and acetate did play a significant role in the methane formation.

It has been known that methanogenesis can be inhibited either by a methyl-coenzyme M analogue, BES [15], or CHCl<sub>3</sub>, an inhibitor of both methyl-coenzyme M reductase and corrinoid enzymes [21]. As both methanol and acetate were accumulated in the methanogenesis inhibited soil

samples, their contribution to methane formation can be calculated according to the conversion stoichiometry shown by Eqs. 1 and 2. Thus, based on the production of  $31.04 \pm 2.84$  mmol L<sup>-1</sup> CH<sub>4</sub> in non-inhibited soil sample (Fig. 2a) and  $7.14 \pm 1.86$  mmol L<sup>-1</sup> of methanol accumulated in BES-inhibited soil sample (Fig. 2b), methanol-derived methane could account for a large portion in *E. vallecuculosa* soil. Acetate-derived CH<sub>4</sub> could also contribute a high proportion in the soil, according to the stoichiometry of Eq. 2 and the accumulated acetate ( $20.80 \pm 2.30$  mmol L<sup>-1</sup> soil). While in the in situ soil, the levels of acetate (1–5 mmol L<sup>-1</sup>) and methanol (0.2–2 mmol L<sup>-1</sup>) were all much lower than in the methanogenesis-inhibited soil sample that can be attributed to the active metabolisms of methanogens.

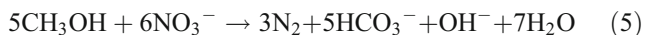
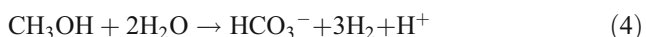
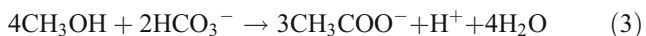


However, by comparing the level of acetate and methanol accumulated in the two inhibitor-treated soil samples, one could see higher methanol (12.09 mmol L<sup>-1</sup>) in BES- than in CHCl<sub>3</sub>-treated soil, while acetate level was just in reverse. Hence, it could be postulated that a part of the acetate in the BES-inhibited soil was derived from methanol, which could be implemented by some bacteria, like acetogens. However, in CHCl<sub>3</sub>-treated soil, conversion of methanol either to acetate or methane would be suppressed attributed to CHCl<sub>3</sub> inhibition of acetyl-CoA activity [22]. Furthermore, methanol-derived acetogenesis was observed in the soil (Fig. 3), and acetogenic bacterial 16S rRNA genes were also detected in the highly diluted soil samples. Those were mainly affiliated to *A. bakkii*, a psychrophilic acetogen using various saccharides and alcohols including methanol [13] and *T. pasteurii*, an aerotolerant lactate-producing spherical bacterium originally isolated from anoxic digester sludge [10]. *T. pasteurii* J-9 isolated from the soil enrichment in this study did convert methanol to acetate at 15°C with a similar lag phase (<5 days) as the soil slurry, but with lower conversion at 30°C, implying that strain J-9 plays a role in psychrophilic methanol conversion to acetate in the soil. In our previous study, a psychrophilic methyl-trophic methanogen *Methanobolus psychrophilus* R15 was isolated from the same soil [30]. Strain R15 grew and produced methane optimally at 18°C (specific growth rate of 0.063 h<sup>-1</sup>) and even at 0°C. Moreover, it produced methane from methanol more efficiently at 15°C (specific growth rate of 0.051 h<sup>-1</sup>) than at 30°C. The proportion of R15 in the soil accounted for 17.2% of the total archaea. This suggested that psychrophilic methanogens in Zoige wetland were likely to be methylotrophic. Compared with the acetogenesis rate of *T. pasteurii* ( $0.96 \pm 0.08$  mmol L<sup>-1</sup> d<sup>-1</sup>), strain R15

displayed a much higher methanol conversion rate of  $9.36 \pm 0.96 \text{ mmol L}^{-1} \text{ d}^{-1}$  at  $18^\circ\text{C}$ .

To understand the plantation effect on methanogenic pathway in the soil, a parallel inhibition experiment was done for *C. muliensis* soil sample. Quite different from the *E. vallecuculosa* soil, low level of methanol ( $2.28 \text{ mmol L}^{-1}$ ) accumulation was only detected in  $\text{CHCl}_3$ -treated soil, but not at all in BES-treated one. Meanwhile, pronounced acetate accumulation was detected in BES treated soil ( $17.32 \text{ mmol L}^{-1}$ ), but acetate was constantly low in the  $\text{CHCl}_3$  treated one (about  $3.59 \text{ mmol L}^{-1}$ ). This implied that acetate as the main methanogenic precursor could originate from the glycolysis pathway in *C. muliensis* soil. The composition difference between *E. vallecuculosa* and *C. muliensis* is that the former is a tuber plant and rich in pectin. Pectin is a complex mixture of homogalacturonan (polymer of galacturonic acid) and rhamnogalacturonans. Methanol in soil was mainly decomposed from pectin because of the activity of pectin methylesterase acting on methoxylesters of galacturonic acid [29]. Addition of pectin in the soil sample grown by *E. vallecuculosa* did promote methanogenesis (data not shown). By the collective data, it can be concluded that methanol contributes a higher proportion to methane formation than expected and previous assessments in some wetlands grown with pectin-rich plants.

In the inhibition experiments, methanol was even degraded in  $\text{CHCl}_3$ -inhibited soil. Here, according to the known chemical reactions, we speculate the possible fates of methanol in fresh water wetlands to channel to acetate,  $\text{CO}_2$ , or denitrification as an electron donor based on the chemical reactions as follows.



The extreme low  $\text{H}_2$  level ( $0.07 \text{ mmol L}^{-1}$ ) in the BES-inhibited soil samples in this study indicated methanol conversion to  $\text{CO}_2$  (Eq. 4) was unlikely. Since denitrification could occur with the presence of  $\text{CHCl}_3$  [1], methanol was probably involved in denitrification (Eq. 5), and we did detect nitrate at  $12.8\text{--}20.8 \text{ (mg kg}^{-1}\text{)}$  in the soil. Though the presence of nitrate could inhibit methanogenesis in waste water reactors in where the concentration of methanol was very high. While methanol level in cold wetland was much lower, and according to the methanol thresholds of denitrification and methanogenesis, methanogens could be competitive over denitrifiers in the wetland. Accumulated acetate in the inhibited soil was gradually

decreased though no  $\text{CH}_4$  formation, implying that acetate could be channeled to other metabolic pathway, like syntrophic degradation or denitrification.

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