High Concentrations of Methyl Fluoride Affect the Bacterial Community in a Thermophilic Methanogenic Sludge

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Abstract

To precisely control the application of methyl fluoride (CH3F) for analysis of methanogenic pathways, the influence of 0–10% CH3F on bacterial and archaeal communities in a thermophilic methanogenic sludge was investigated. The results suggested that CH3F acts specifically on aceticlastic methanogenesis. The inhibitory effect stabilized at an initial concentration of 3–5%, with around 90% of the total methanogenic activity being suppressed, and a characteristic of hydrogenotrophic pathway in isotope fractionation was demonstrated under this condition. However, extended exposure (12 days) to high concentrations of CH3F (>3%) altered the bacterial community structure significantly, resulting in increased diversity and decreased evenness, which can be related to acetate oxidation and CH3F degradation. Bacterial clone library analysis showed that syntrophic acetate oxidizing bacteria Thermacetogenium phaeum were highly enriched under the suppression of 10% CH3F. However, the methanogenic community did not change obviously. Thus, excessive usage of CH3F over the long term can change the composition of the bacterial community. Therefore, data from studies involving the use of CH3F as an acetoclast inhibitor should be interpreted with care. Conversely, CH3F has been suggested as a factor to stimulate the enrichment of syntrophic acetate oxidizing bacteria.

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Introduction

Methane is both a greenhouse gas and a bioenergy gas. The metabolism of methane formation has recently generated a great deal of interest due to the greenhouse effect and the energy crisis. Methane formation can be realized via acetoclastic or hydrogenotrophic methanogenesis, which predominates in anaerobic digestion of biomass. The ratio of the two pathways can be influenced by various environmental factors such as pH, acetate or ammonia concentrations [1–2], the oxidation state of labile organic carbon and competing microbial processes [3].

To better understand the controlling factors for CH4 production in anoxic or anaerobic environments, methyl fluoride (CH3F) has been frequently used and regarded as a specific inhibitor for aceticlastic methanogenesis [3–7]. The inhibitory effect of CH3F has been validated in pure culture studies [8], as well as in rice fields [4], [6], [9], [10], lake sediments [11] and anaerobic reactors [12], [13]. Inhibitor experiments combined with isotope probing and signature were conducted to confirm the specific inhibition on the acetoclastic pathway [4], [10–13]. According to the results of the aforementioned experiments, a CH3F concentration of 1–3% was suggested to be used in various environments including the rhizosphere [4], [10], lake sediments [11] and anaerobic digestors under thermophilic [12] and mesophilic [13] conditions to specifically suppress the acetoclastic pathway without inhibiting the hydrogenotrophic pathway.

To further investigate the effect of CH3F on the microbial community, the inhibition of CH3F (approx. 1%) on the growth of acetotrophic Methanosaeta sp. and Methanosarcina sp. was also confirmed in pure cultures [8] and in the rhizosphere [6]. Other microbes, including homoacetogenic, sulfate reducing bacteria (e.g., Desulfitomaculum spp.) and fermentative bacteria (e.g., Acetobacterium spp.), as well as a methanogenic mixed culture based on hydrogen syntrophy, were not inhibited [8]. Thus, it is often assumed that other organisms in anoxic or anaerobic mixed cultures are not affected by CH3F, and data are interpreted accordingly to infer the roles of methanogens and other microorganisms in the consortium.

However, microbial populations may act differently in complicated environmental microcosms compared with that in pure culture due to redistribution of the substrates and competition among various populations. Daebeler et al. [3] recently demonstrated that CH3F affects methanogenic activity rather than community composition of methanogenic archaea in a rice field soil, which was in accordance with the findings by Hao et al. [14]. Conversely, the syntrophic acetate oxidizing (SAO) bacteria and hydrogenotrophic methanogens were in higher abundance in the CH3F (3%) treatments after an attack by pH disturbance [1].
Moreover, CH₃F can inhibit CH₄- or ammonium-oxidizing bacteria [15], [16]. Therefore, the assumption that bacteria are not affected by CH₃F is questionable, and further research is necessary to better understand the influence of CH₃F on the microbial community structure in mixed cultures, especially on bacterial community structure.

Additionally, the above conclusions are only based on experiments conducted at a relatively low CH₃F concentration of 0–3%, while nearly no information regarding changes in microbial community structure has been reported for wider ranges of CH₃F concentration. Considering that the appropriate dose of CH₃F differs under different environmental conditions [13], the influence of higher concentrations of CH₃F on micro-ecology needs to be studied.

In the present study, to appropriately control the application of CH₃F for analysis of methanogenic pathways, the influence of 0–10% CH₃F on the bacterial and archaeal community structure was investigated using denaturing gradient gel electrophoresis (DGGE). Since bacterial DGGE fingerprints were found to change significantly at high CH₃F levels, while archaeal ones were not, a bacterial clone library was constructed at CH₃F (10%) to probe into the mechanism for this alteration. Process measurement and isotope signature were applied to identify the inhibitory effect in the inhibitor experiment. Additional evidence was presented that, extended exposure to high concentration of CH₃F affected bacterial community structure in a thermophilic methanogenic sludge.

Materials and Methods

Experimental Set-up

Freshly collected methanogenic granular sludge, which was previously cultivated at 55°C in an anaerobic sequenced batch reactor, was used as the inoculum. The inoculum was added to serum bottles (250 mL) with 100 mL basal medium at a volatile solid (VS) concentration of about 3 g L⁻¹. The basal medium contained per L: 1.0 g NH₄Cl, 0.4 g KH₂PO₄, 3H₂O, 0.2 g MgCl₂·6H₂O, 0.08 g CaCl₂·2H₂O, 0.1 g yeast extracts, 0.2 g Na₂S·9H₂O, 10 mL trace element solution (containing per L: 400 mg FeCl₃·4H₂O, 19 mg H₂BO₃, 100 mg ZnCl₂, 20 mg CuCl₂·2H₂O, 100 mg MnCl₂·H₂O, 10 mg Na₂MoO₄·H₂O, 90 mg AlCl₃·6H₂O, 170 mg CoCl₂·6H₂O, 20 mg NiCl₂·6H₂O, 194 mg Na₂SeO₃·3H₂O, 1.0 g EDTA-2Na) and 5 mL stock vitamin solution (containing per L: 10 mg biotin, 50 mg pyridoxine·HCl, 25 mg thiamineHCl, 25 mg D-calcium pantothenate, 10 mg folic acid, 25 mg riboflavin, 25 mg nicotinic acid, 25 mg 4-Aminobenzic acid and 0.5 mg vitamin B₁₂). Sodium acetate was then added as the substrate to an initial concentration of 54 mM. Bicarbonate buffer was used and the pH of the bulk liquid was adjusted to 6.8 with 1 M HCl. Reagents were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

After the headspace was filled with a N₂/CO₂ (4/1, V/V) gas mixture (1 atm), CH₃F (99%, Shanghai Chinyu Special Gas Co., Ltd, China) was injected into the headspace and then mixed by manual shaking. A total of 12 different reactors were set up at different initial CH₃F concentrations of 0, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 4.0%, 5.0%, 6.0%, 8.0% and 10% (V/V). The sludge was incubated statically at 55°C in the dark for 12 days, during which time period liquid and gas samples were taken periodically for analysis.

Physical-chemical Analysis of the Samples

Gas composition, including CH₄, CO₂, H₂ and CH₃F, was periodically monitored. The stable carbon isotopic compositions of CH₄ (δ¹³CH₄) and CO₂ (δ¹³CO₂) in the headspace were measured on day 6. The liquid samples were analyzed for pH, total organic carbon, total inorganic carbon and volatile fatty acids. Samples were analyzed using methods that have been described in detail elsewhere [13].

DNA Extraction and PCR-DGGE for Bacteria and Archaea

Sludge samples were collected at the end of the incubation period on day 12 and DNA was extracted from the seed and incubated sludge. Polymerase chain reaction (PCR), and DGGE of the PCR products for bacteria and archaea were conducted as previously described [12]. Sequencing of single DGGE bands and the subsequent analyses were conducted using the methods described by Ye et al. [17].

Establishment of Bacterial 16S rRNA Gene Library

A bacterial clone library was generated from PCR-amplified 16S rRNA gene using bacterial primers 27 f (5’-AGA GTT TGA TCC TGG CTC AG-3’ [18]) and 1492r (5’-GTT TAC CTT GGG CTC AG-3’) [18]. Each PCR reaction (50 μL) contained 1 μL of template, 10 pmol of each primer, 5 μL of 10 × Red Hot PCR buffer, 100 μmol of dNTPs, 20 mmol of MgCl₂ and 1 μL of Taq DNA polymerase (Shanghai BioColor BioScience and Technology Company, China). PCR amplification was conducted as follows: 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min and then a single final extension at 72°C for 10 min. PCR reactions were performed on a Mastercycler ep realplex² qPCR thermal cycler (Eppendorf AG, Hamburg, Germany).

To minimize PCR artifacts, reconditioning PCR was conducted according to the method described by Thompson et al. [19] after initial amplification of the bacterial 16S rRNA gene. Briefly, the PCR-amplified reaction was diluted 10-fold in a fresh reaction mixture of the same composition and cycled three times using this program. ssDNA and heteroduplex DNA were minimized by adding excess primer during the reaction [20].

Cloning and Sequencing

Bacterial reconditioning PCR products of 0.2 pmol were electrophoresed on 1.0% agarose and bands of the correct size (>1.5 kb for bacteria) were purified using an EZ-Spin Column DNA Gel Extraction Kit (Sangon Biotech Co., China).

Finally, the purified product was cloned into the pUCm-T vector (Sangon Biotech Co., China) following the manufacturer’s instructions. The ligated products were transformed into Escherichia coli competent cells (SK2301, Sangon Biotech Co., China) with ampicillin and blue/white screening, after which positive clones were arrayed in 96-well plates and stored at −80°C for long-term storage. Plasmid inserts were checked by PCR amplification using an M13 PCR set and 103 bacterial positive insert-containing clones were randomly selected for gene sequencing. The template DNA was prepared from overnight cultures of selected clones using a UNIQ-10 Column Plasmid Mini-Preps Kit (Sangon Biotech Co., China) sequencing, was performed on an ABI 3730 DNA sequencer (Applied Biosystems, CA, USA) with BigDye Terminator chemistry according to the manufacturer’s instructions.

Sequence Analysis

Sequences were analyzed manually to remove vector and ambiguous sequences at the ends by scanning the individual chromatograms using Chromas software ver.2.23 (Technelyx, Shanghai, China). Chimeras were checked by the CHIMERA_
Figure 1. Temporal changes in gas composition in the headspace. (A) percentage of CH₄, (B) percentage of CO₂, (C) H₂ partial pressure and (D) percentage of CH₃F in different reactors: 0% CH₃F (solid circle), 0.5% CH₃F (open circle), 1.0% CH₃F (solid inverted triangle), 1.5% CH₃F (open triangle), 2.0% CH₃F (solid square), 2.5% CH₃F (open square), 3.0% CH₃F (solid diamond), 4.0% CH₃F (open diamond), 5.0% CH₃F (solid triangle), 6.0% CH₃F (open inverted triangle), 8.0% CH₃F (solid hexagon), 10.0% CH₃F (open hexagon).

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Figure 2. Temporal changes in acetate concentration under different CH₃F levels. 0% CH₃F (solid circle), 0.5% CH₃F (open circle), 1.0% CH₃F (solid inverted triangle), 1.5% CH₃F (open triangle), 2.0% CH₃F (solid square), 2.5% CH₃F (open square), 3.0% CH₃F (solid diamond), 4.0% CH₃F (open diamond), 5.0% CH₃F (solid triangle), 6.0% CH₃F (open inverted triangle), 8.0% CH₃F (solid hexagon), 10.0% CH₃F (open hexagon).

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CHECK program [21] in Ribosomal Database Project (RDP) firstly, and were further firms by DECIPHER and Bellerophon program on the Greengenes website [22]. After eliminating low-quality sequences and chimeric sequences, 89 sequences were used for the subsequent analyses. All reference sequences were obtained from the GenBank (NCBI) and RDP. Bacterial nucleotide sequences obtained in this study are available in the GenBank database under accession numbers: KF990054–KF990120 and KJ003859–KJ003880.

Figure 3. Temporal changes in $\delta^{13}$CH$_4$, $\delta^{13}$CO$_2$ and $\alpha_c$ at day 6 under different CH$_3$F levels. $\delta^{13}$CH$_4$ (solid circle), $\delta^{13}$CO$_2$ (open circle), $\alpha_c$ (solid triangle).
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Figure 4. Denaturing gradient gel electrophoresis (DGGE) fingerprints of the microbial communities. (A) bacterial and (B) archaeal communities in the sludge after incubation under different CH$_3$F levels.
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The inhibition efficiency and apparent stable carbon isotope fractionation factor \((\alpha_c)\) were calculated according to the methods described by Hao et al. [13]. Diversity and evenness of the microbial community structure based on the DGGE profiles were assessed using the methods as described previously [14], [23–26].

### Results and Discussion

#### Metabolites and Inhibitory Effect

As shown in the profiles of the biogas composition (Figure 1) and acetate concentration (Figure 2), the inhibitory effect of CH₃F could be clearly seen in the kinetics of CH₄ formation and acetate degradation when compared with that of the control experiment in which no CH₃F was added. Increasing CH₃F concentrations led to greater inhibition of mineralization of acetate and higher hydrogen partial pressure in the headspace.

However, along with incubation time, the inhibitory effect was not consistent. CH₄ production was obviously accelerated after 7 days of incubation at high CH₃F concentrations \((\geq 3\%)\) when compared with that observed in the earlier period. Since an apparent decrease in CH₃F concentration was observed after 7 or 8 days (initial CH₃F concentrations \(3\%\)), the resumption of methanogenic activity could be related to the loss of CH₃F. To evaluate the acute toxicity of CH₃F, the degree of inhibition was quantified at day 6, with a consistent inhibitory effect occurring during the period of 0–6 days. The fitted dose-response curve had ED₉₀ (effective dose for 90% inhibition on methanogenic activity) concentrations of 3.8% CH₃F. When the CH₃F concentration was as high as 6–10%, the anaerobic consortium still showed 5%

### Data Processing

The inhibition efficiency and apparent stable carbon isotope fractionation factor \((\alpha_c)\) were calculated according to the methods described by Hao et al. [13]. Diversity and evenness of the microbial community structure based on the DGGE profiles were assessed using the methods as described previously [14], [23–26].

#### Table 1. Summarized information of denaturing gradient gel electrophoresis (DGGE) profiles in the present study.

| Sample          | Bacteria          | Archaea          |
|-----------------|-------------------|------------------|
|                 | H*                | E                | S                | 20% PL Evennessd |
| Seed            | 2.41              | 0.89             | 15               | 0.49             |
| 0% CH₃F         | 2.56              | 0.85             | 20               | 0.55             |
| 1% CH₃F         | 2.69              | 0.91             | 19               | 0.44             |
| 1.5% CH₃F       | 2.87              | 0.92             | 23               | 0.46             |
| 3% CH₃F         | 2.86              | 0.90             | 24               | 0.47             |
| 5% CH₃F         | 2.76              | 0.89             | 22               | 0.47             |
| 10% CH₃F        | 2.88              | 0.86             | 28               | 0.57             |

*Shannon index of general diversity \((H)\) as described by Tiwar et al. [23].

The indice \((E)\) to evaluate the evenness of microbial structure as calculated by Pielou [24].

*Based on the Pareto principle, the cumulative y axis value (in this case the proportion of intensities) corresponding with the 20% level on the x axis (in this case the cumulative proportion of band numbers) (20%PL Evenness) used to evaluate the Lorenz curves by Wittebolle et al. [25].

*Richness \((S)\) of the bacterial and archaeal communities as described by Chau et al. [26], which is the number unique taxa present in the sample (here represented by the number of bands in the DGGE lanes).

#### Table 2. Phylogenetic sequence affiliation of amplified 16S rRNA gene sequences excised from bacterial denaturing gradient gel electrophoresis (DGGE) gels.

| Band ID | Length (bp) | Phylogenetically most closely related organism (Accession No.) | Max identity | Phylum     |
|---------|-------------|---------------------------------------------------------------|--------------|------------|
| 1       | 190         | Uncultured Bacteroidetes bacterium (CU918707.1)              | 100%         | Bacteroides|
| 5       | 204         | Uncultured bacterium (JN707963.1)                             | 99%          | unclassified|
| 7       | 204         | Thermotogaceae bacterium (JX088344.1)                         | 99%          | Thermotogae|
| 10      | 179         | Uncultured bacterium (JQ423781.1)                             | 100%         | unclassified|
| 12      | 200         | Uncultured bacterium (EF515355.1)                             | 98%          | unclassified|
| 13–1    | 176         | Thermoanaerobacteriaceae bacterium (GU129121.1)               | 100%         | Firmicutes |
| 13–2    | 199         | Uncultured bacterium clone thermophilic_alkaline-14 (GU455254.1) | 97%          | unclassified|
| 14      | 201         | Bacteroidetes bacterium (JN836378.1)                          | 100%         | Bacteroides|
| 16      | 182         | Uncultured bacterium (JQ519265.1)                             | 100%         | unclassified|
| 17      | 216         | Synergistetes bacterium (JX473564.1)                          | 100%         | Synergistetes|
| 20      | 198         | Bacteroidetes bacterium (JN836378.1)                          | 100%         | Bacteroides|
| 21      | 195         | Uncultured bacterium (JQ316632.1)                             | 98%          | unclassified|
| 26      | 212         | Uncultured bacterium (HQ267061.1)                             | 97%          | unclassified|
| 28      | 176         | Uncultured bacterium (HE804957.1)                             | 100%         | unclassified|
| 29      | 177         | Uncultured Firmicutes bacterium (JQ433814.1)                  | 99%          | Firmicutes |
| 30      | 193         | Uncultured bacterium (AB374122.1)                             | 98%          | unclassified|

*Shannon index of general diversity \((H)\) as described by Tiwar et al. [23].

The indice \((E)\) to evaluate the evenness of microbial structure as calculated by Pielou [24].

*Based on the Pareto principle, the cumulative y axis value (in this case the proportion of intensities) corresponding with the 20% level on the x axis (in this case the cumulative proportion of band numbers) (20%PL Evenness) used to evaluate the Lorenz curves by Wittebolle et al. [25].

*Richness \((S)\) of the bacterial and archaeal communities as described by Chau et al. [26], which is the number unique taxa present in the sample (here represented by the number of bands in the DGGE lanes).
methanogenic activity of that displayed in the control experiment (Figure S1).

Higher resistance of hydrogenotrophic methanogens to CH3F has frequently been reported [4], [6], [8], [13]. In a previous experiment [3], hydrogenotrophic methanogenesis was found to be unaffected, even at 4% CH3F. Thus, the hydrogenotrophic pathway was suggested to contribute to partial CH4 formation in the current study.

Isotopic Signature

The influence of CH3F on methanogenic pathways can also be elucidated by the change in isotopic fractionation factor, δ, along with the elevated CH4F concentration (Figure 3). δ was around 1.01 at CH3F concentrations of 0–1.5%, indicating a predominant pathway of acetoclastic methanogenesis. δCH4 gradually decreased to −62.8‰ and δ13CO2 increased to −28.7‰, resulting in an increase of δ to about 1.04 at CH3F concentrations of 2.5–5%. An increase of the contribution of the hydrogenotrophic pathway was thus indicated, and even dominance could be possible as reported by Hao et al. [13]. As the CH3F concentration was further increased to 8–10%, δCH4 and δ13CO2 quickly decreased to −73.4‰ and increased to −24.3‰, respectively, with δ increasing to 1.05. Hence, the hydrogenotrophic pathway might be further concentrated, or the functional microbial players could have changed under higher stress from 8–10% CH3F, resulting in different isotopic fractionation [5].

Bacterial and Archaeal DGGE Fingerprinting

Use of CH3F (1 kPa, around 1% in the headspace) is generally assumed to eliminate archaea while having little effect on bacteria [6–8]. However, DGGE analyses in this study revealed additional information.

As shown in Figure 4A, long-term (12 days) exposure to various levels of CH3F (0–10%) led to surprising alterations in the bacterial community. The bacterial DGGE profiles demonstrated a gradual change at high CH3F concentrations (1–5%), while they stayed stable at lower CH3F levels (0–1.5%). It can be seen that, along with the elevated CH3F concentration, bands 14, 15 and 27 were first promoted (CH3F 0–3%) and then eliminated (CH3F 3–5%). Band 17 initially appeared at a CH3F of 3%, and then gradually faded out at higher CH3F concentrations. New bands 13, 14, and 29 appeared and bands 13, 14 and 29 had high intensity at 10% CH3F. Correspondingly, in this treatment, the diversity and evenness of the bacterial community structure increased to 2.88 and decreased to 0.86, respectively, when compared with the treatments using lower CH3F levels (Table 1). These findings indicate that more bacterial populations exist under these conditions, but that only a few are active functional players related to the metabolism of acetate or CH3F.

Table 3. Phylogenetic sequence affiliation of amplified 16S rRNA gene sequences excised from archaean denaturing gradient gel electrophoresis (DGGE) gels.

| Band ID | Length (bp) | Phylogenetically most closely related organism (Accession No.) | Max identity | Order |
|---------|-------------|-------------------------------------------------------------|--------------|-------|
| 1       | 151         | Uncultured Methanobacterium sp. (AB602627.1) | 98%          | Methanobacteriales |
| 2       | 152         | Uncultured Methanobacterium sp. (JX576095.1) | 99%          | Methanobacteriales |
| 4       | 153         | Methanobacterium kanagasei (AB368917.1) | 97%          | Methanobacteriales |
| 5       | 152         | Methanoseta sp. (JX088310.1) | 99%          | Methanosarcinales |
| 8       | 152         | Methanothermobacter thermotrophicus (NR_074260.1) | 95%          | Methanobacteriales |

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Sequencing of these bands showed that bands 13–1 and 29 were affiliated with the Firmicutes phylum, while band 14 corresponded to the Bacteroidetes phylum (Table 2). Most of the other bands (such as band 10 and 16), however, can only match to the uncultured bacteria, except that band 7 was assigned to the Thermotogae phylum and band 17 belonged to the Synergistetes phylum. According to previous studies, 4 of the 5 identified SAO bacteria belong to the Firmicutes phylum [27], including the Clostridium ultunense strain BST [28], Syntrophobacter chini [29], Tepidanaerobacter acetatoxydans [30] and Thermacetogenium phaena strain PB [31], while Thermotoga lettingae strain TMO [32] is affiliated with the Thermotogae phylum. Uncultured bacteria in the Thermanaerobacteriaceae family were also found to possess the SAO function [33]. Thus, the members of Firmicutes and Thermotogae in the microcosm could be related to the SAO bacteria. Sequencing of some other bands (like bands 15 and 27) failed; hence, no specific information was available for these bands.

Incubation at CH3F of 0–10% did not alter the archaen DGGE profiles significantly, which is in accordance with the results of a study by Daebeler et al. [3], who found that the suppression of acetoclastic methanogenesis by CH3F caused little differences in the community composition of active methanogenic archaea in a rice field soil. While, it could be seen that, the intensity of bands 1 and 2 slightly increased at CH3F of 3–5%. Sequencing results showed that bands 1 (98%) and 2 (99%) corresponded to uncultured archaean in the Methanobacterium genus (Table 3), while band 5 (99%) was closely related to the acetotrophic Methanoseta genus. These findings suggested that hydrogenotrophic methanogens were enriched to some extent in response to high concentrations of CH3F.

Bacterial Community Confirmed by Clone Library

Acetate metabolism can proceed via SAO in methanogenic systems without electron acceptors such as sulphate, nitrate and chloride. Analysis of the DGGE profiles indicated that the highly concentrated bands might be related to the SAO bacteria at high CH3F concentrations. To obtain in-depth knowledge regarding the bacterial community under these conditions, a clone library was constructed for the microcosm adapted to the treatment with 10% CH3F, and the results confirmed the enrichment of SAO bacteria under pressure from high concentrations of CH3F.

The 89 sequences of 16S rRNA genes were classified into 29 operational taxonomic units (OTUs) as shown in Table S1. Overall, 87% of the sequences were classified as Firmicutes, 4% as Bacteroidetes and 4% as Synergistetes. The taxonomic information of 13 representative OTUs, which were phylogenetically different, was listed in Table 4.

In the sequences assigned to the Firmicutes phylum, the most abundant group of OTUs, with BS01 as the most representative,
Table 4. Taxonomic relationship of bacterial 16S rRNA gene sequences in 13 representative OTUs from 10% CH$_3$F treatment compared (BLAST) with public databases (RDP and NCBI).

| OTU | % of Total | Length (bp) | Phylogenetically most closely related organism (Accession No.) | Accession No. | Phylum       | Similarity (%) | Function                | Source                  |
|-----|------------|-------------|---------------------------------------------------------------|--------------|--------------|----------------|------------------------|-------------------------|
| BS01| 46         | 1593        | *Thermacetogenium phaeum* (NR_074723.1)                       | KF990054     | Firmicutes   | 94%            | Syntrophic acetate oxidation | DSM 12270               |
| BS24| 2          | 1504        | *Syntrophacetus schinkii* (EU386162.1)                        | KF990067     | Firmicutes   | 94%            | Syntrophic acetate oxidation | Mesophilic anaerobic filter |
| BS21| 0          | 1502        | Uncultured *Thermacetogenium* sp. (HQ183380.1)                | KF990065     | Firmicutes   | 94%            | Syntrophic acetate oxidation | Leachate sediment in landfill |
| BS28| 4          | 1485        | *Lutispora thermophila* (NR_041236.1)                        | KF990071     | Firmicutes   | 97%            | Protein-fermenting Strain EBR46 |                          |
| BS84| 1          | 1486        | *Clostridium albidii* (X71846.1)                              | KF990107     | Firmicutes   | 93%            | Cellulolytic            | DSM 6159               |
| BS66| 1          | 1487        | Uncultured bacterium (JF808030.1)                             | KF990092     | Firmicutes   | 99%            | —                      | Produced fluid from Yabase oilfield |
| BS22| 4          | 1296        | *Anaerobaculum mobile* (CP003198.1)                          | KJ003866     | Synergistetes| 97%            | Peptide-fermenting      | DSM 13181               |
| BS40| 1          | 1257        | *Bacteroidetes* bacterium (AY548787.1)                       | KF990076     | Bacteroidetes| 98%            | —                      | Fluidized-bed reactors: acidic wastewater |
| BS70| 1          | 1503        | Uncultured bacterium (AB696265.1)                            | KF990096     | Bacteroidetes| 99%            | —                      | Anaerobic digester sludge |
| BS79| 1          | 1488        | Uncultured *Cytophaga*ales bacterium (FJ516908.1)            | KF990102     | Bacteroidetes| 97%            | —                      | The semiarid ‘Tablas de Daimiel National Park’ wetland |
| BS96| 1          | 1488        | Uncultured bacterium (AB192126.1)                            | KJ003880     | Chlorobi     | 96%            | —                      | Gut homogenate of termites |
| BS94| 1          | 1494        | Uncultured bacterium (FJ462092.1)                            | KJ003878     | uncultified  | 99%            | —                      | Anaerobic reactor: effluent from the chemical industry |
| BS87| 1          | 1489        | Uncultured bacterium (EF0255585.1)                           | KF990110     | uncultified  | 99%            | —                      | Geothermal spring mat    |

OTU, operational taxonomic unit. 

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matched the pure culture (DSM 12270) of Thermacetogenium phaeum (NR_074723.1) with a similarity of 93–94%, which occupied 61% of the total sequences. Another group of OTUs, with BS24 as the most representative, were closely related to Syntrophaceticus schinkii (EU386162.1), which was isolated from a mesophilic anaerobic filter [29]. Thermacetogenium phaeum was reported as a SAO bacterium grown under thermophilic conditions [31]. Syntrophaceticus schinkii also oxidize acetate to support their growth under mesophilic condition [29], but has ever been found in thermophilic reactors as well [2]. It was thus indicated that bacteria with SAO function dominated the methanogenic sludge incubated with 10% CH3F. Notably, there were still 5 OTUs (with BS21 as the most representative) contributing to 9% of the total sequences, which were closely affiliated to the uncultured Thermacetogenium sp. (HQ183800.1) [34]. These bacteria populations could be new, previously undescribed species with SAO function grown under thermophilic conditions.

The other OTUs, including BS22, BS28 and BS84, were related to fermentative bacteria functioning at protein and cellulose degradation, which accounted for 4%, 4% and 1% of the total sequences, respectively. These OTUs might represent some of the aboriginal bacterial populations related with endogenous metabolism and cellulose degradation, since the sludge originated from an anaerobic digester treating wastewater from a paper mill before acclimatization. Most other OTUs could only be matched to uncultured bacteria without clearly defined functions.

Influence of CH3F on Microbial Community

Analyses of the microbial community structure demonstrated that long-term exposure to high concentrations of CH3F (10%) induced the enrichment of SAO bacteria, which has seldom been reported in the literature. Thermacetogenium phaeum-related bacteria predominated the microcosm (Table 4), and diversity of the bacterial community structure increased while the evenness decreased (Table 1). Faster growth of SAO bacteria was also indicated in CH3F treatments (3%) after an attack by high pH in a previous study [1]. Based on these findings, it could be speculated that high concentrations of CH3F strongly inhibited the aceto trophic methanogens [8]; without their competition for acetate, SAO bacteria that were unaffected or less affected by CH3F became active and enriched. The consequently promoted SAO reaction generated more H2 and CO2, resulting in increased hydrogen partial pressure (Figure 1C). The hydrogenotrophs might be thus activated, leading to stronger isotope fractionation (Figure 3). Methanogenic activity resumed along with the incubation time and possibly with the decreasing CH3F concentrations (Figure 1D). By contrast, the microbial community structure did not change obviously at CH3F concentrations of 0–1.5%, which was similar to the results of an experiment conducted by Hao et al. [14]. These results indicated that CH3F usage should be carefully controlled to prevent significant changes in the microbial community structure if the methanogenic function of the microbial community is intended to be evaluated under in-situ conditions.

However, from the perspective of bioaugmentation, use of high concentrations of CH3F may be an efficient tool to stimulate the enrichment of SAO bacteria. Then, the acclimatized consortium can be used as inoculum to address the inhibition problems that occur in anaerobic digestion processes as a result of ammonia or other acetoclast inhibitors [35].

Biodegradation of CH3F

The CH3F concentration decreased quickly in the thermophilic methanogenic system, especially at high initial CH3F levels of 4–10%, which decreased to 3–4.5% after 7 or 8 days of incubation (Figure 1D). Similar phenomena were observed in the previous experiments [4], [12], [35]. It is thus suggested that microbial degradation of CH3F occurs in anaerobic and anoxic systems. Although new DGGE bands appeared after 8 days of incubation under high levels of CH3F, the phylogenetic affiliations of the CH3F-degrading microbes that may be present are still not clear. Therefore, further research using stronger analytical tools such as stable isotope probing are warranted.

Conclusions

The influence of 0–10% CH3F on the microbial community in a thermophilic methanogenic sludge was investigated. The results suggested that, CH3F acted specifically on acetoclastic methanogenesis, and the inhibitory effect stabilized at an initial concentration of 3–5%, with around 90% of the total methanogenic activity being suppressed and a characteristic of hydrogenotrophic pathway in isotope fractionation occurring. However, extended exposure (12 days) to high concentrations of CH3F (>3%) altered the bacterial community structure significantly, with the diversity increased while the evenness decreased. These changes were likely related to acetate oxidation and CH3F degradation. Bacterial clone library analysis showed that, syntrophic acetate oxidizing bacteria (Thermacetogenium phaeum) were highly enriched under the pressure from 10% CH3F, but the methanogenic community did not change obviously. Thus, excessive use of CH3F over a long term can change the composition of the bacterial community. Therefore, data from studies involving the use of CH3F as an acetoclast inhibitor should be interpreted with care. However, from the perspective of bioaugmentation, CH3F can be considered as a factor that stimulates the enrichment of SAO bacteria.

Supporting Information

Figure S1 Dose–response curve for the inhibitory effect of CH3F. The solid line describes the inhibition of methanogenesis as a function of the initial CH3F concentration for anaerobic granules. (DOCX)

Table S1 Bacterial community analyzed by clone library. Taxonomic relationship of bacterial 16S rRNA gene sequences in 29 OTUs from 10% CH3F treatment compared (BLAST) with public databases (RDP and NCBI). (DOC)

Author Contributions

Conceived and designed the experiments: FL LH PH LS. Performed the experiments: LH QW. Analyzed the data: LH FL. Contributed reagents/materials/analysis tools: LH FL. Wrote the paper: LH FL. Reviewed the manuscript: FL PH LS.

References

1. Hao LP, Liu F, Li L, Wu Q, Shao LM, et al. (2013a). Self-adaptation of methane-producing communities to pH disturbance at different acetate concentrations by shifting pathways and population interaction. Bioresour Technol 140: 519–327.

2. Westerholm M (2012) Biogas production through the syntrophic acetate-oxidising pathway: Characterization and detection of syntrophic acetate-oxidising bacteria. Upsala: Swedish University of Agricultural Sciences. 45 p.
3. Daebeler A, Gansen M, Frenzel P (2013) Methyl fluoride affects methanogenesis rather than community composition of methanogenic archaea in a rice field soil. PLoS One 8(1): e53656.

4. Conrad R, Klöse M (1999) How specific is the inhibition by methyl fluoride of aceticlastic methanogenesis in anoxic rice field soil? FEMS Microbiol Ecol 30: 47–56.

5. Conrad R (2005) Quantification of methanogenic pathways using stable carbon isotopic signatures: A review and a proposal. Org Geochem 36: 739–752.

6. Penning H, Conrad R (2006) Effect of inhibition of aceticlastic methanogenesis on growth of archaeal populations in an anoxic model environment. Appl Environ Microbiol 72: 178–184.

7. Penning H, Conrad R (2007) Quantification of carbon flow from stable isotope fractionation in rice field soils with different organic matter content. Org Geochem 38: 2058–2069.

8. Janssen PH, Frenzel P (1997) Inhibition of methanogenesis by methyl fluoride: Studies of pure and defined mixed cultures of anaerobic bacteria and archaea. Appl Environ Microbiol 63: 4532–4537.

9. Penning H, Conrad R (2007) Quantification of carbon flow from stable isotope fractionation in rice field soils with different organic matter content. Org Geochem 38: 2058–2069.

10. Conrad R, Klöse M, Claus P (2002) Pathway of CH₄ formation in anoxic rice field soil and rice roots determined by ¹³C-stable isotope fractionation. Chemosphere 47: 797–806.

11. Conrad R, Klöse M, Claus P, Enrich-Prast A (2010) Methanogenic pathway, ¹³C isotope fractionation, and archaeal community composition in the sediment of two clear-water lakes of Amazonia. Limnol Oceanogr 55: 689–702.

12. Hao LP, Li F, He PJ, Li L, Shao LM (2011a) Predominant contribution of syntrophic acetate oxidation to thermophilic methane formation at high acetate concentrations. Environ Sci Technol 45: 508–513.

13. Hao LP, Li F, He PJ, Li L, Shao LM (2011b) Quantify the inhibitory effect of methyl fluoride on methanogenesis in anaerobic granular systems. Chemosphere 84: 1194–1199.

14. Hao LP, Li F, Li L, Shao LM, He PJ (2013) Response of anaerobes to methyl fluoride, 2-bromoethanesulfonate and hydrogen during acetate degradation. J Environ Sci (China) 25(5): 857–864.

15. Overland RS, Culbertson CW (1992) Evaluation of methyl fluoride and dimethyl ether as inhibitors for aerobic methane oxidation. Appl Environ Microbiol 58: 2983–2992.

16. Bodelier PLE, Frenzel P (1999) Contribution of methanotrophic and nitrifying bacteria to CH₄ and NH₃ oxidation in the rice rhizosphere using new methods for discrimination. Appl Environ Microbiol 65: 1826–1833.

17. Ye NF, He PJ, Li F, Shao LM (2007) Effect of pH on microbial diversity and product distribution during anaerobic fermentation of vegetable waste. Ying Yong Yu Huan Jing Sheng Wu Xue Bao 33(3): 238–242.

18. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York: John Wiley & Sons. pp. 115–175.

19. Thompson JR, Marcelino LA Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by ‘reconditioning PCR’. Nucleic Acids Res 30: 2083–2088.

20. Zhang XL, Yan X, Gao PP, Wang LH, Zhou ZH, et al. (2005) Optimized sequence retrieval from single bands of TGG (temperature gradient gel electrophoresis) profiles of the amplified 16S rDNA fragments from an activated sludge system. J Microbiol Methods 60: 1–11.

21. Colón JR, Chai B, Marsh TL, Parris RJ, Wang Q, et al. (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31: 442–443.

22. DeSantis TZ, Hugesholz P, Larsen N, Rojas M, Brodie EL, et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72: 5069–5072.

23. Tiwari SC, Mishra RR (1993) Fungal abundance and diversity in earthworm casts and in ungested soil. Biol Fert Soils 16(2): 131–134.

24. Pielou EC (1966) The measurement of diversity in different types of biological collections. J Theor Biol 13: 131–144.

25. Wittbolle L, Vervaeken H, Verstraete W, Boon N (2008) Quantifying Community Dynamics of Nitrifiers in Functionally Stable Reactors. Appl Environ Microbiol 74(1): 286–293.

26. Chau JF, Raptazoglou AC, Willig MR (2011) The effect of soil texture on richness and diversity of bacterial communities. Environ Forensics 12: 333–341.

27. Hatton S (2008) Minireview: Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ 23: 118–127.

28. Schnürer A, Schink B, Svensson BH (1996) Clostridium ultunense sp nov, a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. Int J Syst Bacteriol 46(4): 1145–1152.

29. Westerholm M, Roos S, Schnürer A (2010) Syntrophus aciditrophicus gen. nov., sp. nov., an anacrobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. FEMS Microbiol Lett 309(1): 100–104.

30. Westerholm M, Roos S, Schnürer A (2011) Tepidanaerobacter acetatoxydans sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from two clear-water lakes of Amazonia. Limnol Oceanogr 55: 689–702.

31. Kamagata Y, Mikami E (1989) Diversity of acetotrophic methanogens in anaerobic digester. In: Hattori T, Ishida Y, Matsumura Y, Morita KY, Uccelli A, editors. Recent advances in microbial ecology. Tokyo: Japan Scientific Societies Press. pp. 459–464.

32. Balk M, Weijma J, Stams AJ (2002) Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 52(4): 1361–1366.

33. Liu F, Conrad R (2010) Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 72: 2633–2640.

34. Liu J, Wu W, Chen C, Sun F, Chen Y (2011) Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems. Appl Microbiol Biotechnol 91 (6): 1659–1675.

35. Fotidis IA, Karakashev D, Angelidaki I (2013) Bioaugmentation with an acetate-utilizing bacterium isolated from two clear-water lakes of Amazonia. Limnol Oceanogr 55: 689–702.

36. Balk M, Weijma J, Stams AJ (2002) Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 52(4): 1361–1366.

37. Liu F, Conrad R (2010) Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 72: 2633–2640.

38. Kamagata Y, Mikami E (1989) Diversity of acetotrophic methanogens in anaerobic digester. In: Hattori T, Ishida Y, Matsumura Y, Morita KY, Uccelli A, editors. Recent advances in microbial ecology. Tokyo: Japan Scientific Societies Press. pp. 459–464.

39. Balk M, Weijma J, Stams AJ (2002) Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 72: 2633–2640.

40. Liu J, Wu W, Chen C, Sun F, Chen Y (2011) Prokaryotic diversity, composition structure, and phylogenetic analysis of microbial communities in leachate sediment ecosystems. Appl Microbiol Biotechnol 91 (6): 1659–1675.

41. Fotidis IA, Karakashev D, Angelidaki I (2013) Bioaugmentation with an acetate-utilizing consortium as a tool to tackle ammonia inhibition of anaerobic digestion. Bioresource Technol 146: 57–62.

42. Frenzel P, Bosse U (1996) Methyl fluoride, an inhibitor of methane oxidation and methane production. FEMS Microbiol Ecol 21: 25–36.