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Regular Article

Studies on the inhibition of methanogenesis and dechlorination by (4-hydroxyphenyl) chloromethanesulfonate

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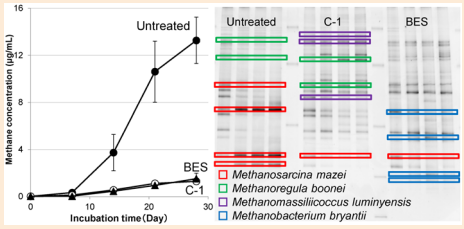
Supplementary material

The purpose of this study was to demonstrate the inhibitory effect of chemicals on methane emissions in paddy soil. We found that (4-hydroxyphenyl) chloromethanesulfonate (C-1) has a methanogenic inhibition activity, and we studied its inhibition mechanism using laboratory tests. The study found that C-1 treatment of flooded soil did not significantly affect the bacterial community but rather the archaeal community; particularly, *Methanosarcina* spp. C-1 strongly inhibited the aceticlastic methanogenesis route. It was suggested that the inhibitory target of C-1 was different from the well-known methanogenic inhibitor 2-bromoethanesulfonate, which targets methyl-coenzyme M reductase of methanogen. In addition, C-1 had a secondary effect of inhibiting the dechlorination of chlorophenols. Although field trials are required as the next development step, C-1 can be used to reduce methane emissions from paddy fields, one of the largest sources in the agricultural sector.



Keywords: (4-hydroxyphenyl) chloromethanesulfonate, 2-bromoethanesulfonate, methanogenic inhibitor, methanogenesis, dechlorination, dichlorophenol.

Introduction

Methane, the second most important anthropogenic greenhouse gas after carbon dioxide, is responsible for approximately 20% of the net increase in radiative forcing since the preindustrial era (*circa* 1750).[1–3] Large amounts of methane are released into the atmosphere as the end product of archaeal metabolism under anaerobic conditions. The major anaerobic sites of methanogenesis are paddy fields, ruminants, natural wetlands, and sediment,[4] with paddy fields contributing about 5–19% of total global methane emissions.[5] According to the National Greenhouse Gas Inventory Report of Japan 2021,[6] total methane emission rates in Japan (28.5 Mt CO_2 eq. in 2019) have decreased by 35.2% since 1990. Greenhouse gas emissions from the agricultural sector decreased by 13.7% in 2019 as compared with levels in 1990. However, methane emissions from rice cultivation—the largest source from the agricultural sector in Japan, accounting for 38%—decreased by only 1.5% in 2019 (11,946 kt CO_2 eq.) from the 1990 levels. Therefore, it is important to reduce methane emissions from paddy fields. Two strategies often proposed are limiting the period of soil submergence (*i.e.*, draining the field) and reducing carbon inputs (through residue management).[7] A pot experiment with 2-bromoethanesulfonate (BES)[8,9] showed that application at 80 mg/kg of BES caused a 49% reduction in methane emission without affecting plant growth and productivity during rice cultivation.[5]

Many studies on methanogenic inhibitors have been conducted to mitigate methane emissions from ruminants, with the most successful compounds tested *in vivo* including BES, bromochloromethane, chloroform, and cyclodextrin.[10] In addition, amichloral, trichloroacetamide, trichloroethyl adipate, 9,10-anthraquinone, 3-bromopropanesulfonate, lumazine, propynoic acid, and ethyl 2-butynoate have been studied for their effects on methane production.[11,12] A novel methanogenic inhibitor, 3-nitrooxypropanol, has been reported to inhibit methanogenesis *in vitro* and *in vivo* in livestock studies, and the target for 3-nitrooxypropanol and BES is methyl-coenzyme M reduc-

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tase (MCR).[13,14]

On the other hand, methanogen involvement in the dechlorination of chlorine-containing compounds has been reported using its specific inhibitor, BES.[15–19] To understand the characteristics of methanogenic inhibitors, it is important to investigate the inhibition of dechlorination.

This study focused on reducing methane emissions with methanogenic inhibitors as agricultural materials. We found by the screening of chemicals that (4-hydroxyphenyl) chloromethanesulfonate can inhibit the production of methane. Clarifying the difference between (4-hydroxyphenyl) chloromethanesulfonate and BES targets through changes in microbial communities and the consumption of methanogenic substrates provides insights into the mechanism inhibiting methane production.

Materials and methods

1. Chemicals

The compounds (4-hydroxyphenyl) chloromethanesulfonate (C-1), 4-(chloromethylsulfonyl)phenol (C-2), (4-hydroxyphenyl) methanesulfonate (C-3), 1-bromo-4-(chloromethylsulfonyl)-benzene (C-4) and (4-bromophenyl)sulfonylmethanol (C-5) were synthesized at Kumiai Chemical Industry Co., Ltd. (Japan) with 95% or higher purities. 4-Chlorophenol (MCP), 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TCP), and sodium 2-bromoethanesulfonate (BES) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Sodium chloromethanesulfonate (CMS) was purchased from Fluorochem Ltd. (United Kingdom). The water was prepared by Milli-Q (Merck, Germany). Other chemicals were purchased from FUJIFILM Wako Pure Chemical (Japan), unless otherwise specified. The nuclear magnetic resonance (^{1}H NMR) data, mp, and the form of the substance of C-1 to C-5 were as follows: C-1: δ 7.21 (2H, m), 6.85 (2H, m), 5.07 (1H, s), 4.65 (2H, s), 1.67 (1H, s), mp 36–39°C, colorless crystalline solid; C-2: δ 7.86 (2H, m), 7.01 (2H, m), 5.89 (1H, s), 4.51 (2H, s), mp 107–110°C, white crystalline solid; C-3: δ 7.15 (2H, m), 6.83 (2H, m), 5.09 (1H, s), 3.12 (3H, s), mp 70–73°C, colorless crystalline solid; C-4: δ 7.84 (2H, m), 7.76 (2H, m), 4.53 (2H, s), 4.65 (2H, s), mp 146–149°C, colorless crystalline solid; and C-5: δ 7.80 (2H, m), 7.74 (2H, m), 4.61 (2H, s), 2.70 (1H, s), mp 112–115°C, white powder.

2. Preparation of precultured soil suspension

Soil samples were collected from a paddy field in Kikugawa, Shizuoka, Japan (0–15 cm sampling depth, 2 mm sieve, clay loam, 34°44′03.3″N, 138°05′05.5″E). To investigate the methanogenic inhibition activity of various compounds, a precultured soil suspension of paddy soil–derived microorganisms was prepared: a 30 g soil sample (dry weight) was weighed in a 100 mL incubation flask; 50 mL of water was added to it, and it was incubated in the dark at 30°C for 4 weeks. After incubation, 1 mL of the soil suspension was transferred to sterilized flooded soil (a mixture of 30 g of soil and 50 mL of distilled water, which had been autoclaved at 121°C for 30 min) and incubated at 30°C for 4 weeks in the dark. After this subculture procedure was performed more than five times, the precultured soil suspension was used for subsequent inhibition tests.

3. Methanogenic inhibition test and the analysis of microbial community structure

To investigate methanogenesis and the microbial community in test solutions, 100 μL of the preculture solution was transferred to sterilized flooded soil (a mixture of 2 g of soil and 4 mL of water, which had been autoclaved at 121°C for 30 min) in 20 mL glass vials with or without C-1 (1 mg/kg dry soil), BES (10 mg/kg dry soil), or CMS (10 mg/kg dry soil). These vials were sealed with sterilized silicone septa and incubated at 30°C for 7, 14, 21, and 28 days. To analyze methane production, 500 μL of headspace gas in the vials was sampled and measured by gas chromatography (GC). The test was conducted in duplicate and performed three times for a total of six separate experiments. To determine changes in the community structure of bacteria and archaea, each solution was aliquoted and centrifuged at 10,000 rpm for 2 min (KITMAN-24, Tomy, Japan). DNA was extracted from the sediment and analyzed using polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE).

4. Characterization of the methanogenic inhibition mechanism using methanogenic substrate

To characterize the methanogenic inhibition mechanism by C-1, methanogenic inhibition tests were performed using methanol and acetate as methanogenic substrates. One hundred microliters of the preculture solution was transferred to sterilized flooded soil in 20 mL glass vials with or without C-1 (1.0 mg/kg dry soil) or BES (40 mg/kg dry soil). These vials were sealed with sterilized silicone septa and incubated at 30°C. After 7 days of incubation, 100 μL of 5% (v/v) methanol solution or 100 μL of 102.5 g/L sodium acetate solution was added to the flooded soils and incubated at 30°C. The generated methane was analyzed by GC. The test was conducted in duplicate and performed three times for a total of six separate experiments.

5. Inhibition activity of TCP dechlorination

To investigate the inhibition activity of TCP dechlorination in test solutions with C-1 and BES treatment, 100 μL of the preculture solution was transferred to TCP (20 mg/kg dry soil, 101 μmol/kg dry soil) containing sterilized flooded soil in 20 mL glass vials with or without C-1 (1.0 mg/kg dry soil) and BES (40 mg/kg dry soil). These vials were sealed with sterilized silicone septa and incubated at 30°C for 7, 14, 21, and 28 days. To analyze the methane production, 500 μL of headspace gas in the vials was sampled and measured by GC. MCP, DCP, and TCP in the flooded soil samples were extracted with 4 mL of acetonitrile by shaking for 30 min and centrifuging at 2,000 rpm for 5 min (LC-121, Tomy, Japan). The supernatants were filtrated through filter vials (0.45 μm PTFE, Thomson, USA), of which 10 μL was used for high-performance liquid chromatography (HPLC) analysis. The test was conducted in duplicate and performed

three times for a total of six separate experiments.

6. Inhibition activity of DCP dechlorination and methanogenesis by C-1 and its analogs

To investigate the inhibition activities of DCP dechlorination and methanogenesis by C-1 and its analogs, 100 μL of the preculture solution was transferred to DCP (20 mg/kg dry soil, 123 μmol/kg dry soil) containing sterilized flooded soil in 20 mL glass vials with or without C-1 to C-5, BES, or CMS (2 μmol/kg dry soil). These vials were sealed with sterilized silicone septa and incubated at 30°C for 7, 14, 21, and 28 days. The generated methane was analyzed by GC, and the DCP and its dechlorinated MCP were analyzed by HPLC. The test was conducted in duplicate and performed three times for a total of six separate experiments.

7. GC conditions

Methane concentrations in the sample bottles were measured by GC (HP5890 Series II, Hewlett Packard, USA) equipped with a HayeSep DB column (30 ft×1/8 in., 100/120 mesh; Agilent Technologies, USA) and a flame ionization detector. GC was programmed as follows: injector temperature, 150°C; column temperature, 100°C; detector temperature, 180°C; helium carrier flow rate, 25 mL/min; injection volume, 500 μL; and retention time, 4.3 min. The calibration curves for methane were prepared by serially diluting 99.9% pure methane by air and measuring by GC. The plotting of the methane amount (abscissa) *versus* observed peak areas (ordinate) was followed by linear regression to calculate the slope of this regression line.

8. HPLC conditions

MCP, DCP, and TCP concentrations were measured by HPLC (Nexera-i, Shimadzu, Japan). Analyte separation was conducted on a Kinetex C18 column (2.6 μm, 100×4.6 mm, Phenomenex, USA) maintained at 40°C. UV absorbance at 285 nm was monitored. Solvents A (0.1% acetic acid in distilled water) and B (0.1% acetic acid in acetonitrile) were used as the mobile phase. The flow rate was 0.4 mL/min in a binary gradient mode with the following elution program: 0 min, 70:30 (A:B ratio (v/v)); 1 min, 70:30; 11 min, 20:80; 15 min, 20:80; 15.1 min, 70:30; 20 min, 70:30. The retention times were as follows: MCP, 7.3 min; DCP, 9.0 min; and TCP, 10.6 min. The calibration curves for the analytes were prepared by serially diluting each compound and analyzing by HPLC. The plotting of the chemical amount (abscissa) *versus* observed peak areas (ordinate) was followed by linear regression to calculate the slope of this regression line.

9. PCR–DGGE analysis

DNA was extracted from sediment samples (0.25 g) using the DNeasy PowerSoil Kit (QIAGEN, Netherlands) in accordance with the manufacturer's instructions. The purity and quantity of the DNA preparation were measured using the NanoDrop UV absorption spectrum (Thermo Fisher Scientific Inc., USA). The experimental procedures of 16S rRNA gene-based PCR-DGGE analysis were followed as described by Hosoda *et al.*[20] Bacterial PCR primers 341F-GC and 534R amplified the variable V3 region of the bacterial 16S rRNA genes (corresponding to positions 341–534 in the *Escherichia coli* 16S rRNA gene sequence) connected to a GC-clamp.[21] Archaeal PCR primers 787F-GC and 1059R amplified a 273 bp from microorganisms of the Archaea domain connected to a GC-clamp.[22] PCR was performed using Premix Taq polymerase (TaKaRa version 2.0, TaKaRa, Japan) in accordance with the manufacturer's instructions (100 ng template DNA and 25 pmol of each primer in a 50 μL reaction volume). The reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at variable temperatures for 30 sec, and extension at 72°C for 1 min. In the first cycle, the annealing temperature was set to 60°C, and for each of the 10 subsequent cycles, the annealing temperature was reduced by 1°C. This was followed by 20 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. DGGE analysis was performed with a DCode™ instrument (Bio-Rad, USA) in accordance with the manufacturer's instructions. Gels were made using a gradient of denaturants between 30% (containing 2.1 M urea and 12.0% (v/v) formamide) and 60% (containing 4.2 M urea and 24.0% (v/v) formamide). Next, 500 ng of each PCR amplicon was subjected to electrophoresis on a 10% (w/v) polyacrylamide gel at 160 V for 5 hr at a running temperature of 60°C in 1×TAE electrophoresis buffer. After electrophoresis, the gel was stained using Gel-Red (FUJIFILM Wako Pure Chemical, Japan) for 30 min in accordance with the manufacturer's instructions. The DGGE profile of sediments was representative of triplicate samples because there were no significant differences in the results, as shown in Fig. 2, and resulting replicates.

A gel slice containing a DGGE fragment was excised and transferred into 100 μL of sterilized water. DNA bands were eluted at 4°C for 12 hr. One microliter of DNA solution was subjected to a second PCR. PCR amplification conditions were as

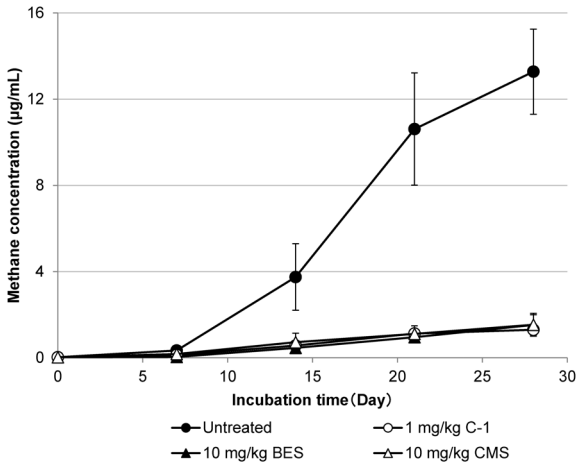


Fig. 1. Effect of C-1, BES, or CMS treatment on methane production in flooded soil as compared with untreated soil samples.

follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and final extension at 72°C for 10 min. The sequencing reaction was conducted using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and a Genetic Analyzer 3130 (Applied Biosystems, USA) in accordance with the manufacturer's instructions.

The BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program was used for homology analysis. The sequences reported in this study have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under GenBank/EMBL/DDBJ accession numbers LC663225 to LC663250 (DGGE bands from enrichment culture).

Results

1. *Methanogenic inhibition tests and analysis of the microbial community structure*

To clarify the inhibitory activity of methanogenesis by C-1, BES, and CMS in flooded soils, a methanogenic inhibition test was conducted. Since CMS is a partial structure of C-1 and is a structural analog of BES, it may contribute to the inhibition of methanogenesis. The effects of C-1, BES, and CMS on methanogenesis are shown in Fig. 1. In samples not treated with C-1, BES, or CMS, notable production of methane was confirmed after 14 days of incubation, reaching a maximum concentration of 13.3 μg/mL after 28 days of incubation. In samples treated with C-1 (1 mg/kg dry soil), BES (10 mg/kg dry soil), or CMS (10 mg/kg dry soil), methane production was suppressed to about 10% of that in the untreated samples, even after 28 days of incubation, and was detected at concentrations of 1.3, 1.5, and 1.5 μg/mL, respectively.

To characterize the microorganisms associated with methanogenesis, changes in the microbial community structure during the methanogenic inhibition tests were investigated. DNA was extracted, and a DGGE analysis was conducted to elucidate the bacterial and archaeal communities. No significant differences in the bacterial communities were found between the samples with and without C-1 or BES (Fig. S1), but a significant change was observed in the archaeal community (Fig. 2).

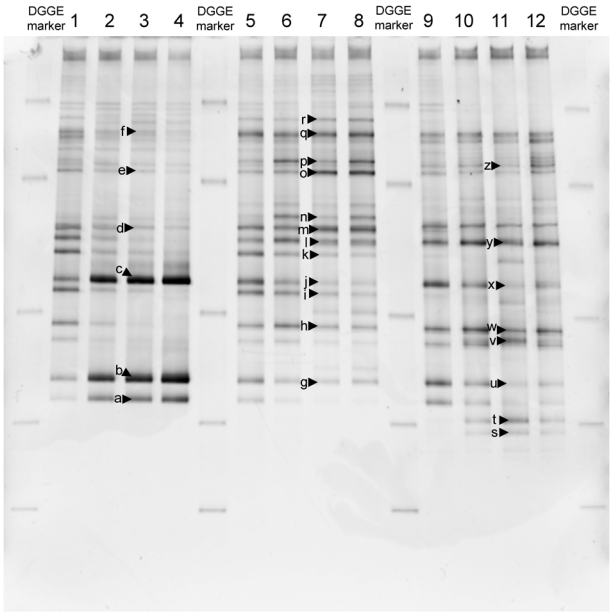


Fig. 2. The 16S rRNA gene-based PCR-denaturing gradient gel electrophoresis (DGGE) profiles of archaeal populations in flooded soil. Archaeal populations from untreated samples for 7, 14, 21, and 28 days (Lanes 1–4, respectively), C-1 treatment for 7, 14, 21, and 28 days (Lanes 5–8, respectively), and BES treatment for 7, 14, 21, and 28 days (Lanes 9–12, respectively). The small letters show the bands excised for DNA sequencing analysis.

According to the sequence analysis of the archaeal DGGE bands (Table 1), in the untreated samples, the sequences of major bands (bands a, b, and c) were highly homologous to those of *Methanosarcina mazei*. The sequences of minor bands (bands e and f) were highly homologous to those of *Methanoregula boonei*. In the C-1-treated samples, the sequence of band g, with decreased density, was highly homologous to that of *M.*

Table 1. BLAST analysis of 16S rRNA gene sequences of archaeal DGGE bands excised from the gel, as shown in Fig. 2

Band No.	Highest similarity	NCBI Accession No.	Identity (%)
a, b, g, u	*Methanosarcina mazei*	KP231494	235/235 (100%)
c	*Methanosarcina mazei*	KP231494	231/232 (99%)
d	*Methanosarcina mazei*	MK734107	230/235 (98%)
e	*Methanoregula boonei*	EU887826	220/235 (94%)
f	*Methanoregula boonei*	EU887826	227/236 (96%)
h, l, w, y, z	*Thermofilum carboxyditrophus*	KX355878	230/235 (98%)
i, j, n	*Thermofilum carboxyditrophus*	KX355878	221/235 (94%)
k	*Methanomassiliicoccus luminyensis*	KX355873	224/237 (95%)
m, o	*Methanoregula boonei*	EU887826	232/235 (99%)
p	*Thermofilum carboxyditrophus*	KX355878	223/235 (95%)
q	*Methanomassiliicoccus luminyensis*	KX355868	210/219 (96%)
r	*Methanomassiliicoccus luminyensis*	KX355873	217/237 (92%)
s, t, v	*Methanobacterium bryantii*	MK680235	233/235 (99%)
x	*Methanobacterium bryantii*	MK680235	229/235 (97%)

Table 2. Effect of methanogenic substrates on methane production in flooded soil

Compounds	Concentration of methane (μg/mL)			
	Day 7	Day 14	Day 21	Day 28
Untreated	0.4	5.6	17.7	28.4
Acetate	NA	33.6	137.2	135.6
Methanol	NA	112.1	112.9	105.7
C-1	0.2	0.7	0.9	1.3
C-1+Acetate	NA	0.4	0.7	1.1
C-1+Methanol	NA	9.5	100.4	93.5
BES	0.0	0.9	2.1	2.8
BES+Acetate	NA	0.2	1.4	6.7
BES+Methanol	NA	0.8	1.9	3.4

NA: Not applicable

mazei, whereas the sequences of DGGE bands with increased densities as compared with those in the untreated sample were highly homologous to those of *M. boonei* (bands m and o), *Methanomassiliicoccus luminyensis* (bands q and r), and *Thermofilum carboxyditrophus* (bands h, l, n, and p). In the BES-treated samples, the sequence of band u, with decreased density, was highly homologous to that of *M. mazei*, whereas the sequences of the DGGE bands with increased densities as compared with those in the untreated sample, were highly homologous to those of *Methanobacterium bryantii* (bands s, t, and v) and *T. carboxyditrophus* (bands w and y). These results revealed that C-1 and BES strongly inhibited the growth of *Methanosarcina* spp.

2. Characterization of the methanogenic inhibition mechanism using a methanogenic substrate

The microbial community structural analysis showed that both C-1 and BES treatments suppressed the growth of *Methanosarcina* spp., with varying effects on the growth of other methanogens. Generally, acetate is used as a substrate for methane production. Dridi *et al.* reported that *M. luminyensis* strain B10^T used hydrogen as an electron donor to reduce methanol to methane.[23] To characterize the mechanism of methanogenic inhibition by C-1, tests were conducted using acetate and methanol as methanogenic substrates. Assuming that one molecule of methanol and one molecule of acetate produce one molecule of methane each, the maximum concentration of methane derived from the substrate is 121 μg/mL. In untreated samples, the methane concentrations were 17.7 and 28.4 μg/mL after 21 and 28 days of incubation, respectively (Table 2). The addition of acetate and methanol increased the methane concentration to 137.2 μg/mL after 21 days of incubation and to 112.1 μg/mL after 14 days of incubation, and most of the treated acetate and methanol were used for methanogenesis. In C-1-treated samples, aceticlastic methanogenesis was inhibited, and the methane concentration was 1.3 μg/mL after 28 days of incubation. However, methylotrophic methanogenesis was delayed but not inhibited, and the methane concentration reached 100.4 μg/mL after 21 days of incubation. In BES-treated samples, both aceticlastic and methylotrophic methanogenesis were significantly inhibited, with concentrations of 6.7 and 3.4 μg/mL after 28 days of incubation, respectively. CMS treatment (40 mg/kg dry soil) also inhibited aceticlastic and methylotrophic methanogenesis as in the BES treatment (Table S1). These results revealed that the mechanism of methanogenic inhibition by C-1 was different from that by BES or CMS.

3. TCP dechlorination tests

Our results have shown that C-1 inhibits methane production by suppressing the growth of *Methanosarcina* spp. To understand the characteristics of C-1, the inhibition activity against dechlorination was investigated. In this study, TCP, commonly used as fungicide,[24] was used as the test substance.

In the untreated samples, TCP was rapidly dechlorinated to DCP (Fig. 3A). It reached a maximum concentration of 36.8 μmol/kg dry soil after 7 days of incubation and gradually decreased to 0.4 μmol/kg dry soil after 28 days of incubation. MCP, a dechlorinated compound of DCP, reached a maximum concentration of 36.4 μmol/kg dry soil after 14 days of incubation as the concentration of DCP decreased, gradually decreasing to 17.8 μmol/kg dry soil after 28 days of incubation. In C-1-treated flooded soil, the dechlorination of TCP to DCP was delayed as compared to untreated samples, and the DCP concentration reached 43.6 μmol/kg dry soil after 14 days of incubation. The dechlorination of DCP to MCP was strongly inhibited, and the MCP concentration was 3.2 μmol/kg dry soil after 28 days of incubation (Fig. 3B). In the BES-treated flooded soil, the results of TCP dechlorination tests were similar to those of untreated samples, and BES treatment had almost no effect on TCP dechlorination (Fig. 3C).

The methane concentrations in these TCP dechlorination tests were 12.5, 0.6, and 1.0 μg/mL in the untreated, C-1-treated, and BES-treated samples after 28 days of incubation, respectively. These results are similar to those shown in Fig. 1, and TCP treatment had almost no effect on methanogenesis.

4. Inhibition activities of DCP dechlorination and methanogenesis by C-1 and its analogs

Since C-1 showed both DCP dechlorination inhibition and methanogenic inhibition activities, DCP dechlorination tests using C-1 and its analogs were conducted to investigate the relationship between these activities. The results of the DCP dechlorination tests in which the treatment concentration of C-1 analogs was unified to 2 μmol/kg dry soil showed that treatment with C-1 (0.45 mg/kg dry soil), C-2 (0.41 mg/kg dry soil), and C-4 (0.54 mg/kg dry soil) almost completely inhibited DCP dechlorination (Table 3). Treatment with C-3 (0.38 mg/kg dry soil) and C-5 (0.50 mg/kg dry soil) in flooded soils did not inhibit DCP dechlorination, and MCP reached a maximum concentration of 101.3 and 106.3 μmol/kg dry soil after 21 days of incubation, respectively, which was comparable to that of untreated samples (93.0 μmol/kg dry soil).

In the C-1-treated samples, 2.0 μg/mL of methane was de-

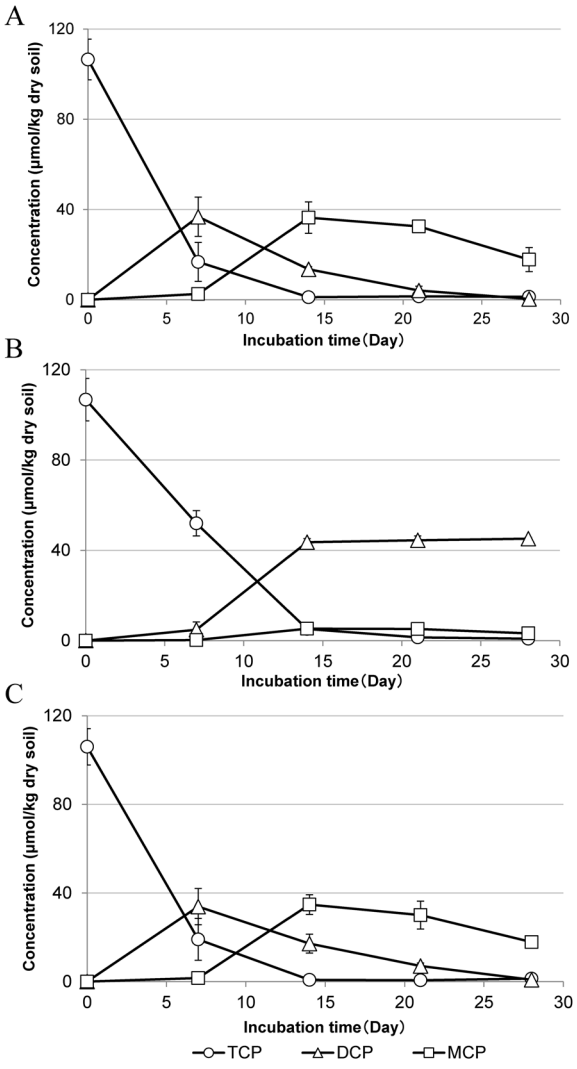


Fig. 3. Dechlorination of TCP in flooded soil for 28 days. (A) Untreated; (B) 1 mg/kg dry soil of C-1 treatment; (C) 40 mg/kg dry soil of BES treatment.

tected after 28 days of incubation, and methanogenesis was significantly inhibited throughout the test period as compared with the untreated samples (27.0 μg/mL). In the C-2- and C-4-treated samples, methane concentrations reached 18.3 and 9.1 μg/mL after 28 days of incubation, respectively, and methanogenesis was inhibited but inferior to that in the C-1-treated samples. In the C-3- and C-5-treated samples, methane concentrations reached 39.0 and 23.2 μg/mL after 28 days of incubation, respectively.

On the other hand, in the 2 μmol/kg dry soil treatment of BES (0.42 mg/kg dry soil) or CMS (0.31 mg/kg dry soil) that did not inhibit DCP dechlorination, the methane concentrations reached 1.3 and 37.8 μg/mL after 28 days of incubation, respectively. BES cannot freely diffuse through the cytoplasmic membrane of methanogens due to the negatively charged sulfonate group and is generally a poor inhibitor of methanogenesis *in vivo*.[24] More than 1 mM of BES is required to inhibit growth and methanogenesis.[11] However, in this study, 2 μmol/kg dry soil treatment of BES showed methanogenic inhibition activity that was comparable to that exhibited by the same amount of C-1. Although, at a high concentration of 10 mg/kg dry soil, CMS inhibited methanogenesis (Fig. 1), the methanogenic inhibition activity of CMS is clearly lower than that of equivalent amounts of C-1 and BES.

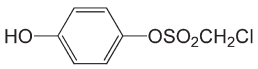
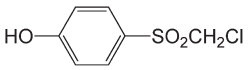
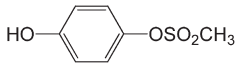
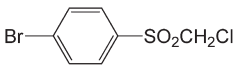
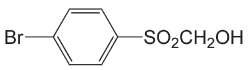
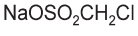
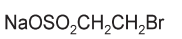
Discussion

In this study, we focused on the mechanism of methanogenic inhibition by C-1, which has a structure different from the existing inhibitors, and we investigated the microorganisms associated with methanogenesis and their characteristics through changes in the microbial communities and the consumption of methanogenic substrates.

C-1 treatment did not significantly affect the microbial community structure of bacteria, but it did affect the archaeal community structure, especially that of methanogens (Fig. 2, Table 1). Although *Methanosarcina* spp. were the dominant species in the untreated sample, C-1 or BES treatment strongly inhibited their growth. With the decrease of *Methanosarcina* spp., *Methanomassiliicoccus* spp. and *Methanoregula* spp. became the main species with C-1 treatment, and *Methanobacterium* spp. became the main species with BES treatment. *Methanosarcina* spp. utilizes acetate, methanol, methylamines, and H_2/CO_2 as substrates for growth and the production of methane.[25,26] *Methanoregula* spp. and *Methanobacterium* spp. utilize H_2/CO_2 to produce methane by hydrogenotrophic methanogenesis, but they do not utilize acetate, methanol, or trimethylamine.[25–27] Methylotrophic methanogenesis is the conversion of a methyl compound (*e.g.*, methanol) to methane and CO_2 as follows: $4CH_3OH \rightarrow CO_2+2H_2O+3CH_4$. However, *M. luminyensis* strain B10^T used hydrogen as an electron donor and reduced methanol to methane as follows: $CH_3OH+H_2 \rightarrow CH_4+H_2O$.[23,28] *T. carboxyditrophus*, a thermophilic CO-oxidizing hydrogenogenic prokaryote, has been detected in both BES and C-1 treatment groups and may supply H_2 to methanogens as follows: $CO+H_2O \rightarrow CO_2+H_2$.[29]

In this study, methanogenic inhibition tests were conducted by adding methanogenic substrates with a focus on aceticlastic methanogenesis and methylotrophic methanogenesis. C-1 treatment inhibited aceticlastic methanogenesis, whereas BES treatment inhibited both aceticlastic and methylotrophic methanogenesis (Table 2). BES, a structural analog of 2-mercaptoethanesulfonate (coenzyme M), competitively inhibits the binding of methyl groups to coenzyme M, a common step in all methanogenic pathways, and inhibits the methanogenic activity of methanogens (*i.e.*, MCR inhibitor).[5] However, C-1 does not inhibit methylotrophic methanogenesis but inhibits aceticlastic methanogenesis, suggesting that it may inhibit enzymes differently from MCR in the methanogenesis pathway (*e.g.*, tetrahydromethanopterin *S*-methyltransferase[30] and acetyl-CoA decarbonylase/synthase[31]). Early studies of anoxic paddy soils have shown anaerobic microbial methane production operating at a ratio of about 67% aceticlastic and 33% hydrogenotrophic meth-

Table 3. Effect of 2 μmol/kg dry soil of C-1 and its analogs on the dechlorination of DCP (123 μmol/kg dry soil) and methanogenesis in flooded soil

Compounds	Structure	Analytes	Concentration (MCP, DCP: μmol/kg dry soil, Methane: μg/mL)				
			Day 0	Day 7	Day 14	Day 21	Day 28
Untreated	—	DCP	123.3	105.8	10.7	3.1	0.6
		MCP	0.0	4.3	91.2	93.0	73.0
		Methane	0.0	0.4	2.9	8.3	27.0
C-1		DCP	121.8	116.8	114.6	115.3	114.2
		MCP	0.2	0.0	0.2	0.4	0.3
		Methane	0.0	0.2	0.7	1.3	2.0
C-2		DCP	126.2	115.4	114.3	115.4	111.4
		MCP	0.2	0.0	0.3	1.7	2.4
		Methane	0.0	0.4	2.1	5.2	18.3
C-3		DCP	126.5	108.3	12.0	3.1	0.9
		MCP	0.3	6.8	99.4	101.3	85.0
		Methane	0.0	0.5	2.5	9.9	39.0
C-4		DCP	120.5	113.1	114.0	114.1	110.4
		MCP	0.4	0.0	0.2	0.5	0.3
		Methane	0.0	0.3	1.1	2.8	9.1
C-5		DCP	126.6	111.9	9.4	3.1	0.6
		MCP	0.3	6.7	104.3	106.3	73.1
		Methane	0.0	0.5	2.8	9.1	23.2
CMS		DCP	122.4	105.6	11.3	2.0	0.6
		MCP	0.2	7.7	98.7	97.4	74.9
		Methane	0.0	0.5	3.2	13.7	37.8
BES		DCP	122.6	106.1	11.2	2.9	0.5
		MCP	0.2	9.5	98.8	96.5	83.1
		Methane	0.0	0.1	0.4	1.0	1.3

anogenesis.[28,32] If tetrahydromethanopterin S-methyltransferase was an inhibitory target, both aceticlastic and hydrogenotrophic methanogenesis might be inhibited. Further research is needed to elucidate the inhibition mechanism.

The inhibitory effect of chlorophenols on methanogenesis has been widely studied. The half maximal effective concentration (EC_{50}) values strongly differed in many studies, with a range of 26–550, 80–300 and 41–117 mg/L for MCP, DCP, and TCP, respectively.[33] In this study, since the maximum concentrations of DCP and TCP were 10 mg/L in the dechlorination tests, it was unnecessary to consider the effects of chlorophenols on methanogenesis.

In studies on dechlorination by methanogens, *Methanosarcina* sp. DCM[34] and *M. mazei* S6[15] have been reported to reductively dechlorinate tetrachloroethene to trichloroethene. Several reports have stated that BES inhibits the dechlorination of chloroethene in methanogenic cultures, concluding that methanogens are directly or indirectly involved in the observed dechlorination reactions.[15,16,19] In contrast, BES acts on microorganisms in the absence of methanogens to inhibit dechlorination.[17,18] In this study, BES did not affect the dechlorination of TCP and DCP (Fig. 3), so it was considered that methanogens (at least *Methanosarcina* spp.) were not involved in the dechlorination of chlorophenols.

Dehalobacter sp. TCP1,[24] *Desulfitobacterium dehalogenans* JW/IU-DC1^T,[35] and *Dehalococcoides* sp. CBDB1[36] are known dechlorinating bacteria for chlorophenols, and TCP1 is capable of dechlorinating TCP to MCP with H_2 as the sole electron donor and acetate as the carbon source. Our study did not reveal whether these bacteria are involved in dechlorination. Therefore, further studies are essential for clarifying this relationship. C-1 is a unique compound with both aceticlastic methanogenesis and DCP dechlorination inhibition activities and is a useful compound for studying the mechanisms of methanogenesis and dechlorination.

Inhibition tests with C-1 and its analogs were conducted to understand the relationship between the structure of C-1 and the inhibition activities of methanogenesis and dechlorination (Table 3). C-1, C-2, and C-4 strongly inhibited the activity of dechlorination, but C-3 and C-5 did not. The common substructure of the previous three compounds is $-SO_2CH_2Cl$. However, CMS, which has the same substructure but not an aromatic ring structure, showed no inhibition activity of dechlorination. Therefore, it was found that $-SO_2CH_2Cl$ or $-OSO_2CH_2Cl$ with an aromatic ring was essential for the expression of the inhibition activity of dechlorination. On the other hand, C-2 and

C-4 inhibited methanogenesis but were significantly inferior to C-1, and C-3 and C-5 did not. There was a certain correlation between the two inhibitory activities, but they may be independent. Taken together, these results revealed that the phenyl chloromethanesulfonate moiety contributed to both methanogenic and dechlorination inhibition activities.

In conclusion, we have studied methanogenic inhibitors and discovered that (4-hydroxyphenyl) chloromethanesulfonate (C-1) suppresses methane production in flooded soil. Although the mechanism of methanogenic inhibition by C-1 has not yet been fully elucidated, C-1 strongly inhibits aceticlastic methanogenesis. Field trials are required as the next development step; however, C-1, which can suppress methane production at a low concentration, has potential as a compound for reducing methane emissions from paddy fields.

Electronic supplementary materials

The online version of this article contains supplementary materials (Supplemental Fig. S1 and Supplemental Table S1), which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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