Geochemical Journal, Vol. 46, pp. 193 to 200, 2012

Hydrogen and carbon isotope fractionation by thermophilic hydrogenotrophic methanogens from a deep aquifer under coculture with fermenters

SHOHEI HATTORI,[1]* HIROAKI NASHIMOTO,[2] HIROYUKI KIMURA,[2] KEISUKE KOBA,[3] KEITA YAMADA,[4] MIKIO SHIMIZU,[2] HIROSHI WATANABE,[3] MUNEOKI YOH[3] and NAOHIRO YOSHIDA[1,4]

[1]Department of Environmental Science and Technology, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Yokohama 226-8502, Japan
[2]Department of Geosciences, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan
[3]Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
[4]Department of Environmental Chemistry and Engineering, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Yokohama 226-8502, Japan

(*Received December 7, 2011; Accepted February 9, 2012*)

To elucidate the isotope geochemistry of CH_4 production in deep subsurface environments, we investigated the relation between H_2 concentration and hydrogen and carbon isotope fractionation by CO_2 reduction using microbial communities obtained from groundwater in a deep aquifer associated with an accretionary prism. Incubation experiments were conducted under anaerobic culture conditions of two types. In one experiment, a coculture of H_2-producing fermenters and hydrogenotrophic methanogens was established in groundwater treated with organic substrates. The other experiment used groundwater under $H_2 + CO_2$ (80:20, v/v) to produce CH_4 under high H_2 concentrations. In the cocultures, H_2 concentrations increased in the initial phases, then decreased gradually and remained low during CH_4 production, indicating H_2 consumption by hydrogenotrophic methanogens to produce CH_4. This study revealed for the first time that cocultures with fermenters and hydrogenotrophic methanogens producing CH_4 in low H_2 concentration cause smaller hydrogen isotope fractionations ($0.663 < \alpha_H < 0.725$) than in monocultures under high H_2 concentrations ($0.629 < \alpha_H < 0.656$). Carbon isotope fractionation in cocultures was greater ($1.052 < \alpha(CO_2–CH_4) < 1.074$) than in monocultures under high H_2 concentrations ($1.021 < \alpha(CO_2–CH_4) < 1.023$). The large carbon fractionation was thought to result from low levels of H_2, supporting the hypothesis of differential reversibility of multiple enzymatic processes in CH_4 production. Although lack of agreement remains between results of incubation experiments and field observations especially in hydrogen isotope fractionations, both hydrogen and carbon isotope fractionation in cocultures were close to the fractionations of field observation in which CO_2 reduction is a dominant pathway in CH_4 production compared with those in monoculture.

Keywords: methane, isotope fractionation, hydrogenotrophic methanogen, syntrophic cooperation, hydrogen concentration

INTRODUCTION

Stable hydrogen and carbon isotope ratios in microbial CH_4 vary widely, with $\delta D–CH_4$ values from –400‰ to –150‰, and $\delta^{13}C–CH_4$ values from –110‰ to –50‰ (Whiticar, 1999). Isotope fractionation factors should be considered when explaining such high variation in the δD and $\delta^{13}C$ values of CH_4. However, reported hydrogen and carbon isotope fractionation factors in CO_2 reduction, which is as important a pathway as acetate fermentation because of its dominance in natural environments, also vary widely. Fractionation factors have been investigated using different approaches such as pure culture experiments using isolated microbes (e.g., Balabane *et al*., 1987; Botz *et al*., 1996; Valentine *et al*., 2004) and incubation experiments using environmental samples (e.g., Sugimoto and Wada, 1995; Chidthaisong *et al*., 2002; Fey *et al*., 2004). The basic mechanisms of hydrogen and carbon isotope fractionations in CO_2 reduction are not yet fully understood.

Less information related to hydrogen isotope fractionation in CO_2 reduction is available than for carbon isotope fractionation. Hydrogen isotope fractionation achieved from a laboratory incubation study of CO_2 reduction with pure cultures (>300‰; Balabane *et al*., 1987) was greater than those observed in natural environments such as deep-sea sediments in which CO_2 reduction is the dominant pathway in CH_4 production (approximately 160‰; Whiticar *et al*., 1986). Incubation experiments have never indicated hydrogen isotope fractionation factors equivalent to those observed in natural environments.

*Corresponding author (e-mail: hattori.s.ab@m.titech.ac.jp)

Although this discrepancy remains unexplained, it has been suggested that it results from the use in experiments of H_2 concentrations that are much higher than those occurring in the natural environment: hydrogen isotope fractionation between CH_4 and H_2O increases continuously with increasing H_2 concentration (Burke, 1993; Sugimoto and Wada, 1995). This interpretation has been supported by field observations in natural wetlands (Sugimoto and Fujita, 2006). However, incubation experiments using *Methanothermobacter thermoautotrophicus* with fermentative bacteria revealed that culture conducted with low H_2 concentrations produced larger hydrogen isotope fractionations than those obtained from pure cultures conducted with higher H_2 concentrations (Yoshioka *et al*., 2008). The effect of H_2 concentration on hydrogen isotope fractionation must therefore be clarified to elucidate δD–CH_4 values.

Carbon isotope fractionation factors of approximately 1.02–1.08 were obtained in various laboratory studies of CO_2 reduction (Conrad, 2005). Valentine *et al*. (2004) reported that carbon isotope fractionation in CO_2 reduction is related to H_2 supply. They proposed a differential reversibility hypothesis dependent on the Gibbs free energy (ΔG). Although this relation between carbon isotope fractionation and H_2 supply has been confirmed in laboratory coculture experiments under various conditions (Penning *et al*., 2005), it has not been tested under thermophilic conditions using natural microbial communities.

As described above, the variations of hydrogen and carbon isotope fractionations in CO_2 reduction are attributable to the high H_2 concentration used in most laboratory experiments. Experiments using low H_2 concentration must be conducted to obtain better estimates of hydrogen and carbon isotopic fractionation factors in CO_2 reduction for use in the interpretation of δD and $\delta^{13}C$ values of CH_4.

Our previous study showed that past and ongoing microbial CH_4 production contribute to the CH_4 reservoirs in a deep aquifer associated with an accretionary prism in southwestern Japan (Kimura *et al*., 2010). We also incubated a microbial community in groundwater obtained from the deep aquifer by coculture of H_2-producing fermenters and hydrogenotrophic methanogens, and by monoculture of hydrogenotrophic methanogens in thermophilic conditions (Kimura *et al*., 2010). This system enables us to investigate hydrogen and carbon isotope fractionation in CO_2 reduction using culture of two types and to compare the fractionation factors under low H_2 concentrations in cocultures relative to those under high H_2 concentrations in monocultures. In this study, we conducted concentration and isotopic measurements in this incubation system to determine the relations between hydrogen and carbon isotopic fractionation factors and H_2 concentration.

Table 1. Culture conditions of each batch undertaken in this study

Batch	Substrate	Headspace gas	Temperature (°C)	Predicted culture
A	YPG[a]	N_2[b]	55	Coculture[e]
B	YPG	N_2	65	Coculture
C	YPG	$N_2 + CO_2$[c]	55	Coculture
D	YPG	$N_2 + CO_2$	65	Coculture
E	—	$H_2 + CO_2$[d]	55	Monoculture[f]
F	—	$H_2 + CO_2$	65	Monoculture

[a]*YPG medium (yeast extract, peptone, and glucose).*
[b]*0.25 MPa.*
[c]*N_2 : CO_2 = 80:20 (0.25 MPa).*
[d]*H_2 : CO_2 = 80:20 (0.25 MPa).*
[e]*CH_4 production by syntrophic cooperation of fermenters and hydrogenotrophic methanogens.*
[f]*CH_4 production by hydrogenotrophic methanogens.*

METHODS

Study site and groundwater sampling

In September 2008, groundwater samples were collected from a deep well (the Ita-Wari well; 34°52.283′ N, 138°09.150′ E) in Shimada, Shizuoka Prefecture, Japan (Kimura *et al*., 2010). The well is located geologically in the Paleogene Setogawa group of the Shimanto Belt, which is a typical and well-studied ancient accretionary prism (Taira *et al*., 1992; Tanabe and Kano, 1996). The Shimanto Belt, which is distributed along southwestern Japan parallel to the Nankai Trough, is composed mainly of non-metamorphosed to weakly metamorphosed thick sequences of sandstone, mudstone, alternating beds of sandstone and mudstone, and locally associated chert and greenstone. The Ita-Wari well is drilled to about 1500 m below the Earth's surface and is reinforced with tight steel casing pipes. The casing pipes are equipped with a strainer at 1188–1489 m below the Earth's surface. Groundwater flows from the deep aquifer through the strainer into the well and rises naturally to about 250 m below the surface; it is then drawn up anaerobically to the ground surface using a pumping system. To prevent contamination by air and water from shallow environments, groundwater was pumped at a flow rate of 116 L min^{-1} for 24 h before sampling. To avoid atmospheric contamination, exactly 120 ml of the groundwater at the bottom of a bucket, with continuous overflowing of groundwater from the well, was injected anaerobically into autoclaved 240-ml serum bottles that had been sealed tightly with sterile butyl rubber stoppers and evacuated. The groundwater samples were stored at 4°C before incubation.

Culture conditions

Cocultures of fermenters and hydrogenotrophic

methanogens were grown by anaerobic cultivation of the deep-aquifer groundwater, and by treatment with organic substrates using a modification method of Kimura *et al*. (2010). Groundwater samples for cocultures were treated with 12 ml of YPG medium (3.0 g of yeast extract, 3.0 g of peptone, and 0.6 g of glucose per 100 ml of distilled water). The gas phase was exchanged to N_2 (0.25 MPa) for batches A and B, and to N_2 + CO_2 (80:20, v/v; 0.25 MPa) for batches C and D.

For batches E and F, the groundwater was not treated with organic substrates and the gas phase was exchanged to H_2 + CO_2 (80:20, v/v; 0.25 MPa); these batches, in which only hydrogenotrophic methanogens were expected to grow, were used as monocultures for comparison with the cocultures.

Incubation temperatures were 55°C for batches A, C, and E, and 65°C for batches B, D, and F because CH_4 production has been observed at these temperatures according to our previous study (Kimura *et al*., 2010). The batch culture conditions are presented in Table 1.

Measurement of H_2, CH_4, and CO_2 concentrations

The H_2, CH_4, and CO_2 concentrations of the gas phase in the headspace were analyzed using a gas chromatograph (GC; GC-2014; Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a packed column (Shin-carbon ST, 6.0 m × 3.0 mm i.d.; Shinwa Chemical Industries Ltd., Kyoto, Japan). Ar was used as the carrier gas.

Analyses of isotope ratios

Headspace gases in the incubated cultures were collected every 2–3 days using a gas-tight syringe. They were then transferred to serum bottles filled with ultrapure He and stored for later isotopic analyses.

Instrumentation for measurement of the hydrogen isotope ratios of CH_4 consisted of an on-line CH_4 extraction system, a GC pyrolysis furnace, and an isotope ratio mass spectrometer (DeltaplusXL; Thermo Fisher Scientific Inc., Bremen, Germany). The GC pyrolysis interface consisted of a GC (6890; Hewlett Packard Co., Palo Alto, CA, USA) equipped with a split/splitless injector and a capillary column (HP-PLOT Q, 30 m × 0.32 mm i.d., 20 μm film; Hewlett Packard Co.), a pyrolysis furnace (Al_2O_3 ceramic tube, 320 mm × 0.5 mm i.d.), a Nafion dryer, and an open split. A similar system was used to measure the carbon isotope ratios of CH_4, but the pyrolysis furnace and the DeltaplusXL mass spectrometer were replaced, respectively, with a combustion furnace (Al_2O_3 ceramic tube packed with CuO, NiO, and Pt wires, 320 mm × 0.5 mm i.d.) and an isotope ratio mass spectrometer (Finnigan MAT 252; Thermo Fisher Scientific Inc.). The operation principle is described in Yamada *et al*. (2003). Briefly, CH_4 was extracted using these systems and introduced on-line into the GC. The separated CH_4 was transferred either into a pyrolysis furnace (at 1440°C) or a combustion furnace (at 960°C) for conversion into H_2 or CO_2 to measure stable hydrogen or carbon isotope ratios.

The water in the culture samples, which was collected from the bottles before incubation, was transferred to 10 ml bottles. Hydrogen isotope ratios of the medium waters were measured using a conventional equilibrium method (Horita *et al*., 1989) with a Finnigan MAT252 mass spectrometer (Thermo Fisher Scientific Inc.).

The carbon isotope ratios of the CO_2 samples were analyzed using a GC-C-IRMS system consisting of a GC-combustion interface (ThermoQuest GC/TC interface; Thermo Fisher Scientific Inc.) and an isotope ratio mass spectrometer (DeltaplusXP; Thermo Fisher Scientific Inc.). The GC-combustion interface was a GC (6890 GC; Hewlett Packard Co.) equipped with a capillary column (PoraPLOT Q, 25 m length, 0.32 mm i.d., 10 μm thickness; Varian Inc., Palo Alto, CA, USA).

Typical uncertainties, quantified by replicate measurements of standards, were $\delta^{13}C$–CH_4 (0.3‰), δD–CH_4 (4‰), $\delta^{13}C$–CO_2 (0.3‰), and δD–H_2O (1‰).

Notation

Stable isotope ratios are expressed in conventional δ notation calculated using Eq. (1) as

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000(‰), \qquad (1)$$

where X denotes a heavy stable isotope such as D or ^{13}C, and R denotes the isotope ratio of samples and standards. The carbon isotope ratio values are given in per mil notation (‰) relative to the Vienna PeeDee Belemnite carbonate (VPDB) standard. The hydrogen isotope ratios are given in per mil notation (‰) relative to the Vienna Standard Mean Ocean Water (VSMOW) standard.

The carbon isotope fractionation factors ($\alpha(CO_2$–$CH_4)$ values) are

$$\alpha(CO_2\text{–}CH_4) = (\delta^{13}C\text{–}CO_2 + 1000)/(\delta^{13}C\text{–}CH_4 + 1000). \qquad (2)$$

The hydrogen isotope fractionation factors (α_H values) are

$$\alpha_H = (\delta D\text{–}CH_4 + 1000)/(\delta D\text{–}H_2O + 1000). \qquad (3)$$

RESULTS

CH_4 production by culture

The enriched cultures were grown in closed systems. Each culture produced CH_4 by CO_2 reduction and had established a steady state within 12 days (Fig. 1). Before

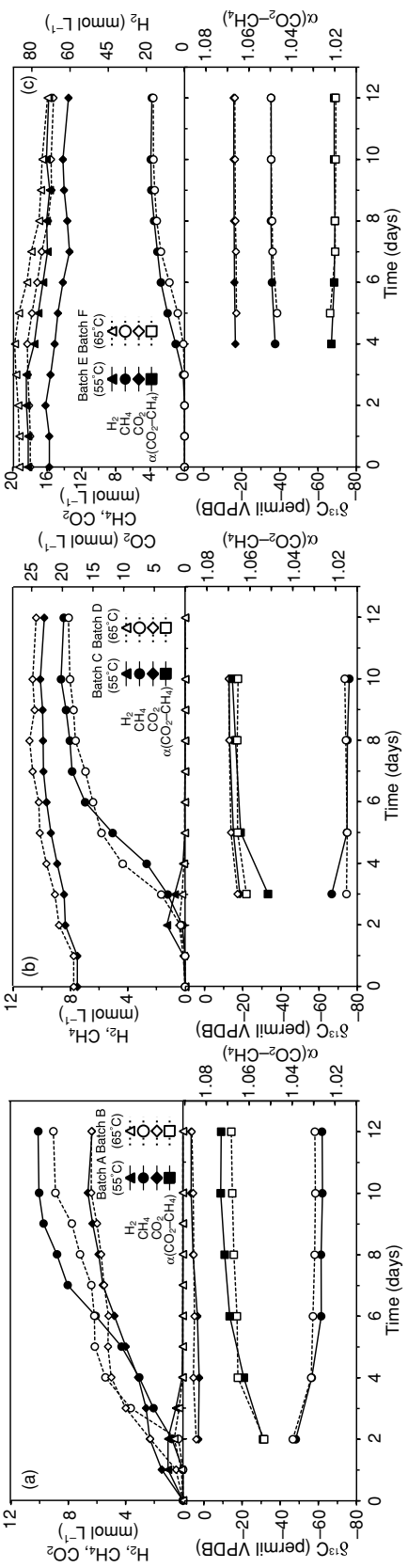

Fig. 1. Changes in concentrations and/or isotope ratios of H_2 (triangles), CH_4 (circles), and CO_2 (diamonds), and $\alpha(CO_2$–$CH_4)$ values (squares) in culture headspaces. (a) Groundwater from deep aquifer + YPG medium under N_2 (0.25 MPa; batch A, 55 °C, and batch B, 65 °C); (b) groundwater from deep aquifer + YPG medium under N_2 + CO_2 (80:20, v/v, 0.25 MPa; batch C, 55 °C, and batch D, 65 °C); (c) groundwater from deep aquifer under H_2 + CO_2 (80:20, v/v, 0.25 MPa; batch E, 55 °C, and batch F, 65 °C).

and after incubation, the pH values of cultures were measured using a pH meter (TPX-90; Toko Chemical Laboratories Co. Ltd., Japan) and were 6.3–6.7 at the final time of incubation. The enriched cultures from groundwater treated with organic substrates (batches A, B, C, and D) showed that CO_2 and H_2 production began first with subsequent CH_4 production starting on the second or third day (Figs. 1a and 1b). In batches A, B, C, and D, the H_2 concentrations increased to a maximum in the initial phase (0–4 days), then decreased gradually and remained under the detection limit of TCD-GC (0.01 mmol L^{-1} in the headspace) (Figs. 1a and 1b). In addition, the increases in CH_4 concentrations were slower or absent at 8–12 days (Figs. 1a and 1b). In cultures with groundwater under H_2 + CO_2 (batches E and F), the CH_4 concentrations increased gradually after 3 or 4 days and then slowed or showed no further increase at 8–12 days (Fig. 1c). These results are generally consistent with the incubation experiment results of our previous study (Kimura *et al*., 2010).

Fractionation of hydrogen isotopes

Changes in δD–CH_4 and α_H values determined from Eq. (1) or Eq. (3) are presented in Table 2. The δD–CH_4 values of all cultures were between –382‰ and –292‰. The average fractionations after steady state in these cultures were, respectively, 0.701, 0.709, 0.711, 0.723, 0.646, and 0.656 for batches A, B, C, D, E, and F (Table 2). Statistical analysis of α_H values using Student's *t*-test between A and B, C and D, and E and F showed no significant difference between these pairs ($p > 0.05$), indicating that hydrogen isotope fractionations did not depend on growth temperatures. In contrast, the α_H values of batches E and F were significantly different ($p < 0.01$) from those of batches A, B, C, and D. Consequently, hydrogen isotope fractionations under high H_2 concentrations (batches E and F) were approximately 10–90‰ larger (0.01–0.09 lower in terms of α_H values) than those in cocultures grown under low H_2 concentrations.

Fractionation of carbon isotopes

Changes in δ^{13}C–CH_4, δ^{13}C–CO_2, and $\alpha(CO_2$–$CH_4)$ values for batches A–D, as determined from Eq. (1) or Eq. (2), are presented in Fig. 1. Botz *et al*. (1996) reported that carbon isotope fractionation during the steady-state period is more representative for natural habitats than that during the early phases. We therefore considered the average carbon isotope fractionation factors in the steady state, i.e., after day 10 for batch A, day 10 for batch B, day 8 for batch C, and day 8 for batch D (Figs. 1a and 1b). The average carbon isotope fractionation factors were, respectively, 1.074 ± 0.000, 1.069 ± 0.000, 1.068 ± 0.001, and 1.067 ± 0.000 for batches A, B, C, and D.

In batches E and F, the carbon isotope fractionation was small ($\alpha(CO_2$–$CH_4)$ = 1.021–1.023), and CH_4 was

Table 2. Hydrogen isotope ratios of CH_4 and the fractionation factors (α_H) in the enrichment cultures

	δD–CH_4 (‰)	α_H[a]		δD–CH_4 (‰)	α_H[a]
Batch A[b] (55°C)			Batch B[b] (65°C)		
Day 2	–350	0.663	Day 2	–339	0.675
Day 4	–335	0.679	Day 4	–311	0.703
Day 6	–323	0.691	Day 6	–307	0.708
Day 8	–311	0.703	Day 8	–310	0.705
Day 10	–311	0.703	Day 10	–305	0.709
Day 12	–318	0.696	Day 12	–306	0.709
Average of all days ± 1σ		0.689 ± 0.016	Average of all days ± 1σ		0.701 ± 0.013
Average after day 8 ± 1σ		0.701 ± 0.003	Average after day 10 ± 1σ		0.709 ± 0.003
Batch C[c] (55°C)			Batch D[c] (65°C)		
Day 3	–338	0.676	Day 3	–308	0.707
Day 5	–322	0.692	Day 5	–295	0.72
Day 8	–315	0.699	Day 8	–290	0.725
Day 10	–292	0.723	Day 10	–294	0.721
Average of all days ± 1σ		0.698 ± 0.019	Average of all days ± 1σ		0.718 ± 0.008
Average after day 8 ± 1σ		0.711 ± 0.017	Average after day 8 ± 1σ		0.723 ± 0.003
Batch E[d] (55°C)			Batch F[d] (65°C)		
Day 4	–382	0.629	Day 4	–369	0.643
Day 6	–378	0.634	Day 7	–370	0.642
Day 8	–372	0.639	Day 8	–355	0.657
Day 10	–364	0.648	Day 10	–356	0.656
Day 12	–361	0.651	Day 12	–356	0.656
Average of all days ± 1σ		0.640 ± 0.009	Average of all days ± 1σ		0.651 ± 0.008
Average after day 8 ± 1σ		0.646 ± 0.004	Average after day 8 ± 1σ		0.656 ± 0.000

[a]*Calculated using equation (δD–CH_4 + 1000)/(δD–H_2O + 1000). δD–H_2O values were measured before incubation and were: –20.1‰, –20.4‰, –20.4‰, –20.5‰, –18.1‰, and –18.1‰, respectively, for batches A, B, C, D, E, and F.*
[b]*Groundwater + YPG medium + N_2 (0.25 MPa).*
[c]*Groundwater + YPG medium + N_2 + CO_2 (80:20, v/v, 0.25 MPa).*
[d]*Groundwater + H_2 + CO_2 (80:20, v/v, 0.25 MPa).*

produced under high concentrations of H_2 (71–86 mmol L^{-1} in the headspace) (Fig. 1c). As with the other cultures, we examined the average carbon isotope fractionation factors in the steady state. They were 1.021 ± 0.000 for batch E (after day 8), and 1.022 ± 0.000 for batch F (after day 8).

No significant difference in α(CO_2–CH_4) values was found between different incubation temperatures, but α(CO_2–CH_4) values of batches E and F were significantly different from those of batches A, B, C, and D.

DISCUSSION

CH_4 production by syntrophic cooperation

In our previous study, a series of culture-dependent and culture-independent microbiological studies was undertaken using groundwater from the same location (Kimura *et al*., 2010). In that study, CH_4 production was observed for enrichment via CO_2 reduction, but it was not observed with other substrate such as acetate, methanol, and formate. Based on phylogenetic analysis, we also demonstrated that the dominant species in this groundwater included hydrogenotrophic methanogens belonging to the genus *Methanobacteriales*, and confirmed that the microorganisms propagated in this groundwater treated with H_2 + CO_2 were closely related to *M. thermoautotrophicus*, which is a thermophilic methanogen using H_2 and CO_2 for growth and CH_4 production (Zeikus and Wolfe, 1972). Therefore, CH_4 production by CO_2 reduction was expected to be observed in these cultures in the present study.

For batches A, B, C, and D, the H_2 concentrations increased to approximately 1–2 mmol L^{-1} (in the headspaces) at 1–4 days. The H_2 concentrations remained low and constant after CH_4 production began (Figs. 1a and 1b). The H_2 and CH_4 dynamics observed in these

cocultures resembled those of syntrophic cocultures of fermentative bacteria and hydrogenotrophic methanogens reported previously (Penning *et al*., 2005; Ishii *et al*., 2005; Kimura *et al*., 2010). Consequently, syntrophic cooperation (symbioses based on nutritional cooperation) between fermenters and hydrogenotrophic methanogens at thermophilic conditions was established for batches A, B, C, and D in which CH_4 was produced under low H_2 concentration (Figs. 1a and 1b). For batches E and F, on the other hand, CH_4 was produced by CO_2 reduction under much higher H_2 concentrations than in batches A, B, C, and D (Fig. 1c). This experimental approach allowed CH_4 production at different H_2 conditions using microbial communities from the deep aquifer under moderately thermophilic conditions (55°C and 65°C).

Several studies have investigated the relation between H_2 concentration and hydrogen or carbon isotope fractionation (e.g., Burke, 1993; Valentine *et al*., 2004). We therefore concentrate mainly on these relations.

Hydrogen isotope fractionation by CO_2 reduction

The cocultures (batches A, B, C, and D) gave hydrogen isotope fractionation values that were approximately 10–90‰ smaller (higher α_H values) than those of batches E and F (Table 2). Therefore, hydrogen isotope fractionations were larger (lower α_H values) in the case of CH_4 production at low H_2 concentrations, which is consistent with the hypothesis proposed by Burke (1993): hydrogen isotope fractionation between CH_4 and H_2O increases (and α_H values decrease) with increasing H_2 concentration. These results are the first to support Burke's hypothesis using incubation experiments.

Yoshioka *et al*. (2008) reported the opposite of Burke's hypothesis: larger hydrogen isotope fractionations (lower α_H values) were observed in cocultures of *M. thermoautotrophicus* and *Syntrophothermus lipocalidus*, which maintained lower H_2 concentrations than in a pure culture of *M. thermoautotrophicus* under higher H_2 concentrations. Their results are not consistent with our results, although their experimental conditions resembled ours. Nevertheless, it is noteworthy that steady-state α_H values in our cocultures (batches A, B, C, and D) were similar to their coculture values: 0.701–0.723 for our cocultures (Table 2), and 0.725 ± 0.003 for their cocultures of *M. thermoautotrophicus* and *S. lipocalidus* (Yoshioka *et al*., 2008). The steady-state α_H values observed in previous laboratory studies in which CH_4 was produced under high H_2 concentrations were 0.602 ± 0.003 for *Methanobacterium formicicum* (Balabane *et al*., 1987), and 0.755 ± 0.014 for *M. thermoautotrophicus* (Yoshioka *et al*., 2008). The steady-state α_H values for batches E and F in this study were 0.646–0.656 (Table 2). Other than those in pure cultures of *M. thermoautotrophicus* (Yoshioka *et al*., 2008), small hydrogen isotope

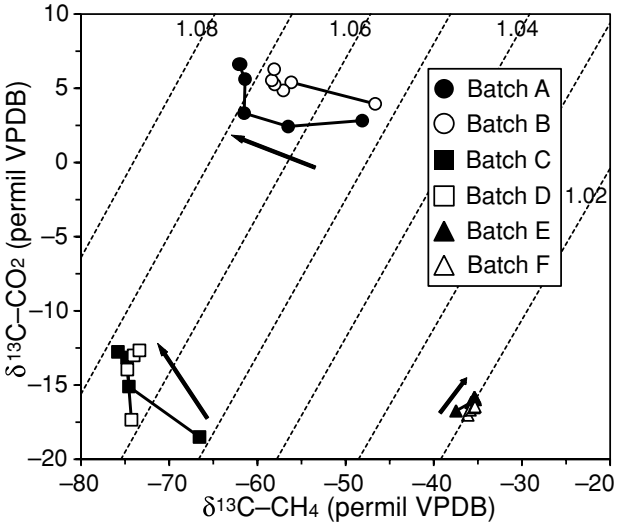

Fig. 2. Stable carbon isotope ratios of CO_2 and CH_4 produced in anaerobic cultures using groundwater obtained from the deep aquifer. Arrows indicate increasing incubation times. Dotted lines show apparent fractionation between CO_2 and CH_4 determined using the ratio $(\delta^{13}C\text{–}CO_2 + 1000)/(\delta^{13}C\text{–}CH_4 + 1000)$. Circles: groundwater + YPG medium (under N_2); ●, batch A (55°C); ○, batch B (65°C). Squares: groundwater + YPG medium (under $N_2 + CO_2$, 80:20, v/v); ■, batch C (55°C); □, batch D (65°C). Triangles: groundwater (under $H_2 + CO_2$, 80:20, v/v); ▲, batch E (55°C); △, batch F (65°C).

fractionations (α_H = 0.701–0.725) were observed in these cocultures of fermenters and hydrogenotrophic methanogens in which CH_4 was produced at low H_2 concentrations, which is consistent with Burke's hypothesis. In addition, low hydrogen isotope fractionation between H_2O and CH_4 was observed in a terrestrial natural wetland with very low H_2 partial pressure (Sugimoto and Fujita, 2006), indicating that the relation between hydrogen isotope fractionation and H_2 concentration proposed by Burke (1993) is generally correct. Although Sugimoto and Fujita (2006) present possible explanations of how H_2 concentration affects variations in hydrogen isotope fractionation between H_2O and CH_4, it is important to investigate how isotopic exchange between H_2 and H_2O is reached and to elucidate its relation to the enzymatic expression of step 4 of multi-enzymatic processes. These mechanisms, which remain unclear, demand further study.

This report describes the first results showing that a coculture in which fermenters and hydrogenotrophic methanogens produced CH_4 under low H_2 concentrations caused smaller hydrogen isotope fractionation (higher α_H values) than did monocultures under high H_2 concentrations. However, the hydrogen isotope fractionations in our cocultures are also large compared with those in natural environments in which CO_2 reduction is dominant (ap-

proximately 160‰; Schoell, 1980; Whiticar *et al*., 1986), and to those obtained in field observations of this deep aquifer (160.6–165.9‰; α_H values 0.831–0.837) by Kimura *et al*. (2010), suggesting the existence of another factor controlling hydrogen isotope ratios of CH_4 and suggesting that future study is necessary.

Carbon isotope fractionation by CO_2 reduction

Changes in $\delta^{13}C$ values of CO_2 and CH_4 and calculated $\alpha(CO_2–CH_4)$ values are presented in Fig. 2. Although the $\alpha(CO_2–CH_4)$ values for batches E and F were much lower than those for the other cocultures, the differences in CO_2 addition between batches A, B and batches C, D did not strongly affect the $\alpha(CO_2–CH_4)$ values (Figs. 1 and 2). This finding suggests that CH_4 production under low H_2 concentrations gives large carbon isotope fractionation.

Valentine *et al*. (2004) found that carbon isotope fractionation was affected by the H_2 supply in a CO_2 reducing culture of *Methanothermobacter marburgensis* at 65°C. They proposed a ΔG-dependent differential reversibility hypothesis for the multistep enzymatic processes, and the extent of reversibility in multistep enzymatic processes is expected to impact the extent to which fractionations are expressed from each enzymatic step as well as shown in dissimilatory sulfate reduction (Kaplan and Rittenberg, 1964; Rees, 1973). Indeed, strong correlations between carbon isotope fractionation by CO_2 reduction and ΔG values for CO_2 reduction have been observed (Penning *et al*., 2005; Takai *et al*., 2008). The H_2 concentration is the most important parameter in control of the ΔG values. Therefore, the relation between carbon isotope fractionation and H_2 concentration in this study was consistent with the differential reversibility hypothesis. According to the differential reversibility hypothesis (Valentine *et al*., 2004), for cocultures (batches A, B, C, and D), the low concentration of H_2 in syntrophic cooperation of fermenters and hydrogenotrophic methanogens is expected to increase reversibility in the multiple enzymatic processes of CH_4 production, resulting in large carbon isotope fractionation ($1.052 < \alpha(CO_2–CH_4) < 1.074$). In batches E and F in this study, CH_4 production under high H_2 concentrations gave small carbon isotope fractionation because of reduced reversibility in the multiple enzymatic processes ($1.021 < \alpha(CO_2–CH_4) < 1.023$).

The $\alpha(CO_2–CH_4)$ values observed in the cocultures are consistent with those obtained from field observations in some terrestrial and marine environments (1.04–1.09; Whiticar *et al*., 1986), and from field observations at the site used in the current study (1.058–1.059; Kimura *et al*., 2010). This result suggests that carbon isotope fractionation via syntrophic cooperation between fermenters and hydrogenotrophic methanogens resembles fractionation in natural environments more closely than monocultures under high H_2 concentration do.

Conclusions

We investigated hydrogen and carbon isotope fractionation factors in CO_2 reduction under coculture and monoculture. For these cocultures, in which CH_4 was produced under low H_2 concentrations, hydrogen isotope fractionations were smaller and carbon isotope fractionations were larger than those observed in monocultures. Although lack of agreement between results of incubation experiments and field observations in hydrogen isotope fractionations remains, both hydrogen and carbon isotope fractionation in cocultures were similar to the fractionations observed *in situ*. Consequently, microbial syntrophic cooperation and the concentration of nutrients such as H_2 might be important for obtaining fractionation factors similar to those obtained from field observations.

Acknowledgments—We thank Mr. Katsuya Yabuzaki and Mr. Masataka Saito (Shimada City Hall) for assistance in groundwater sampling. We also thank Professor K. Kato for assistance with culture experiments and for providing the environments used for the experiments. Dr. S. Toyoda, Dr. Y. Ueno and M. Kobayashi are gratefully acknowledged for their discussion of data. We thank Dr. Susan Waldron for her advice on an early version of this manuscript. We wish to thank two anonymous reviewers for valuable and helpful comments. This work was supported by a Grant in aid for Scientific Research (A) (No. 19201004) and (S) (No. 23224013) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and the Global Environment Research Fund (A094) of the Ministry of the Environment, Japan. S. Hattori is supported by a Grant in Aid for JSPS Research Fellows (DC1 (No. 22-7563)) from MEXT, Japan.

References

Balabane, M., Galimov, E., Hermann, M. and Letolle, R. (1987) Hydrogen and carbon isotope fractionation during experimental production of bacterial methane. *Org. Geochem.* **11**, 115–119.

Botz, R., Pokojski, H. D., Schmitt, M. and Thomm, M. (1996) Carbon isotope fractionation during bacterial methanogenesis by CO_2 reduction. *Org. Geochem.* **25**, 255–262.

Burke, R. A., Jr. (1993) Possible influence of hydrogen concentration on microbial methane stable hydrogen composition. *Chemosphere* **26**, 55–67.

Chidthaisong, A., Chin, K. J., Valentine, D. L. and Tyler, S. C. (2002) A comparison of isotope fractionation of carbon and hydrogen from paddy field rice roots and soil bacterial enrichments during CO_2/H_2 methanogenesis. *Geochim. Cosmochim. Acta* **66**, 983–995.

Conrad, R. (2005) Quantification of methanogenic pathways

using stable carbon isotopic signatures: a review and a proposal. *Org. Geochem.* **36**, 739–752.

Fey, A., Claus, P. and Conrad, R. (2004) Temporal change of ^{13}C-isotope signatures and methanogenic pathways in rice field soil incubated anoxically at different temperatures. *Geochim. Cosmochim. Acta* **68**, 293–306.

Horita, J., Ueda, A., Mizukami, K. and Takatori, I. (1989) Automatic δD and $\delta^{18}O$ analyses of multiwater samples using H_2- and CO_2-water equilibration methods with a common equilibration set-up. *Appl. Radiation. & Isotopes* **40**, 801–805.

Ishii, S., Kosaka, K., Hori, K., Hotta, Y. and Watanabe, K. (2005) Coaggregation facilitates interspecies hydrogen transfer between Pelotomaculum thermopropionicum and Methanothermobacter thermautotrophicus. *Appl. Environ. Microbiol.* **71**, 7838–7845.

Kaplan, I. R. and Rittenberg, S. C. (1964) Microbiological fractionation of sulphur isotopes. *J. Gen. Microbiol.* **34**, 195–212.

Kimura, H., Nashimoto, H., Shimizu, M., Hattori, S., Yamada, K., Koba, K., Yoshida, N. and Kato, K. (2010) Microbial methane production in deep aquifer associated with the accretionary prism in Southwest Japan. *The ISME Journal* **4**, 531–541.

Penning, H., Plugge, C. M., Galand, P. E. and Conrad, R. (2005) Variation of carbon isotope fractionation in hydrogenotrophic methanogenic microbial cultures and environmental samples at different energy status. *Global Change Biol.* **11**, 2103–2113.

Rees, C. E. (1973) A steady-state model for sulphur isotope fractionation in bacterial reduction processes. *Geochim. Cosmochim. Acta* **37**, 1141–1162.

Schoell, M. (1980) The hydrogen and carbon isotopic composition of methane from natural gases of various origins. *Geochim. Cosmochim. Acta* **44**, 649–661.

Sugimoto, A. and Fujita, N. (2006) Hydrogen concentration and stable isotopic composition of methane in bubble gas observed in a natural wetland. *Biogeochemistry* **81**, 33–44.

Sugimoto, A. and Wada, E. (1995) Hydrogen isotopic composition of bacterial methane: CO_2/H_2 reduction and acetate fermentation. *Geochim. Cosmochim. Acta* **59**, 1329–1337.

Taira, A., Byrne, T. and Ashi, J. (1992) *Photographic Atlas of an Accretionary Prism: Geologic Structures of the Shimanto Belt, Japan*. The University of Tokyo Press, 124 pp.

Takai, K., Nakamura, K., Toki, T., Tsunogai, U., Miyazaki, M., Miyazaki, J., Hirayama, H., Nakagawa, S., Nunoura, T. and Horikoshi, K. (2008) Cell proliferation at 122°C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proc. Natl. Acad. Sci. USA* **105**, 10,949–10,954.

Tanabe, H. and Kano, K. (1996) Illite crystallinity study of the Cretaceous Shimanto Belt in the Akaishi Mountains, eastern southwest Japan. *The Island Arc* **5**, 56–68.

Valentine, D. L., Chidthaisong, A., Rice, A., Reeburgh, W. S. and Tyler, S. C. (2004) Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens. *Geochim. Cosmochim. Acta* **68**, 1571–1590.

Whiticar, M. J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **161**, 291–314.

Whiticar, M. J., Faber, E. and Schoell, M. (1986) Biogenic methane formation in marine and freshwater environments: CO_2 reduction vs. acetate fermentation-isotope evidence. *Geochim. Cosmochim. Acta* **50**, 693–709.

Yamada, K., Ozaki, Y., Nakagawa, F., Tanaka, M. and Yoshida, N. (2003) An improved method for measurement of the hydrogen isotope ratio of atmospheric methane and its application to a Japanese urban atmosphere. *Atmos. Environ.* **37**, 1975–1982.

Yoshioka, H., Sakata, S. and Kamagata, Y. (2008) Hydrogen isotope fractionation by *Methanothermobacter thermoautotrophicus* in coculture and pure culture conditions. *Geochim. Cosmochim. Acta* **72**, 2687–2694.

Zeikus, J. G. and Wolfe, R. S. (1972) *Methanobacterium thermoautrophicus* sp. n, an anaerobic, autotrophic, extreme thermophile. *J. Bacteriol.* **109**, 707–713.

Supplementary Materials

URL (http://www.terrapub.co.jp/journals/GJ/archives/data/46/MS161.pdf)