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ORIGINAL ARTICLE

Microbial methane production in deep aquifer associated with the accretionary prism in Southwest Japan

Hiroyuki Kimura[1], Hiroaki Nashimoto[1], Mikio Shimizu[1], Shohei Hattori[2], Keita Yamada[3], Keisuke Koba[4], Naohiro Yoshida[2] and Kenji Kato[1]

[1]*Department of Geosciences, Faculty of Science, Shizuoka University, Shizuoka, Japan;* [2]*Department of Environmental Science and Technology, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Yokohama, Japan;* [3]*Department of Environmental Chemistry and Engineering, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, Yokohama, Japan and* [4]*Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan*

To identify the methanogenic pathways present in a deep aquifer associated with an accretionary prism in Southwest Japan, a series of geochemical and microbiological studies of natural gas and groundwater derived from a deep aquifer were performed. Stable carbon isotopic analysis of methane in the natural gas and dissolved inorganic carbon (mainly bicarbonate) in groundwater suggested that the methane was derived from both thermogenic and biogenic processes. Archaeal 16S rRNA gene analysis revealed the dominance of H_2-using methanogens in the groundwater. Furthermore, the high potential of methane production by H_2-using methanogens was shown in enrichments using groundwater amended with H_2 and CO_2. Bacterial 16S rRNA gene analysis showed that fermentative bacteria inhabited the deep aquifer. Anaerobic incubations using groundwater amended with organic substrates and bromoethanesulfonate (a methanogen inhibitor) suggested a high potential of H_2 and CO_2 generation by fermentative bacteria. To confirm whether or not methane is produced by a syntrophic consortium of H_2-producing fermentative bacteria and H_2-using methanogens, anaerobic incubations using the groundwater amended with organic substrates were performed. Consequently, H_2 accumulation and rapid methane production were observed in these enrichments incubated at 55 and 65 °C. Thus, our results suggested that past and ongoing syntrophic biodegradation of organic compounds by H_2-producing fermentative bacteria and H_2-using methanogens, as well as a thermogenic reaction, contributes to the significant methane reserves in the deep aquifer associated with the accretionary prism in Southwest Japan.
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Introduction

The accretionary prism situated along the Pacific side of Southwest Japan is traceable laterally for some 1800 km and forms a thick sediment that accretes onto the nonsubducting tectonic plate at a convergent plate boundary. The sediment is composed mainly of non- to weakly metamorphosed sequences of sandstone, shale, alternating beds of both, and local associations of chert and greenstone (Taira *et al.*, 1992). The materials are derived from marine sediment scraped from the subducting oceanic crust; therefore, they are rich in complex organic matter (Karasawa and Kano, 1992). The sediment contains layers of water-bearing permeable sandstone and no water-bearing impermeable shale. Groundwater is mainly recharged by rainfall infiltrating into outcrops or faults. The water flows down through the permeable sandstone and is reserved in a deep aquifer. In addition to the groundwater, it has been reported that dissolved natural gases are present in the deep aquifer associated with the accretionary prism in Southwest Japan (Igari and Sakata, 1989).

It is generally believed that natural gas in subsurface environments is formed by microbial production (biogenic origin), nonbiological thermal decomposition of organic matter (thermogenic origin), or geochemical reaction in hydrothermal systems during

Correspondence: H Kimura, Department of Geosciences, Faculty of Science, Shizuoka University, 836 Oya, Suruga-ku, Shizuoka 422-8529, Japan.
E-mail: shkimur@ipc.shizuoka.ac.jp
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water–rock interactions (abiogenic origin). These origins have been interpreted based on chemical compositions of gaseous alkanes (for example C_1–C_3, methane, ethane, propane) and stable carbon and hydrogen isotope ratios of methane ($\delta^{13}C$–CH_4 and δD–CH_4). The chemical and isotopic data have been interpreted by plotting hydrocarbon gas composition $C_1/(C_2+C_3)$ versus $\delta^{13}C$–CH_4 (Bernard *et al.*, 1978) and δD–CH_4 versus $\delta^{13}C$–CH_4 (Schoell, 1983) on widely used conventional diagrams. Numerous microbiological approaches have also been used to understand the methane production processes in subsurface environments such as petroleum-contaminated aquifers (Gray *et al.*, 2009), geothermal aquifers (Kimura *et al.*, 2005; Mochimaru *et al.*, 2007b), and coalbed aquifers (Shimizu *et al.*, 2007; Strąpoć *et al.*, 2008).

Igari and Sakata (1989) measured stable carbon isotopic ratios of methane in natural gases associated with the accretionary prism in Southwest Japan, and concluded that the methane was of thermogenic origin. Exhaustive geochemical and microbiological studies, however, have not yet been performed to determine the pathways of methane production *in situ*. The goals of this study were to identify the origin of the methane using the chemical and stable isotopic signatures of natural gases and groundwater and, using culture-dependent and culture-independent methods, to explore the microbial methane production pathway in the deep aquifer associated with the accretionary prism in Southwest Japan.

Materials and methods

Study site and sample collection

Groundwater and natural gas samples were collected from a deep well (Ita-wari well; 34 °52.283′N, 138 °09.150′E) situated in Shimada, Shizuoka Prefecture, Japan (Figure 1). The well is geologically located in the Setogawa group of the Shimanto Belt, which is an ancient accretionary prism situated along the Pacific side of Southwest Japan (Taira *et al.*, 1992). The Setogawa group was deposited 30–40 million years ago with abyssal sediment of the Nankai Trough, and the materials are composed of non- to weakly metamorphosed thick sequences of sandstone, shale, and alternating bed of both (Karasawa and Kano, 1992). The deep well is drilled down to 1500 m and constructed from tight steel casing pipes including strainers at 1188–1489 m. Groundwater flows into the deep well through parts of the strainers and rises up to 250 m underground by natural water pressure. The groundwater is drawn up to ground level by a water pump under anaerobic conditions.

Groundwater and natural gas were sampled nine times from July 2007 to January 2009. To prevent contamination by air and water from shallow environments, the groundwater was pumped at a flow rate of

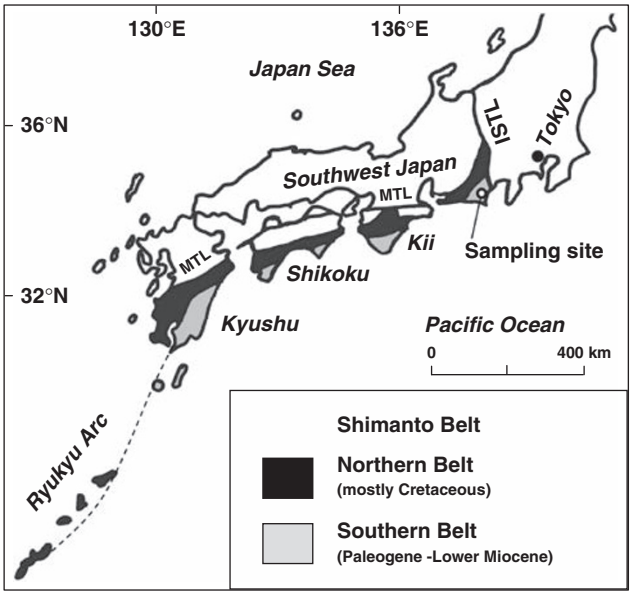

Figure 1 Location of the Shimanto Belt in Southwest Japan. The geological map is according to Kano *et al.* (1991). ISTL, Itoigawa-Shizuoka Tectonic Line; MTL, Median Tectonic Line.

116 l min^{-1} for 24 h before sampling. The groundwater samples were collected under anaerobic conditions into autoclaved glass bottles by using a sterile silicone tube. The concentrations of dissolved natural gases were so high that they were exsolving at ground level. The gases were collected in an inverted funnel underwater and then directed into autoclaved serum bottles. The serum bottles were tightly sealed with sterile butyl rubber stoppers and aluminum crimps underwater to prevent contamination by air.

Measurement of physical and chemical parameters

Physical and chemical parameters of groundwater were measured at the outflow of the deep well. Temperature was measured with a platinum resistance thermometer (Custom, Tokyo, Japan). Oxidation–reduction potential and pH were measured with RM-20P and HM-20P portable meters (DKK-TOA, Tokyo, Japan), respectively. The oxidation–reduction potential was converted to Eh using a reference electrode. Electric conductivity was measured with a CM-21P portable meter (DKK-TOA). The anion (HCO_3^-, Cl^-, Br^-, I^-, SO_4^{2-}, acetate, formate) concentrations in the groundwater were analyzed with an IC-2001 ion chromatograph equipped with a TSKgel SuperIC-AZ column (Tosoh, Tokyo, Japan). The cation (Na^+, K^+, Mg^{2+}, Ca^{2+}) concentrations were analyzed with an IC-2001 ion chromatograph equipped with a TSKgel SuperIC-CR column (Tosoh). Sulfide was analyzed using the methylene blue method. Spectrophotometric analysis of sulfide was performed at the sampling site with a DR/2400 portable spectrophotometer

(Hach, Loveland, CO, USA). Dissolved organic carbon in the groundwater filtered through pre-combusted GF/F glass microfiber filters (Whatman, Maidstone, UK) was measured with a TOC-V total organic carbon analyzer (Shimadzu, Kyoto, Japan). Concentrations of H_2, N_2, O_2, CO_2, CH_4, C_2H_6, and C_3H_8 in the natural gas samples were determined on a GC-2014 gas chromatograph (GC) equipped with a thermal conductivity detector and a flame ionization detector (Shimadzu).

Analysis of stable isotopic ratios

The stable hydrogen isotope ratio (D/H) of methane in the natural gas was measured by an online CH_4 extraction system, a HP6890 GC (Hewlett-Packard, Palo Alto, CA, USA), a ThermoQuest pyrolysis interface (ThermoFinnigan, Bremen, Germany), and a ThermoQuest Delta^{plus}XL isotope ratio mass spectrometer (IRMS) (ThermoFinnigan) (Yamada *et al*., 2003). The same system was used to measure the stable carbon isotope ratio ($^{13}C/^{12}C$) of methane when the pyrolysis furnace and the ThermoQuest Delta^{plus}XP were replaced with a combustion furnace (ceramic tube packed with CuO, NiO, and Pt wires) and a Finnigan MAT 252 IRMS (Finnigan MAT, Bremen, Germany). The $^{13}C/^{12}C$ of total dissolved inorganic carbon (ΣCO_2) in groundwater, mainly bicarbonate, was analyzed according to an earlier described method (Miyajima *et al*., 1995). Groundwater samples for analysis of $^{13}C/^{12}C$ of ΣCO_2 were collected into an autoclaved serum bottles (inner volume, 68.6 ml), fixed with 0.5 ml of saturated $HgCl_2$ solution, and sealed with a sterile butyl rubber stoppers and aluminum crimps with no air bubbles. A 5 ml headspaces were created inside the serum bottles with pure helium gas and acidified by adding 0.5 ml of a CO_2-free HCl solution (6.0N). The sample bottles were left in the dark for at least 24 h, during which time the original dissolved CO_2 became equilibrated with the headspace gas. The CO_2 in this headspace was subsampled, and $^{13}C/^{12}C$ of CO_2 was measured by an online extraction system, HP6890 GC (Hewlett-Packard), the combustion furnace, and a ThermoQuest Delta^{plus}XP IRMS. Groundwater samples for the analysis of stable hydrogen and oxygen isotope ratios (D/H and $^{18}O/^{16}O$) were collected into an autoclaved polycarbonate bottle. The D/H and $^{18}O/^{16}O$ of water were measured using a conventional IRMS according to the earlier described method (Yoshida and Mizutani, 1986).

These stable isotope ratios are expressed in the conventional δ notation calculated from the equation:

$$\delta = [R_{\mathrm{sample}}/R_{\mathrm{standard}} - 1] \times 1000\ [‰]$$

where R is the isotope ratio (D/H, $^{13}C/^{12}C$, or $^{18}O/^{16}O$). All isotope ratios in this paper are reported relative to the international standards: Vienna Standard Mean Ocean Water for δD and $\delta^{18}O$, and Vienna Pee Dee Belemnite for $\delta^{13}C$. The standard deviations of δD of CH_4, $\delta^{13}C$ of CH_4 and CO_2, δD of water, and $\delta^{18}O$ of water were found to be $\pm 4‰$, $\pm 0.3‰$, $\pm 1‰$, and $\pm 0.1‰$, respectively.

Microbial cell staining and counting

The groundwater samples for total cell counts were fixed with formaldehyde (final concentration 2%). Exactly 10 ml of groundwater sample was filtered using pre-blackened polycarbonate filters (pore size, 0.22 μm; diameter, 25 mm; Millipore, Bedford, MA, USA). Microbial cells collected on the filter were stained with SYBR Green I (1:100 dilution, Molecular Probes, Eugene, OR, USA). The microbial cells were observed under a model BX51 epifluorescence microscope (Olympus, Tokyo, Japan), and over 50 microscopic fields were counted for each sample.

Archaeal and bacterial 16S rRNA gene analyses

Groundwater was collected from the deep well on 25 July 2008. Exactly 6 l of groundwater sample was aseptically filtered with Sterivex-GV filter units (pore size, 0.22 μm; Millipore) using sterilized silicone tubing and a pump. Bulk DNA of microorganisms trapped by the filter units was extracted according to the method described by Kimura *et al*. (2007). Archaeal 16S rRNA gene fragments were amplified by PCR from the bulk DNA using *Archaea*-specific primers 109aF (Großkopf *et al*., 1998) and 915aR (Stahl and Amann, 1991). Bacterial 16S rRNA gene fragments were amplified by PCR from the bulk DNA using *Bacteria*-specific primers 8bF and prokaryotic universal primer 1512uR (Eder *et al*., 1999). These reactions were performed using KOD DNA polymerase (Toyobo, Osaka, Japan) in a TaKaRa PCR Thermal Cycler PERSONAL (TaKaRa, Kyoto, Japan) with the following program: 94 °C for 2 min; 25 cycles at 94 °C for 20 s, 56 °C for 30 s, 68 °C for 1 min, and 68 °C for 10 min. PCR products of the archaeal and bacterial 16S rRNA genes were cloned with a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). The sequences of inserted PCR products selected from recombinant colonies were determined with a model RISA-384 capillary DNA sequencer (Shimadzu). Chimeric sequences were identified using the Bellerophon chimera detection program (Huber *et al*., 2004). Sequences that were of potential chimeric origin were excluded from further examination.

The operational taxonomic units (OTUs) for each clone library were determined using DOTUR software version 1.53 (Schloss and Handelsman, 2005). A 2% distance level between sequences was considered the cutoff to consider distinct OTUs. Each OTU was classified using the Classifier program from the RDP II (Wang *et al*., 2007), and the nearest relative of each OTU was determined by a FASTA search (Pearson and Lipman, 1988) against the DNA Data Bank of Japan. The coverage of each clone library was calculated by the formula

$[1-(n_1/N)]$, where n_1 is the number of OTUs represented by only one clone and N is the total number of clones examined (Good, 1953). The 16S rRNA gene sequences obtained in this study were deposited in the DNA Data Bank of Japan/EMBL/GenBank database under accession numbers AB477989 to AB478012.

Measurements of potential microbial methane and H_2 productions

Exactly 30 ml of groundwater samples were anaerobically injected into autoclaved 70 ml serum bottles that were tightly sealed with sterile butyl rubber stoppers and aluminum crimps used to form a vacuum to prevent contamination by air. To determine the potential for methane production by methanogenic archaea, enrichments using groundwater amended with methanogenic substrates were performed. The groundwater was supplemented with acetate (10 mM), methanol (10 mM), formate (10 mM), or H_2+CO_2 (80:20, vol/vol; 0.25 MPa). Except for H_2+CO_2-supplemented bottles, the headspaces of serum bottles were filled with pure N_2 gas at 0.25 MPa. The enrichments were anaerobically incubated without shaking at 35, 45, 55, 65, 75, and 85 °C. H_2, CH_4, and CO_2 concentrations in the headspace were measured every 24 h for the initial 6 days, and every 48 h thereafter, with a GC-2014 GC equipped with a thermal conductivity detector (Shimadzu). The enrichments were carried out in duplicate. The 16S rRNA genes of microorganisms in the enrichments in which methane production was observed were analyzed according to the clone library method described above.

Enrichments using groundwater amended with organic substrates were also performed to determine the potential for microbial H_2 production through anaerobic biodegradation of organic matter. The groundwater samples were supplemented with 3 ml 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) and 20 mM of bromoethanesulfonate, an inhibitor of methanogens (Gunsalus *et al.*, 1978). The headspaces of the serum bottles were filled with pure N_2 at 0.25 MPa. These enrichments were anaerobically incubated without shaking at 35, 45, 55, 65, 75, and 85 °C. H_2, CH_4, and CO_2 concentrations in the headspace were measured every 12 h for the initial 3 days, and every 24 h thereafter. The enrichments were carried out in duplicate. The 16S rRNA genes of microorganisms in the enrichments in which H_2 production was observed were analyzed according to the clone library method described above.

We also measured the potential of methane production by syntrophic biodegradation of fermentative bacteria and methanogenic archaea. The groundwater samples were supplemented with 3 ml of the YPG medium. In these enrichments, the bromoethanesulfonate was not added. The headspaces of the serum bottles were filled with pure N_2 at 0.25 MPa. The enrichments were incubated without shaking at 35, 45, 55, 65, 75, and 85 °C, and were performed in duplicate. H_2, CH_4, and CO_2 concentrations in the headspace were measured every 12 h for the initial 4 days, and every 24 h thereafter.

Results

Physical and chemical parameters

Groundwater temperatures ranged from 40.3 to 44.4 °C, and pH was weakly alkaline. Eh ranged from −106 to −54 mV (Supplementary Table S1). Cl^-, SO_4^{2-}, Na^+, K^+, Mg^{2+}, and Ca^{2+} in groundwater indicated 10–100 times lower concentrations than those found in surface seawater (Millero *et al.*, 2008). Br^- and I^- were much lower than those of ancient seawater samples from natural gas fields (Mochimaru *et al.*, 2007a, b). Sulfide was below the detection limit (<0.01 mg l^{-1}). Dissolved organic carbon ranged from 36.5 to 37.7 mg l^{-1}, indicating 15–30 times higher concentrations than those found in coastal seawater (Kimura *et al.*, 2001) (Supplementary Table S2).

Methane was the predominant component of the natural gas (>97%). Other main components were C_2H_6 and N_2 at 1.3–1.4 vol.% and 0.8–1.2 vol.%, respectively. Hydrocarbon gas composition $C_1/(C_2+C_3)$ of the natural gas ranged from 69.8 to 75.0, which indicated that the natural gas included thermogenic C_{2+} hydrocarbons. H_2, O_2, and CO_2 in natural gas were below the respective detection limits (Supplementary Table S3).

Stable isotopic ratios of natural gas and groundwater

Stable isotope ratios of natural gas and groundwater are summarized in Table 1. Stable carbon isotope ratios of methane ($\delta^{13}C$–CH_4) ranged from −39.1‰ to −38.2‰, with an average of −38.8‰. These values are relatively close to those derived from the accretionary prism in Southwest Japan reported earlier: −34.3‰ to −33.8‰ (Igari and Sakata, 1989). When the hydrocarbon gas composition $C_1/(C_2+C_3)$ was plotted versus the $\delta^{13}C$–CH_4 diagram according to Bernard *et al.* (1978), the natural gases examined in this study fell within the range of thermogenic origin (Figure 2a). The stable hydrogen isotope ratio of methane (δD–CH_4) ranged from −183‰ to −177‰, with an average of −180‰. When $\delta^{13}C$–CH_4 was plotted versus the δD–CH_4 diagram according to Schoell (1983), the observed methane fell in the range of thermogenic gases with condensate (Figure 2b).

Carbon isotopic ratios of total dissolved inorganic carbon ($\delta^{13}C$–ΣCO_2) in groundwater were moderately enriched $\delta^{13}C$ in the range of +17.7‰ to +18.3‰. We also plotted the observed isotopic values in $\delta^{13}C$–ΣCO_2 versus the $\delta^{13}C$–CH_4 diagram according to Smith and Pallasser (1996). These values fell on the boundary between biogenic origin

Table 1 Stable isotopic ratios of natural gas and groundwater samples

Date	*Gas*		*Liquid*				
	CH_4		ΣCO_2	H_2O		*Stable isotopic offsets*	
	$\delta^{13}C$ (‰)	δD (‰)	$\delta^{13}C$ (‰)	δD (‰)	$\delta^{18}O$ (‰)	$\Delta^{13}C_{\Sigma CO_2-CH_4}$ (‰)	$\Delta D_{H_2O-CH_4}$ (‰)
19 February 2008	−39.0	−179	ND	−16.8	+0.9	—	162.2
25 July 2008	−38.2	−177	+17.7	−16.4	+0.8	55.9	160.6
25 September 2008	−39.1	−183	+18.3	−17.1	+0.9	57.4	165.9

Abbreviation: ND, not determined.

by CO_2 reduction and mixed biogenic/thermogenic origins (Figure 2c). Stable hydrogen and oxygen isotopic ratios of groundwater ranged from −17.1‰ to −16.4‰ and from +0.8‰ to +0.9‰, respectively, which were in good agreement with those of formation waters obtained from the southeastern Illinois Basin in the mid-continental region of the United States (Taylor, 1974).

Microbial cell density

Microbial cell density in the groundwater sample ranged from 5.8×10^4 to 1.1×10^5 cells ml^{-1}, with an average of 8.0×10^4 cells ml^{-1} (Supplementary Table S1). The cell density was in good agreement with those in other geothermal aquifers reported by Kimura *et al.* (2005) and Mochimaru *et al.* (2007b).

Phylogenetic analysis of archaeal and bacterial 16S rRNA genes

A total of 41 clones in an archaeal 16S rRNA gene clone library were sequenced and divided into four OTUs (SMD-A01 to SMD-A04) (Table 2). The coverage of the clone library was 97.6%. Phylogenetic analysis revealed that 98% of the clones in the library belonged to the order *Methanobacteriales* (SMD-A01, SMD-A02, and SMD-A03), which is known as a group of methanogenic archaea able to use H_2 and CO_2 for growth and methanogenesis. SMD-A01 was closely related to the 16S rRNA gene from the cultured organism *Methanobacterium aarhusense* (94.8% 16S rRNA sequence identity), which is a hydrogenotrophic methanogen (Shlimon *et al.*, 2004). SMD-A02 showed the highest identity to the 16S rRNA gene from the cultured organism *M. alcaliphilum* (94.8% identity), another hydrogenotrophic methanogen (Worakit *et al.*, 1986). SMD-A03 showed the closest match to the 16S rRNA gene from the cultured organism *Methanothermobacter thermautotrophicus* (99.4% identity), a thermophilic methanogen that uses H_2 and CO_2 for methanogenesis (Zeikus and Wolfe, 1972). The remaining OTU, SMD-A04, was closely related to the 16S rRNA gene from the cultured organism *Thermococcus gammatolerans* (82.3% identity), which is known as a thermophilic heterotrophic archaeon isolated from a deep-sea hydrothermal vent (Jolivet *et al.*, 2003).

A total of 63 clones in a bacterial 16S rRNA gene clone library were sequenced and divided into 16 OTUs (SMD-B01 to SMD-B16) (Table 2). The coverage of the clone library was 90.5%. The most abundant OTU was SMD-B01, which was closely related to the 16S rRNA gene from the cultured organism *Thermodesulfovibrio yellowstonii* (96.8% 16S rRNA sequence identity), known as sulfate-reducing or fermentative bacterium (Henry *et al.*, 1994). The SMD-B01 clones accounted for 30% of the clones in the library. SMD-B02 showed the highest identity to the 16S rRNA gene from the cultured organism *Desulfotomaculum salinum* (85.8% identity), which is able to grow by sulfate reduction or fermentation (Nazina *et al.*, 2005), and accounted for 14% of all the clones in the library. SMD-B03 showed the closest match to the 16S rRNA gene from the cultured organism *Burkholderia fungorum* (99.6% identity), which is known as an aerobic bacterium (Coenye *et al.*, 2001). The SMD-B03 clones accounted for 13% of the clones in the library. SMD-B04 was closely related to the 16S rRNA gene from the cultured organism *Thermotoga lettingae* (94.6% identity), which is able to degrade methanol to H_2 and CO_2 (Balk *et al.*, 2002). SMD-B05 was closely related to the 16S rRNA gene from the cultured organism *Moorella thermoacetica* (86.7% identity), which is known as an anaerobic bacterium-producing H_2 and CO_2 through fermentation (Jiang *et al.*, 2009). SMD-B06 showed the highest identity to the 16S rRNA gene from the cultured organism *Syntrophus gentianae* (93.8% identity), which is able to ferment benzoate to H_2 and CO_2 (Schöcke and Schink, 1998). The remaining low-abundance OTUs showed closest matches to the 16S rRNA genes from anaerobic heterotroph and fermentative bacterium (SMD-B07 to SMD-B16).

Potential for microbial methane production

Methane production was observed only in the enrichment amended with $H_2 + CO_2$, similar to earlier observations found in enrichments using formation waters from a gas field in the North Sea (Gray *et al.*, 2009). In

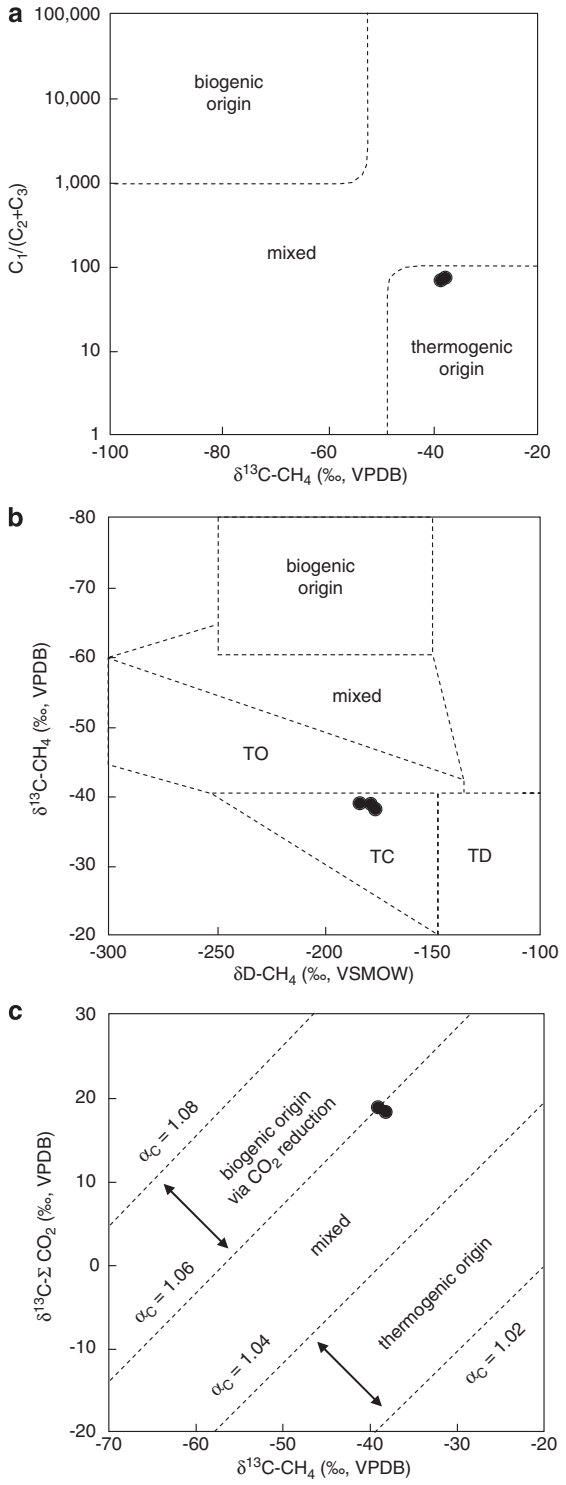

Figure 2 (**a**) Methane carbon isotope versus hydrocarbon gas composition diagram. The categorization of methane origin is according to Bernard *et al.* (1978). VPDB, Vienna Pee Dee Belemnite. (**b**) Methane carbon and hydrogen isotope diagram according to Schoell (1983). TO, thermogenic methane with oil; TC, thermogenic methane with condensate; TD, dry thermogenic methane; VSMOW, Vienna Standard Mean Ocean Water. (**c**) Carbon isotope compositions of methane and dissolved inorganic carbon (mainly bicarbonate; ΣCO_2). The broken lines of equal carbon isotopic fractionation, $\alpha_C = (\delta^{13}C-\Sigma CO_2 + 10^3)/(\delta^{13}C–CH_4 + 10^3)$, are drawn for $\alpha_C = 1.02$, 1.04, 1.06, and 1.08. The categorization of methane origin is according to Smith and Pallasser (1996).

particular, high rates of methane production were found in the enrichments incubated at 55 and 65 °C (Figure 3a). Consumption of H_2 and CO_2 in the enrichments was in good agreement with the values expected from methane production in terms of stoichiometry of H_2/CO_2-using methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) (data not shown). The bulk DNA of propagated microorganisms in the enrichment incubated at 55 °C was extracted, and then archaeal 16S rRNA genes were amplified by PCR. The archaeal 16S rRNA gene fragments were then cloned and sequenced. A total of 40 clones were randomly sequenced and were found to belong to only one OTU (H2CO2-A01) (Table 3). H2CO2-A01 was closely related to the 16S rRNA gene from the thermophilic H_2-using methanogen *M. thermautotrophicus* (Zeikus and Wolfe, 1972), and the sequence of the 16S rRNA gene of this OTU was 99.9% identical to the SMD-A03-derived sequence from the clone library for the archaeal community in the groundwater. The SMD-A03 was not the predominant OTU in the clone library for the archaeal community in groundwater. This may be due to the strong selective pressure caused by using very high concentrations of H_2 and CO_2.

Potential for H_2 production by fermentative bacteria

H_2 production by fermentative bacteria was observed in the enrichments incubated at 35, 45, 55, 65, and 75 °C. In particular, high H_2 production was indicated in enrichments incubated at 55 and 65 °C (Figure 3b). The bulk DNA of propagated microorganisms in the enrichment at 55 °C was extracted, bacterial 16S rRNA genes were amplified by PCR, and the 16S rRNA gene fragments were cloned and sequenced. A total of 37 clones were randomly sequenced and divided into three OTUs (YPGBES-B01, YPGBES-B02, and YPGBES-B03) (Table 3). Nearly 95% of clones in the clone library (YPGBES-B01) were most closely related to the 16S rRNA gene from the cultured organism *Clostridium thermocellum*, which is known as a thermophilic fermentative bacterium and is able to degrade various organic compounds, including cellulosic materials, to H_2 and CO_2 (Ng *et al.*, 1977). The remaining 5% of clones (YPGBES-B02 and YPGBES-B03) belonged to the genus *Desulfotomaculum*. The clones derived from this enrichment were not detected in the clone library for the bacterial community in groundwater. This result may be due to the selective pressure caused by using YPG medium, which contains very high concentrations of organic compounds. Archaeal 16S rRNA genes derived from the enrichment were not amplified by PCR after repeated attempts.

Potential for methane production by syntrophic consortium of H_2-producing fermentative bacteria and H_2-using methanogens

To confirm whether or not methane is produced from organic matter by cooperative catabolism of

Table 2 Archaeal and bacterial 16S rRNA gene sequences derived from microbial community in groundwater sample

OTU	*No. of clones*	*Phylogenetic group*	*Closest cultivated species (% identity)*	*Predicted metabolism*
Archaea				
SMD-A01	24	*Methanobacteriales*	*Methanobacterium aarhusense* (94.8)	ME
SMD-A02	9	*Methanobacteriales*	*Methanobacterium alcaliphilum* (94.8)	ME
SMD-A03	7	*Methanobacteriales*	*Methanothermobacter thermautotrophicus* (99.4)	ME
SMD-A04	1	*Thermococcales*	*Thermococcus gammatolerans* (82.3)	Anaerobic HE
Total	41			
Bacteria				
SMD-B01	19	*Nitrospirales*	*Thermodesulfovibrio yellowstonii* (96.8)	SR or FE
SMD-B02	9	*Clostridiales*	*Desulfotomaculum salinum* (85.8)	SR or FE
SMD-B03	8	*Burkholderiales*	*Burkholderia fungorum* (99.6)	Aerobic HE
SMD-B04	6	*Thermotogales*	*Thermotoga lettingae* (94.6)	FE
SMD-B05	4	*Thermoanaerobacterales*	*Moorella thermoacetica* (86.7)	FE
SMD-B06	3	*Deltaproteobacteria*	*Syntrophus gentianae* (93.8)	FE
SMD-B07	2	*Betaproteobacteria*	*Thauera aminoaromatica* (97.9)	Aerobic HE
SMD-B08	2	*Bacteroidetes*	*Prolixibacter bellariivorans* (87.8)	FE
SMD-B09	2	*Alphaproteobacteria*	*Parvibaculum lavamentivorans* (98.6)	Aerobic HE
SMD-B10	2	*Coriobacteriales*	*Olsenella uli* (88.7)	FE
SMD-B11	1	*Clostridiales*	*Syntrophothermus lipocalidus* (90.5)	FE
SMD-B12	1	*Clostridiales*	*Catabacter hongkongensis* (84.7)	Anaerobic HE
SMD-B13	1	*Clostridiales*	*Desulfosporosinus auripigmenti* (83.8)	Anaerobic HE
SMD-B14	1	*Flavobacteriales*	*Brumimicrobium mesophilum* (86.2)	FE
SMD-B15	1	*Solibacterales*	*Solibacter usitatus* (90.0)	Anaerobic HE
SMD-B16	1	*Clostridiales*	*Desulfitibacter alkalitolerans* (92.0)	SR
Total	63			

Abbreviations: FE, fermentation; HE, heterotroph; ME, methanogenesis; OTU, operational taxonomic unit; SR, sulfate reduction.

H_2-producing fermentative bacteria and H_2-using methanogens, we performed anaerobic cultivation using groundwater amended with YPG medium. Consequently, methane production was observed in enrichments incubated at 55 and 65 °C (Figures 4a and b). H_2 accumulated during the first few days, then gradually decreased to below the detection limit. However, methane generation began while H_2 was decreasing, and then methane continued increasing linearly in the stage in which H_2 fell below the detection limit. H_2 and methane were not detected in enrichments incubated at 35, 45, or 85 °C. In contrast, the 75 °C-incubated enrichment indicated only H_2 and CO_2 production (Figure 4c).

Discussion

We determined the chemical and isotopic compositions of the natural gas and the groundwater derived from the deep aquifer associated with the accretionary prism in Southwest Japan, and these compositions suggested that the methane was derived from both thermogenic and biogenic processes. The crossplot of the hydrocarbon gas composition $C_1/(C_2+C_3)$ over $\delta^{13}C$–CH_4 typifies methane of thermogenic origin (Figure 2a). Similarly, the $\delta^{13}C$–CH_4 versus δD–CH_4 plot also indicates methane of thermogenic origin (Figure 2b). However, the carbon isotopic ratio of the total dissolved inorganic carbon (ΣCO_2) in the groundwater ranged from +17.7‰ to +18.3‰, whereas the $\delta^{13}C$ of CO_2 in thermogenic gases ranged from −20‰ to 0‰ (Smith and Pallasser, 1996). Furthermore, this isotopically heavy ΣCO_2 is likely to cause high ^{13}C enrichment of methane in H_2/CO_2-using methanogenesis. We, therefore, took notice of the offsets between $\delta^{13}C$–ΣCO_2 and $\delta^{13}C$–CH_4 ($\Delta^{13}C_{\Sigma CO_2-CH_4}$) (Table 1). The observed $\Delta^{13}C_{\Sigma CO_2-CH_4}$ were in the range of 55.9–57.4‰, which is consistent with the values for H_2/CO_2-using methanogenesis (55 ± 10‰) rather than thermogenic reactions (Smith and Pallasser, 1996). In addition to the carbon isotopic offsets, hydrogen isotopic offsets ($\Delta D_{H_2O-CH_4}$) were calculated based on δD–H_2O and δD–CH_4 (Table 1). $\Delta D_{H_2O-CH_4}$ ranged from 161‰ to 166‰, which agreed with typical fractionation during methanogenesis through CO_2 reduction, within the equation produced by Schoell's (1980) equation:

$$\delta D_{CH_4} = \delta D_{H_2O} - 160 \pm 10‰$$

This semi-quantitative empirical relationship has been reported by Aravena *et al.* (2003) and Strąpoć *et al.* (2007), and describes the overall hydrogen isotopic fractionation along the transfer of water-derived hydrogen through intermediary reactions into biogenic methane produced by H_2-using methanogens. These carbon and hydrogen isotopic fractionations indicate that H_2/CO_2-using methanogenesis is responsible for the gas composition.

To identify methanogenic archaea in the groundwater samples, we determined the phylogenetic positions of members of the archaeal community using 16S rRNA gene clone libraries. The phylogenetic analysis revealed the dominance of species

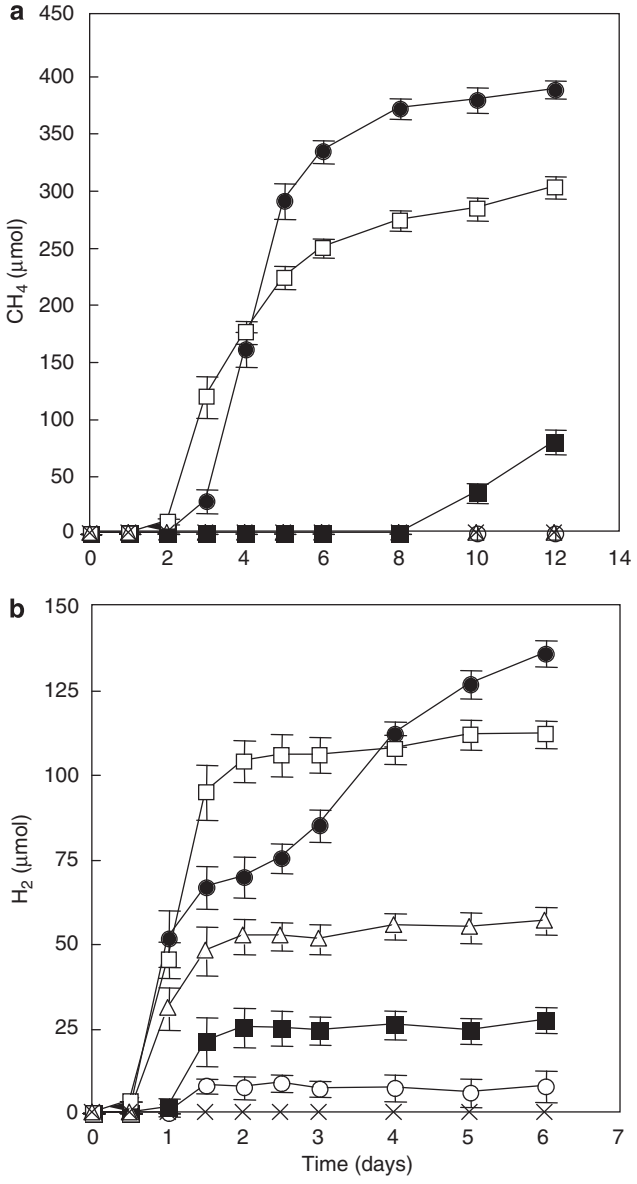

Figure 3 (**a**) Methane production from the groundwater samples amended with $H_2 + CO_2$. (**b**) H_2 production from the groundwater samples amended with YPG medium and BES (a methanogen inhibitor). The enrichments were incubated at 35 °C (○), 45 °C (■), 55 °C (●), 65 °C (□), 75 °C (△), and 85 °C (×). Data points indicate the amount of methane and H_2 accumulated in the headspaces of the culture bottles. Errors bars represent the level of variance calculated from measurements from duplicate incubations.

related to the H_2-using methanogens belonging to the order *Methanobacteriales*. Furthermore, we observed a high rate of methane production in the enrichments using groundwater amended with $H_2 + CO_2$ (Figure 3a). We confirmed that microorganisms propagated on these enrichments were closely related to *M. thermautotrophicus*, which is a thermophilic methanogen using H_2 and CO_2 for growth and methanogenesis (Zeikus and Wolfe, 1972). This combination of phylogenetic and physiological evidence strongly suggests that methane associated with the accretionary prism in Southwest Japan is produced by H_2/CO_2-using methanogenesis.

Here, one question arises: where does the H_2 come from? We determined the phylogenetic positions of bacterial community in the groundwater using 16S rRNA gene clone libraries. We detected several clones that were closely related to fermentative bacteria that can degrade organic matter to H_2 and CO_2 (SMD-B04, SMD-B05, SMD-B06, and SMD-B11). However, the dominant OTUs in the clone library were sulfate-reducing bacteria related to *T. yellowstonii* and *D. salinum* (SMD-B01 and SMD-B02). It is known that these sulfate reducers are able to grow by fermentation in low-sulfate environments (Henry *et al.*, 1994; Nazina *et al.*, 2005). In addition, a few members of the genus *Desulfotomaculum* degrade organic matter to H_2 and CO_2 through fermentation process and grow in syntrophic association with hydrogenotrophic methanogens in the absence of sulfate (Imachi *et al.*, 2006). The dominant species related to sulfate-reducing bacteria in the clone library (SMD-B01 and SMD-B02) may grow by fermentation and degrade organic matter to H_2 and CO_2 in the deep aquifer, because the groundwater has low concentrations of sulfate and sulfide (Supplementary Table S2). We further determined the potential for H_2 production by fermentative bacteria based on enrichments using groundwater amended with organic matter (as glucose, peptone, and yeast extract). Consequently, high rates of H_2 production were observed in the enrichments incubated at 55 and 65 °C (Figure 3b). Microorganisms propagated in the 55 °C-incubated enrichment were closely related to *C. thermocellum* (Ng *et al.*, 1977), confirming the growth of fermentative bacteria that degrades organic matter to H_2 and CO_2. In addition to the microbiological assays, stable

Table 3 Archaeal and bacterial 16S rRNA gene sequences derived from enrichments using groundwater amended with substrates

Substrate	*OTU*	*No. of clones*	*Phylogenetic group*	*Closest cultivated species (% identity)*	*Predicted metabolism*
H_2+CO_2	H2CO2-A01	40	*Methanobacteriales*	*Methanothermobacter thermautotrophicus* (99.4)	ME
	Total	40			
YPG+BES	YPGBES-B01	35	*Clostridiales*	*Clostridium thermocellum* (96.6)	FE
	YPGBES-B02	1	*Clostridiales*	*Desulfotomaculum ruminis* (88.8)	SR or FE
	YPGBES-B03	1	*Clostridiales*	*Desulfotomaculum putei* (93.7)	SR or FE
	Total	37			

Abbreviations: FE, fermentation; ME, methanogenesis; OTU, operational taxonomic unit; SR, sulfate reduction.

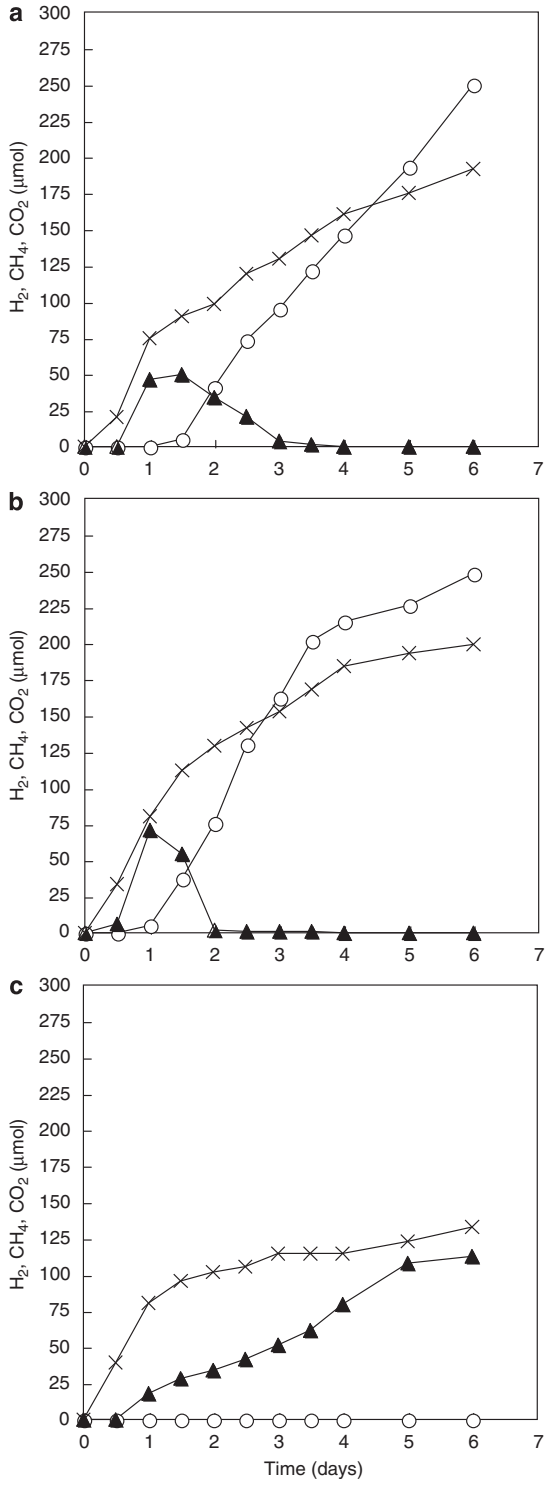

Figure 4 Gas production from the groundwater samples amended with YPG medium. The cultures were incubated at 55 °C (**a**), 65 °C (**b**), and 75 °C (**c**). Data points were obtained from measurement of cumulative H_2 (▲), CH_4 (○), and CO_2 (×) in the headspaces of culture bottles.

carbon isotopic analysis of dissolved inorganic carbon in groundwater (mainly bicarbonate) also suggested bacterial fermentation process in the deep aquifer. The dissolved HCO_3^- discussed in this study had enriched $\delta^{13}C$ values, in the range of +17.7‰ to +18.3‰. Similarly, isotopically heavy dissolved HCO_3^- has been detected from oil and gas fields, and the primary signature of this HCO_3^- is thought to be the result of anaerobic biodegradation of organic matter through fermentation processes (Carothers and Kharaka, 1980; Pallasser, 2000). These findings suggest that bacterial fermentation of organic matter in groundwater or the deep aquifer matrix leads to production of H_2 and CO_2, which can serve as the energy and carbon sources for H_2/CO_2-using methanogenesis. Although it is unclear what chemical components of the organic matter are actually used in fermentation in the subsurface environments, this can be tested in future studies.

Anaerobic biodegradation of organic matter is achieved by cooperative catabolism of diverse bacteria and archaea; in particular, it is known that syntrophic cooperation (symbioses based on nutritional cooperation) between fermentative bacteria and H_2-using methanogens leads to the conversion of diverse organic compounds to methane in anaerobic environments (Schink, 1997; Sakai *et al.*, 2009; Shimoyama *et al.*, 2009). Thus, we measured the potential for methane production by syntrophic biodegradation in enrichments using groundwater amended with organic substrates. Consequently, methane production was high in the enrichments incubated at 55 and 65 °C (Figures 4a and b). The dynamics of H_2 and methane consumption/production in these enrichments were obviously similar to earlier observations in syntrophic co-cultures of fermentative bacteria and hydrogenotrophic methanogens (Hattori, 2008). These results strongly suggest that anaerobic biodegradation supports methanogenesis through a coupled mechanism in which H_2 released from the anaerobic degradation of organic matter is taken up in the transformation of CO_2 to methane, and that these biochemical steps take place simultaneously.

The available microbiological data show that H_2-producting fermentation and H_2/CO_2-using methanogenesis contribute to methane production in the deep aquifer. Overall, our studies conclude that the past and ongoing syntrophic biodegradation of organic matter based on H_2-producing fermentative bacteria and H_2-using methanogens, as well as thermogenic methane production, contributes to the methane reserves in the deep aquifer associated with the accretionary prism in Southwest Japan.

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