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ORIGINAL PAPER

Microbial diversity and methanogenic potential in a high temperature natural gas field in Japan

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Abstract Microbial diversity and methanogenic potential in formation water samples from a dissolved-in-water type gas field were investigated by using 16S rRNA gene libraries and culture-based methods. Two formation water samples (of 46 and 53°C in temperature) were obtained from a depth of 700 to 800 m. Coenzyme F_{420}-autofluorescence indicated that 10^3–10^4 cells per ml of active methanogens were present, accounting for at least 10% of the total cell count. The 16S rRNA gene sequence analysis indicated that the diversity of *Archaea* and *Bacteria* of the two samples was quite limited; i.e., the archaeal libraries were dominated by the sequences related to *Methanobacterium formicicum* and *Methanothermobacter thermautotrophicus*, and the bacterial libraries were dominated by the sequences related to *Hydrogenophilus* and *Deferribacter*. Of the methanogenic substrates tested using the formation water-based medium, only H_2–CO_2 gave rise to methane formation. Those dominant archaeal and bacterial genera have the potential to use hydrogen for growth at the in situ temperatures, suggesting that the formation water of the Pliocene strata in the gas field has been provided with hydrogen, probably from underneath the strata, and thus ongoing active methanogenesis has been occurring to date.

Keywords Methanogen · Hydrogenotroph · Acetotroph · Gas field · Formation water · *Archaea* · Methane · Anaerobes

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Introduction

Biogenic methane is generally distinguishable from thermogenic methane (formed via thermochemical degradation of organic matter) by the stable carbon ($^{13}C/^{12}C$) and hydrogen (D/H) isotopic composition of the methane as well as by the molecular ratio of methane to ethane and propane (Schoell 1980; Bernard et al. 1977; Whiticar et al. 1986). These methods indicated that some natural gases and most marine methane hydrate are primarily composed of the biogenic

methane that was produced by methanogens whereas the great majority of natural gas from oil and gas fields originates from the thermal degradation of organic matter (Rice 1992; Kvenvolden 1995; Noble and Henk 1998; Sakai et al. 1992; Sakata et al. 1997).

Methane gas deposits in interstitial water, classified as a natural gas of the dissolved-in-water type, are found in Japan. Besides such natural gas, there are very few deposits of the dissolved-in-water type of gas that have been commercially in production in the world except for those in Japan. Gas-dissolved formation water has been found in reservoir rocks that originate from the marine sediments of the Miocene to Quaternary periods. Based on geochemical analysis, the methane produced in these geological settings was shown to be microbial (Nakai et al. 1974; Igari and Sakata 1989; Sakata et al. 1997; Kaneko et al. 2002).

Belyayev et al. (1977) showed the activities of microbial methane formation in the Pliocene rocks of the Caspian depression by means of a radioactive tracer technique. However, little is known of the microbial community structure in natural gas fields where biogenic methane has accumulated, though many studies on microbial community structure in oil reservoirs have been conducted (Orphan et al. 2000; Magot et al. 2000; Grabowski et al. 2005).

To better understand the formation process of the gas deposit and to evaluate the current state of the reservoir, we performed microbial community analysis together with determination of the methanogenic potential. Here we report the phylogenetic diversity of microorganisms based on 16S rRNA gene analysis, and methanogenic activities in the high temperature formation water in a gas field in Niigata, Japan.

Materials and methods

Sampling site and formation water samples

Samples of gas-associated formation water were obtained from two commercially gas-water-producing wells on different sandstone reservoir rocks in a gas field, Niigata, Japan, on 8 March 2005. The formation water samples (NAK1 and NAK2) were obtained from the depth of 740 to 797 m and 704 to 805 m, respectively. Two water samples were derived from separate reservoir layer on the Nishiyama Formation, which was deposited in the 1.2–3.7 Ma (million years ago) with bathyal sediments of the Japan Sea. The reservoir layer NAK1 is deeper than NAK2 because of dip. These gas reservoirs are filled with ancient seawater and are not associated with oil, whereas the deeper reservoirs at around one thousand meters are associated with oil. The concentrations of methane in the gases obtained from the reservoir rocks (nearly our sampling point) in the Nishiyama stratum were 91.5% (the other 8.3% was carbon dioxide) (Japan Natural Gas Association 1980). The stable carbon isotope ratio of the methane was –63‰ and the abundance of methane to ethane and propane was 688–1,100 (Kaneko et al. 2002).

The temperature, pH and redox potential of the formation water samples were measured by a Lab pH Meter D-23 (Horiba, Kyoto, Japan) in the field. Formation water samples for measuring the methane formation activities were collected in N_2-filled sterilized glass bottles with butyl rubber stoppers and screw caps, and stored at 4°C before use. Samples for total cell counts were fixed with formaldehyde (final concentration 2%) immediately after sampling and stored at 4°C. About 2.3 l of the formation water was filtered through a 0.22-μm pore size membrane filter unit Sterivex-GV (Millipore, MA, USA) via a sterile silicon tube and a peristaltic pump for molecular analysis. This filter unit filled with water was forced off by air, the inlet and outlet were capped, and the filters were stored at –20°C until extraction.

Chemical analyses of formation water

The chemical analyses of the formation waters were performed according to the Japanese Industrial Standards (JIS K 0102). The total organic carbon (TOC) and dissolved organic carbon (DOC) were measured using a total organic carbon analyzer TOC-V_{CPH} (Shimadzu, Kyoto, Japan). Chemical oxygen demand (COD), alkalinity, and chloride and iodide were determined by titration. Bromide ion was analyzed by ion chromatography ICS-1000 (Dionex, CA, USA) by means of an Ion Pac AS9-HC column. Sulfate, nitrite, nitrate, phosphate, and ammonium were analyzed by spectroscopic methods. The cation (Na, Mg, K and Ca) concentrations were analyzed by using an atomic absorption photometer AA-660 (Shimadzu, Kyoto, Japan). Iron and HBO_2 were analyzed by the inductively coupled plasma emission spectroscope ICPS-8000 (Shimadzu, Kyoto, Japan). Acetate in the water was extracted with diethyl ether/dichloromethane (4:1). The aqueous phase was acidified to pH 1, and extracted three times with diethyl ether/dichloromethane (4:1). The organic phase was subsequently filtered through a column containing anhydrous magnesium sulfate. After the solvent was removed under vacuum, a portion of the acids was butyl-esterified with 14% boron trifluoride butylate. Upon cooling, the reaction was quenched with water, and extracted with

hexane/diethyl ether (9:1). The extracted liquid was measured by a Hewlett Packard 6890 GC system fitted with an on-column injector and flame ionization detector (Agilent Technologies, CA, USA) and a 60 m CPSil-5 CB-Low Bleed/MS 100% dimethylpolysiloxane capillary column (Chrompack Capillary Columns).

Total cell counts and methanogenic cell counts

The total microbial cell density was estimated by the direct total cell count method (Hobbie et al. 1977) except for the use of 4′,6-diamino phenylidole dehydrochloride (DAPI) instead of acridine orange. A volume of 2–30 ml of a fixed formation water sample was filtered with a 0.2-μm pore size Isopore membrane filter (25 mm in diameter, Millipore, MA, USA). The cells on the filter were stained for 10 min with DAPI solution (10 μg ml^{-1}) and examined under an epifluorescence microscope AX80 (Olympus, Tokyo, Japan). Methanogen cells were counted based on F_{420} autofluorescence (Doddema and Vogels 1978) using samples prepared in an anaerobic atmosphere. The number of cells per ml of sample was estimated based on counts of at least ten randomly chosen microscope fields and the volume of the filtered sample.

Determination of methanogenic potential

To determine whether methanogenesis could occur with only formation water, the samples were incubated at in situ temperature without any additives. The samples were dispensed in 500 ml aliquots into 680 ml sterile bottles sealed with sterile butyl rubber stoppers and screw caps in an anaerobic box. The headspace was exchanged with N_2. As a control, bromoethanesulfonate (20 mM), an inhibitor of methanogenesis (Gunsalus et al. 1978), was added to the bottle. Two samples, NAK1 and NAK2, were incubated at the in situ temperature of 55 and 45°C, respectively. All incubations were carried out in duplicate. The concentration of methane in the headspace was measured at appropriate intervals by using a gas chromatograph GC-8A (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and Unibeads C column, 60/80 mesh.

To determine the potential methanogenic activity, the formation water samples (NAK1 and NAK2) were amended with methanogenic substrates and incubated. The formation water (40 ml) was anaerobically dispensed into 70 ml sterile bottles sealed with sterile butyl rubber stoppers and aluminum crimps. Those samples were supplemented with either 4 mM acetate (head space: N_2, 0.2 MPa), 4 mM methanol (head space: N_2, 0.2 MPa), H_2–CO_2 (80:20, v/v; 0.2 MPa) or 4 mM acetate + H_2–CO_2 (80:20, v/v; 0.2 MPa), and were incubated at 55°C (NAK1) and 45°C (NAK2). All incubations were carried out in duplicate. The concentrations of hydrogen and methane in the headspace were measured as described above at appropriate intervals. After 6 weeks of incubation, the samples (10 ml each) that showed methane formation were filtered through a 0.2-μm pore size Isopore membrane filter GTTP (Millipore, MA, USA) for microbial community analysis based on 16S rRNA gene cloning, and the filters were stored at –20°C until DNA extraction.

Phylogenetic analysis of archaeal and bacterial diversity based on a 16S rRNA gene clone library

The bulk DNA of the original formation water was extracted from the filter unit by a method described by Somerville et al. (1989), and the following physical extraction was conducted. The bulk DNA solution was treated with 0.1 mm sterilized glass beads by a bead beater, FastPrep 120 Instrument (Qbiogene, CA, USA), and processed for 30 s at a speed setting of 5. After centrifugation, the supernatant was first extracted with one volume of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0) and then with one volume of chloroform–isoamyl alcohol (24:1). Finally, the extracted bulk DNA was concentrated by ethanol precipitation with distilled water.

The 16S rRNA genes were amplified from the extracted DNA solutions by PCR using AmpliTaq Gold (Applied Biosystems, CA, USA). Archaeal-specific primer Ar109F (5′-AMDGCTCAGTAACACGT-3′) (Imachi et al. 2006) and slightly modified (Weisburg et al. 1991) universal primer 1490R (5′-GGHTAC CTTGTTACGACTT-3′) were used for amplification of archaeal 16S rRNA genes. Bacterial-specific primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal primer 1490R were used for amplification of bacterial 16S rRNA genes (Weisburg et al. 1991). PCR was carried out in triplicate in 50 μl reaction volumes with a Perkin-Elmer GeneAmp PCR System 9700 (Perkin-Elmer, MA, USA). The thermal cycler program included initial denaturation at 95°C for 9 min, 20–35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final elongation step of 72°C for 10 min. To minimize the PCR bias (Kanagawa et al. 2003; Suzuki and Giovannoni 1996), the number of PCR cycles was decreased to 28 and 30 for *Archaea* and 23 and 25 for *Bacteria* in NAK1 and NAK2, respectively. The purified rRNA gene fragments were cloned with a pT7 Blue T-vector kit (Novagen, CA, USA). The clonal DNAs were amplified from ran-

domly selected recombinants by direct PCR with M13 primers (M13, 5′-GTTTTCCCAGTCACGAC-3′; T7, 5′-TAATACGACTCACTATAGGG-3′), purified with a MinElute 96 UF PCR Purification kit (Qiagen, Hilden, Germany), and used as templates for sequencing. Sequencing was performed with primer Ar109F for *Archaea* and 8F for *Bacteria*, a CEQ DTCS-Quick Start kit (Beckman, CA, USA) and a CEQ-2000 automated sequence analyzer (Beckman, CA, USA).

We obtained partial archaeal and bacterial sequences (≥500 bp) from 50 clones of each library (a total of 200 clones). The sequences with greater than 97% similarity were treated as identical. The representative clones were fully sequenced (approximately 1,300 bp for *Archaea* and 1,500 bp for *Bacteria*). The sequences were checked for chimeras using the CHIMERA_CHECK function of the Ribosomal Database Project II (Maidak et al. 2000). The sequences were compared with the similar sequences of reference organisms by BLAST search (Altschul et al. 1997).

Phylogenetic analysis of hydrogen supplemented formation water based on a 16S rRNA gene clone library

The bulk DNA of the incubated formation water with substrate (H_2–CO_2) was extracted from the filter by means of a Fast DNA kit (Qbiogene, CA, USA). The partial archaeal and bacterial sequences (≥500 bp) from 10 clones of each library were determined in the same way as described above. The number of PCR cycles was 35 cycles for *Bacteria*, and 40 and 42 cycles for *Archaea* in NAK1 and NAK2, respectively.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained have been deposited in the GenBank database under accession numbers DQ867042 to DQ867052 and EF012250 to EF012262.

Results

Characteristics of formation water

The physical properties and chemical characteristics of the formation waters were determined (Tables 1, 2). The temperatures of the two formation water samples were 53.1 and 45.8°C in NAK1 and NAK2 wells, respectively. The redox potential indicated extremely reductive conditions (<–300 mV) in the formation water in both wells. The pH values of both formation waters were approximately neutral. The TOC values were 34 and 23 mg l^{-1} in NAK1 and NAK2, respectively. The acetate concentrations of those samples ranged from 3.0 to 3.5 μmol l^{-1} (Table 1). Nitrate was below the detection limit (<0.005 mg l^{-1}) in both waters (Table 2). The concentrations of bicarbonate and ammonium ions were 450–560 mg l^{-1} and 120–130 mg l^{-1} in both samples, respectively (Table 2). The iodine concentrations were approximately 1,600 times higher than that in seawater, and the sulfate concentrations were below the detection limit (<5 mg l^{-1}), while the NaCl concentrations in the formation waters were almost the same as those in general seawater (Table 2).

Table 1 Characteristics of the samples collected in gas wells

Sample	Depth (m)	Temp (°C)	pH	Redox (mV)	TOC (mg l^{-1})	DOC (mg l^{-1})	Acetate (μM)
NAK1	740–797	53.1	6.8	–337	34	32	3.0
NAK2	703–805	45.8	6.6	–307	23	22	3.5

Table 2 Chemical compositions of the formation waters collected from NAK1 and NAK2 wells

Sample	Amount (mg l^{-1}) of:														
	HCO_3^-	Cl^-	I^-	Br^-	SO_4^{2-}	NO_2^-	NO_3^-	PO_4^{3-}	NH_4^+	Ca^{2+}	Mg^{2+}	K^+	Na^+	HBO_2	total Fe
NAK1	560	18000	81	130	<5	0.02	<0.005	0.027	120	990	110	200	11000	200	5.6
NAK2	450	19000	84	140	<5	0.012	<0.005	0.058	130	1100	180	240	11000	120	4.1
Seawater	130[a]	18980[a]	0.05[a]	67[b]	2650[a]	NA[c]	0.42[b]	NA	0.005–0.05[a]	400[a]	1270[a]	380[a]	10780[b]	NA	NA

[a] Sudo 1967

[b] Nozaki 1997

[c] data not available

Direct cell counts

The microbial population density in the formation water samples was estimated by direct epifluorescence microscopic count of the DAPI-stained cells or F_{420} autofluorescent cells. In the NAK1 water sample, the total cell density was 2.5×10^5 cells ml^{-1} and the density of methanogenic cell (mainly rods) was 4.0×10^4 cells ml^{-1}. On the other hand, the total cell density was 5.6×10^4 cells ml^{-1} and the density of methanogenic cells (mainly short rods) was 6.5×10^3 cells ml^{-1} in the NAK2 water sample. These results indicated that methanogens constitute a significant population in both formation waters.

Archaeal sequence diversity

Five unique phylotypes (sequences with greater than 97% similarity were treated as identical) were sequenced from the two libraries constructed ($N = 100$) using archaeal specific PCR primers, and all of them were closely related to methanogenic *Archaea* (Table 3). Approximately 70% of the archaeal clones from NAK1 were most closely related to the cultured organism *Methanothermobacter thermautotrophicus*, a hydrogenotrophic methanogen, and their homology was 96%. The remaining 30% of the clones were related to the genus *Methanosaeta*, which is known to be an aceticlastic methanogen generating methane from acetate. Almost all clones from NAK2 were closely related to the cultured organism *Methanobacterium formicicum*, a hydrogenotrophic methanogen, and their homology was 98% (Table 3).

Bacterial sequence diversity

Six unique phylotypes were sequenced from the two libraries constructed ($N = 100$) using bacterial specific PCR primers. In the NAK1 library, most of the sequences were closely related to *Deferribacter thermophilus* and *D. desulfuricans* (the homology was 99%, respectively), with clones related to *Pelotomaculum thermopropionicum* as minor populations (Table 4). By contrast, in the NAK2 library, all of the clonal sequences were allocated to the phylum *Proteobacteria*. The phylotype closely related to the cultured organism *Hydrogenophilus thermoluteolus* (sequence similarity: 98%) accounted for 80% of the total clones and the remaining sequences affiliated with the classes *Gammaproteobacteria* and *Deltaproteobacteria* (Table 4).

Methanogenic potential of the formation water samples

The formation water samples were anaerobically incubated in order to estimate the methane-producing potential using dissolved indigenous organic matter in the formation water. For this purpose, the water samples were not amended with additional substrates and nutrients. Methane accumulation was not detected in any of the 500 ml water samples for 3 months of

Table 3 Archaeal phylotypes retrieved from NAK1 and NAK2 samples

Sample	Type sequence	No. of clones	Phylogenetic group	Closest cultivated species	Sequence similarity (%)
NAK1	NAK1–a1	34	*Methanobacteriales*	*Methanothermobacter thermautotrophicus*	96
	NAK1-a2	2	*Methanobacteriales*	*Methanothermobacter wolfeii*	99
	NAK1-a3	14	*Methanosarcinales*	*Methanosaeta thermophila*	99
NAK2	NAK2-a1	49	*Methanobacteriales*	*Methanobacterium formicicum*	98
	NAK2-a2	1	*Methanobacteriales*	*Methanothermobacter thermautotrophicus*	99

Table 4 Bacterial phylotypes retrieved from NAK1 and NAK2 samples

Sample	Type sequence	No. of clones	Phylogenetic group	Closest cultivated species	Sequence similarity (%)
NAK1	NAK1-b1	49	*Deferribacteres*	*Deferribacter thermophilus*	99
			Deferribacteres	*Deferribacter desulfuricans*	99
	NAK1-b2	1	*Firmicutes*	*Pelotomaculum thermopropionicum*	99
NAK2	NAK2-b1	40	*Betaproteobacteria*	*Hydrogenophilus thermoluteolus*	98
	NAK2-b2	3	*Gammaproteobacteria*	*Thioalcalovibrio denitrificans*	93
	NAK2-b3	3	*Gammaproteobacteria*	*Marichromatium indicum*	92
	NAK2-b4	4	*Deltaproteobacteria*	*Desulfobulbus propionicus*	93

incubation (data not shown), suggesting that there were no remaining substrates for methanogenesis.

The formation waters were then amended with methanogenic substrates and incubated to determine the potential methanogenic activity (Fig. 1). Methane production was observed in both formation water samples when they were amended with H_2–CO_2 and acetate + H_2–CO_2. Methanogenesis occurred exponentially concomitant with the growth of methanogens as a result of amendment of the substrates. Hydrogen was completely consumed after 2 weeks (NAK1) and 3 weeks (NAK2) of incubation. There was no significant difference in the methane production between H_2–CO_2 and acetate + H_2–CO_2 in either sample, suggesting that methanogenesis was not stimulated by the presence of acetate. Based on the stoichiometry of methanogenesis from H_2–CO_2 ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$), a quarter equivalent of methane was expected to be produced from H_2. However, as shown in Fig. 1, approximately 15 and 30% of hydrogen seemed to have been used by the non-methanogenic microorganisms in NAK1 and NAK2, respectively.

The microbial communities of the two formation water samples amended with H_2–CO_2 were analyzed based on 16S rRNA gene sequencing. In the NAK1 and NAK2 samples, the predominant archaeal clones were closely related to the cultured organisms *M. thermautotrophicus* (sequence similarity: 100%) and *M. formicicum* (98%), respectively (Table 5). The bacterial clones obtained from the H_2–CO_2 supplemented formation water were closely related to *Caloranaerobacter azorensis* (sequence similarity: 92%) and *Deferribacter thermophilus* (99%) in NAK1, and *Caminicella sporogenes* (93%) and *D. thermophilus* (99%) in NAK2 (Table 6).

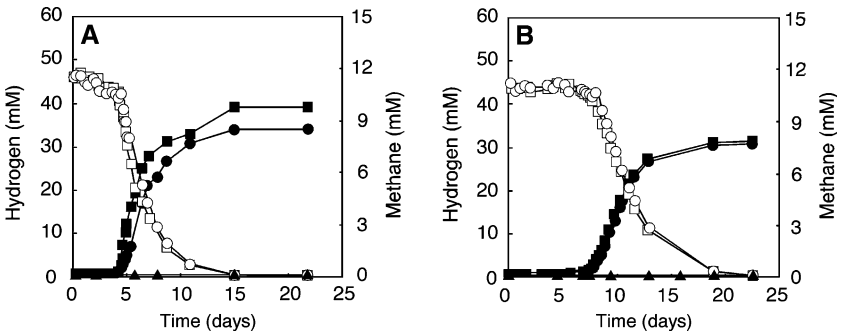

Fig. 1 Time course of CH_4 formation from the formation water samples amended with substrates. **a** NAK1; **b** NAK2. Methanogenesis from: H_2/CO_2 amended (*filled squares*), acetate + H_2/CO_2 amended (*filled circles*), and unamended (*filled triangles*) formation water samples. Hydrogen concentration: H_2/CO_2 (*open squares*); acetate + H_2/CO_2 (*open circles*). The experiments were performed in duplicate and the mean values of duplicate experiments are shown

Table 5 Archaeal phylotypes retrieved from NAK1 and NAK2 samples amended with H_2–CO_2

Sample	Substrate	Type sequence	No. of clones	Phylogenetic group	Closest species or clone name	Sequence similarity (%)
NAK1	H_2–CO_2	NAK1-aH1	8	*Methanobacteriales*	*Methanothermobacter thermautotrophicus*	100
		NAK1-aH2	2	*Thermoplasmata*	Uncultured archaeon clone 7C08	99
NAK2	H_2–CO_2	NAK2-aH1	10	*Methanobacteriales*	*Methanobacterium formicicum*	98

Table 6 Bacterial phylotypes retrieved from NAK1 and NAK2 samples amended with H_2–CO_2

Sample	Substrate	Type sequence	No. of clones	Phylogenetic group	Closest species or clone name	Sequence similarity (%)
NAK1	H_2–CO_2	NAK1-bH1	7	*Firmicutes*	*Caloranaerobacter azorensis*	92
		NAK1-bH2	3	*Deferribacteres*	*Deferribacter thermophilus*	99
NAK2	H_2–CO_2	NAK2-bH1	7	*Firmicutes*	*Caminicella sporogenes*	93
		NAK2-bH2	2	*Deferribacteres*	*Deferribacter thermophilus*	99
		NAK2-bH3	1	*Firmicutes*	*Thermovirga lienii*	92

Discussion

In this report, we determined the chemical compositions, microbial community structure, and culture-dependent methanogenic potential of natural gas-associated waters, which are characteristic of natural gas fields in Japan, and are referred to as the dissolved-in-water type of natural gas. The chemical analyses revealed that the chemical composition of the formation water was characterized by high amounts of iodine, bicarbonate and ammonia, and a negligible amount of sulfate (Table 2). Such a characteristic was commonly observed in formation waters from oil and gas field in Japan (Sudo 1967). In particular, the iodine concentration in the formation water was significantly higher than that in general seawater. The accumulation mechanisms of iodine is, however, still controversial and the impact of such high iodine concentrations on in situ methane formation remains unclear.

Direct cell count by epifluorescence microscopy revealed that the original formation water samples harbored 10^3–10^4 cells of methanogens per ml that accounted for approximately 10% of the total cells in both samples. Considering the fact that many *Methanosaeta*, an aceticlastic methanogen, exhibit no or very weak autofluorescence (Patel 1984; Zinder et al. 1987), the F_{420}-based autofluorescence microscopic count may have underestimated the total methanogenic populations, particularly in the case of the NAK1 sample that contained a significant number of *Methanosaeta* clones (Table 3). In fact, thick sheathed rods morphologically resembling *Methanosaeta* but showing no autofluorescence were present in the NAK1 formation water.

The major archaeal groups in the NAK1 formation water sample were closely related to the cultured organisms, *Methanothermobacter thermautotrophicus* (Zeikus and Wolfe 1972) and *Methanosaeta thermophila* (Kamagata and Mikami 1991), whose optimum temperatures for growth are 65–70 and 55–65°C, respectively. The major archaeal constituent in the NAK2 formation water sample was closely related to *Methanobacterium formicicum*, which is capable of utilizing hydrogen as an energy source. The optimum temperature for the growth of *M. formicicum* is 37–45°C (Bryant and Boone 1987). The optimum temperatures of those related strains were well correlated with the temperatures of the environments from which those clones were obtained (Table 1).

We also determined bacterial communities in the two formation water samples based on 16S rRNA gene cloning analysis. Very interestingly, both formation waters harbored simple bacterial communities. In sample NAK1, almost all of the clonal sequences were affiliated with *D. thermophilus* and *D. desulfuricans* (Table 4). The culture representative of the genus *Deferribacter* (*D. thermophilus*) was isolated from a submarine oil reservoir and could utilize hydrogen, acetate, some complex organic compounds as electron donors, and Fe(III), Mn(IV) and nitrate as electron acceptors (Greene et al. 1997). In the sample NAK2, all of the clones were within the phylum *Proteobacteria* and clones closely related to *H. thermoluteolus* accounted for 80% of the total clones analyzed (Table 4). The culture representative of the genus *Hydrogenophilus* (*H. thermoluteolus*) is known to be an aerobic, facultatively hydrogen-oxidizing chemolithotrophic, thermophilic bacterium (Hayashi et al. 1999). However, another species within the same genus, *H. hirschii* (Stohr et al. 2001), was later isolated and found to grow not only on H_2–CO_2 under microaerophilic conditions, but on H_2–CO_2 with nitrate reduction under anaerobic conditions. The closely related sequences of the genus were also obtained from anaerobic deep aquifer of the Great Artesian Basin in Australia (Kimura et al. 2005).

D. thermophilus and *H. thermoluteolus* share several common features: first, they are thermophilic (optimum temperatures for growth are 60 and 50–52°C, respectively) hydrogenotrophs; second, they are not strictly anaerobic, but *D. thermophilus* and other species of *Hydrogenophilus* prefer anoxic conditions for their growth. These results strongly suggest that the dominant bacteria present in the formation waters are involved in hydrogen metabolism.

Our community analyses strongly suggest that formation water environments form special niches for particular thermophilic methanogens and hydrogenotrophic bacteria. Since there was no remarkable difference in chemical constituent between the two samples, the major factor affecting the microbial community structure was considered to be in situ temperature in the gas field. In addition, the bacterial community might be affected by an electron acceptor. However, what type of material could be used as an electron acceptor in the reductive environment is uncertain. In particular, thermophilic methanogens play a key role in producing methane, although methane was not produced simply by incubating the water samples at the in situ temperatures, probably because the substrates in the samples were already depleted. We therefore determined the methanogenic potential using the formation water samples amended with several methanogenic substrates. Of the substrates tested, methane production was clearly observed with H_2–CO_2 regardless of the presence or

absence of acetate that possibly acts as a carbon source, and methanogenesis continued until the hydrogen was completely consumed (Fig. 1). Also, the experiments showed there was competition between hydrogenotrophic methanogens and hydrogenotrophic bacteria in terms of hydrogen consumption. These results suggested that the formation water taken from the deep subsurface environment contained sufficient inorganic elements except for hydrogen. Here the most important question arises: where do the substrates, in particular hydrogen, come from? There are several possibilities: (1) High microbial activity was reported at the sandstone-shale interfaces, since a relatively high concentration of organic matter is present within shale (Krumholz et al. 1997). Organic matter might be degraded by microorganisms at the interface resulting in hydrogen formation. (2) The thermal alteration of organic matter (catagenesis) could occur at the ambient temperature in this site, and the condensation of aromatics could result in the formation of pericondensed polycyclic aromatics concomitant with hydrogen release in a reservoir (Hunt 1979). (3) Cataclasis of silicate minerals associated with faulting can produce hydrogen (Sugisaki et al. 1983, Apps and Kamp 1993), and that hydrogen might diffuse through the sediment column. (4) Radiolysis of water by radioactive isotopes (uranium and thorium and their daughters) can release hydrogen (Apps and Kamp 1993).

Our study demonstrates that the formation waters in the high temperature natural gas field harbor active methanogens and bacteria, and that on-going methanogenesis may be occurring to date. In addition, the high temperature interstitial water environment together with other strata that could supply hydrogen may form a special niche for those particular organisms to actively grow.

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