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Physicochemical impacts associated with natural gas development on methanogenesis in deep sand aquifers

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The Minami-Kanto gas field, where gases are dissolved in formation water, is a potential analogue for a marine gas hydrate area because both areas are characterized by the accumulation of microbial methane in marine turbidite sand layers interbedded with mud layers. This study examined the physicochemical impacts associated with natural gas production and well drilling on the methanogenic activity and composition in this gas field. Twenty-four gas-associated formation water samples were collected from confined sand aquifers through production wells. The stable isotopic compositions of methane in the gases indicated their origin to be biogenic via the carbonate reduction pathway. Consistent with this classification, methanogenic activity measurements using radiotracers, culturing experiments and molecular analysis of formation water samples indicated the predominance of hydrogenotrophic methanogenesis. The cultivation of water samples amended only with methanogenic substrates resulted in significant increases in microbial cells along with high-yield methane production, indicating the restricted availability of substrates in the aquifers. Hydrogenotrophic methanogenic activity increased with increasing natural gas production from the corresponding wells, suggesting that the flux of substrates from organic-rich mudstones to adjacent sand aquifers is enhanced by the decrease in fluid pressure in sand layers associated with natural gas/water production. The transient predominance of methylotrophic methanogens, observed for a few years after well drilling, also suggested the stimulation of the methanogens by the exposure of unutilized organic matter through well drilling. These results provide an insight into the physicochemical impacts on the methanogenic activity in biogenic gas deposits including marine gas hydrates.

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Introduction

Methanogenesis, the biological formation of methane, is the terminal process of the degradation of organic matter in anoxic environments where electron acceptors other than CO_2 are depleted. Although methanogenic archaea form methane from very limited substrates (H_2/CO_2, methylated compounds and/or acetate), these microorganisms have a central role in the global carbon cycle (Kotelnikova, 2002; Thauer *et al.*, 2008). Methane in gas hydrates, found extensively offshore in deep continental margin sediments, is suggested to be primarily derived by methanogenesis via carbonate reduction (Kvenvolden, 1995; Milkov, 2005). Despite the presence of large methane hydrate reserves in the marine subsurface (Kvenvolden, 1993), the detailed mechanisms of microbially mediated gas hydrate formation are poorly understood, primarily due to their low metabolic activities over geological timescales (D'hondt *et al.,* 2002) and limited access.

The Minami-Kanto gas field is the largest natural gas deposit of the dissolved-in-water type in Japan, accounting for ~90% (in volume) of the total domestic production of natural gas of this type. The reservoir rocks of the gases and associated formation water consist of turbidite sand and mud sediments that originated as eroded debris

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transported by rivers and deposited in deep marine environments by turbidity currents during the Plio-Pleistocene period. Oil is not associated with the gas due to low geothermal heat flow and short-term sediment diagenesis (Uyeda, 1972). The organic matter in the gas-bearing sediments is thus composed predominantly of low-maturity type III kerogen (Yoshioka *et al.*, unpublished data), which is derived from terrestrial higher plants. The methane in the natural gas is thought to be of biogenic origin based on its light carbon isotopic composition ($^{13}C/^{12}C$) and the predominance of methane over ethane and propane (as high as 99%) (Igari and Sakata, 1989). Previous studies have suggested that methanogenesis is the terminal step of anaerobic degradation in the aquifers of this gas field (Mochimaru *et al.*, 2007). However, the *in situ* methanogenic pathway and the extent of its activity remain uncharacterized. As the rock facies (turbidite), type of organic matter (type III kerogen) and origin of methane (biogenic) in the Minami-Kanto gas field are all common among marine gas hydrate areas such as the Nankai Trough (Waseda and Uchida, 2004; Fujii *et al.*, 2009) and the Cascadia Margin (Adams, 1990; Pohlman *et al.*, 2009); the aquifers of this gas field are a potential analogue for the habitats of microorganisms that produce methane in marine gas hydrates.

In this gas deposit, gas-bearing sand aquifers are distributed in multiple geological formations, which gently dip towards the northwest. The depth of gas-associated formation water collection thus depends on the location, resulting in variations in the water chemistry and in the natural gas productivity of the well. Well drilling and subsequent natural gas production cause physicochemical changes in subsurface environments. In addition to geochemical parameters, these physicochemical changes may affect methanogenesis in confined sand aquifers. However, the physicochemical impact on the abundance, diversification and activity of indigenous methanogens in deep subsurface environments remains poorly understood (Kirk *et al.*, 2012), despite the worldwide exploration and development of biogenic gas deposits.

The objectives of this study were to determine the methanogenic activity rate and the pathways and diversity within the gas-bearing aquifers in the Minami-Kanto gas field and to determine their changes due to the development of natural gas, that is, well drilling and natural gas production. We have examined the methanogenic activities in gas-associated formation water samples collected from commercial wells using a ^{14}C-radiotracer and cultivation-based techniques, combined with a community structure analysis through the 454 pyrosequencing of archaeal 16S rRNA genes. We have compared the results with those of other subsurface samples, particularly sub-seafloor sediments, and discussed their implications for the formation of methane in marine gas hydrates.

Materials and methods

Descriptions of site and wells

Gas-associated formation water samples were collected from 24 commercial production wells at the Minami-Kanto gas field in Chiba prefecture, located in central Japan (Supplementary Figure S1 in the Supplementary Material). The reservoir rocks of this gas field are repeating sequences of turbidite (alternating beds of sandstone and mudstone) from the Kokumoto to Namihana formations in the Kazusa group. The depth ranges of the tops and bottoms of well strainers are 200–1500 m and 400–1800 m, respectively. Other well properties possessed by the natural gas company (that is, the natural gas productivity, age and geological formations) were used as parameters for comparison.

The details of natural gas productivity in a well are as follows: in this dissolved-in-water type gas field, the natural gas productivity of a commercial well is represented by the ratio of natural gas to associated water (hereafter referred to as the gas–water ratio). In many wells except for the regions of BHC1 and HSY1 (Supplementary Figure S1 in the Supplementary Material), the gas–water ratio increases, peaks and gradually decreases during the gas production period. As discussed in the Discussion section, the phase of this gas–water ratio profile is taken into account for consideration of its physical impact on the gas-bearing sediments. The 24 wells used for sample collection were divided into three groups. At the time of sampling, the gas–water ratios of the AOY2, AOY3, BOM6, GOT3, KDO1, KNS1, MCI2 and MOB7 wells were in the increasing phase, those of the AOY1, BOM6, BRS1, GOT1, GOT2, GOT4, GOT5, HBS1, HBS2, HKN1, MCI1, MOB4 and NTK1 wells were in the decreasing phase (after the peak), that of the SRT1 well was in the terminating phase, and those of the remaining wells (BHC1 and HSY1) did not show this profile. In the areas of BHC1 and HSY1, the gas–water ratio was nearly constant throughout the gas production period. In this study, the gas–water ratio at the time of sampling was defined as that measured for 1 month immediately before sample collection. The gas–water ratios in the commercial wells were measured by the company according to Japanese Industrial Standards. The ratio in the SRT1 well was not available.

Sample collection

Water samples were collected in sterilized tubes connected to the well valve after the first several litres of water were discarded. The samples for methanogenic activity measurement and cultivation were collected in sterilized glass bottles with butyl rubber stoppers and screw caps. Four litres of water samples was used for molecular analysis filtered using a 0.2-μm pore Millipore Express Plus membrane filter (Millipore, Billerica, MA, USA) and

stored at −20 °C. The samples used for total cell counts were fixed with formaldehyde at a final concentration of 2% (v/v) immediately after sampling.

Geochemical analysis and direct cell counts

The stable carbon and hydrogen isotope ratios of methane were measured using a Trace GC Ultra gas chromatograph connected to a DELTA V plus isotope ratio mass spectrometer via a GC IsoLink combustion/pyrolysis interface (Thermo Fisher Scientific, Bremen, Germany), using NGS3 as an isotope reference material. The concentrations of total organic carbon and chemical compositions of the sample water were measured according to the methods described by Mochimaru *et al.* (2007). Nitrate and nitrite were measured by absorption spectrophotometry using an autoanalyzer (AACS II, Bran + Luebbe, Norderstedt, Germany).

A fixed water sample was filtered through a 0.2-μm pore size Isopore membrane filter (Millipore), stained for 10 min with SYBR green solution (10 μg ml^{-1}) and observed under an epifluorescence microscope (Olympus, Tokyo, Japan).

Methanogenic activity measurement

The methane production rates were measured using radiotracer experiments as described previously (Yoshioka *et al.*, 2009). A 20 ml water sample in a 50-ml serum vial sealed with a butyl rubber stopper and aluminium crimp was injected with either the radiotracers ^{14}C-bicarbonate (10 μl, 199 kBq) or [2-^{14}C]-acetate (10 μl, 81 kBq). Incubation was conducted under an atmosphere of N_2/CO_2 (80:20, v/v) for 14, 28 and 42 days. The temperature was set to 35 °C or 25 °C for BHC1 or the other samples, respectively. The reactions were terminated by the addition of 1 M NaOH. The produced $^{14}CH_4$ was oxidized to $^{14}CO_2$ by flushing the bottle headspace with He through a furnace containing CuO. $^{14}CO_2$ was collected in vials containing 2-phenethylamine and mixed with 10 ml Permafluor E$^+$ scintillation cocktail (Perkin Elmer, Waltham, MA, USA). The total ^{14}C-activities were determined in triplicate for each cultivation period with a Tri-Carb 3100TR liquid scintillation counter (Perkin Elmer). The ^{14}C-activity at zero time was used as a control.

The methane production rate was calculated using the equation $a_p/(a_r t)C$, where a_p and a_r are the activities of the product and added reactant (that is, CO_2 or acetate), respectively; t is the incubation period; and C is the *in situ* concentration of the reactant.

Cultivation of formation waters amended with methanogenic substrates

The water samples were dispensed in 60-ml aliquots into 100-ml serum vials sealed with butyl rubber stoppers and aluminium crimps under an atmosphere of N_2/CO_2 (80:20, v/v). The cultures were supplemented with either a mixture of H_2/CO_2 (80:20; 0.1 MPa), 20 mM acetate, 20 mM methanol and 20 mM trimethylamineor 20 mM 2-bromoethanesulphonic (BES) acid. The cultivation temperatures were the same as those for the ^{14}C-radiotracer experiments. The time courses of methane production and hydrogen consumption were measured by gas chromatography with a thermal conductivity detector. After the methane production was terminated, 10 ml of the sample cultures were collected by filtration and used for the molecular analysis. The microbial cell density in the H_2/CO_2-amended cultures was measured by total cell counting.

DNA extraction and 454 pyrosequencing of archaeal 16S rRNA amplicons

DNA extractions from the filtered water samples and methanogenic cultures were performed using the PowerWater kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol.

The V3 and V4 regions of the archaeal 16S rRNA genes were amplified from the original formation waters (Supplementary Text S1). The primers used in this study are described in Supplementary Table S1 in the Supplementary Material. Pyrosequencing was performed using a 454 Life Sciences GS FLX Titanium platform (Roche, Basel, Switzerland) at Hokkaido System Science (Sapporo, Japan).

Clone library construction and Sanger sequencing from methanogenic cultures

DNA extracted from the methanogenic cultures was subjected to clone library analysis. The archaeal 16S rRNA genes were amplified using the primer pair Arc109F and Univ1492R with 20 PCR cycles. The products were purified, cloned into the pCR4–TOPO vector (Life Technologies, Carlsbad, CA, USA) and sequenced by the dideoxynucleotide chain-termination method using BigDye terminator reagents (Life Technologies).

Sequence analysis

The 454 pyrosequencing reads were analyzed using Mothur ver. 28 (Schloss *et al.*, 2009) according to the standard operating procedure (http://www.mothur.org/wiki/Schloss_SOP) (For details, see Supplementary Text S2). The quality-filtered pyroreads combined with the Sanger sequencing reads were aligned and clustered into operational taxonomic units (OTUs). Taxonomic classification of each unique OTU was performed using a Bayesian classifier based on the Silva taxonomy (Pruesse *et al.*, 2007). The taxonomic information of the OTUs (97%) was obtained from the majority consensus taxonomy of the sequences within the OTU. Differences in the archaeal community

structures among the original water samples were visualized using nonmetric multidimensional scaling plots. The representative sequences in each OTU of the methanogenic culture clones were aligned with their relatives and used for the contraction of phylogenetic trees.

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences are AB819999 to AB820007. The 454 sequences were deposited in the DDBJ Sequence Read Archive database under accession number DRA001051.

Results

Geochemical characteristics of gas-associated formation water samples

The physicochemical properties of the water samples are summarized in Supplementary Table S2 in the Supplementary Material. The stable carbon isotopic ratios of methane (δ^{13}C-CH_4) ranged from -67.2 to -60.8‰, which is consistent with the values obtained from different wells in the same gas field (Igari and Sakata, 1989). The hydrogen isotopic ratios of methane (δD-CH_4) ranged from -182 to -171‰. Both isotopic ratios are similar to those of gas hydrates from the Nankai Trough (Waseda and Uchida, 2004) and Cascadia Margin (Pohlman *et al.*, 2009). When these methane isotopic compositions were plotted using the diagram of Whiticar (1999), the gas samples were assigned a biogenic origin by carbonate reduction (Figure 1), as were the gas hydrates.

The water temperature ranged from 15.8–35.3 °C with an average of 22.8 °C. The highest water temperature was measured in BHC1. The redox potential ranged from -313 to -139 mV. Overall, the water samples were characterized as paleo seawater with higher concentrations of HCO_3^-, Br^-, I^-, NH_4^+, total organic carbon and almost complete depletion of SO_4^{2-} compared with the present seawater. The sulphate concentration was lower than 3.1 mg l^{-1} (detection threshold) except in the KNS1 (26 mg l^{-1}), BHC1 (47 mg l^{-1}) and AOY1

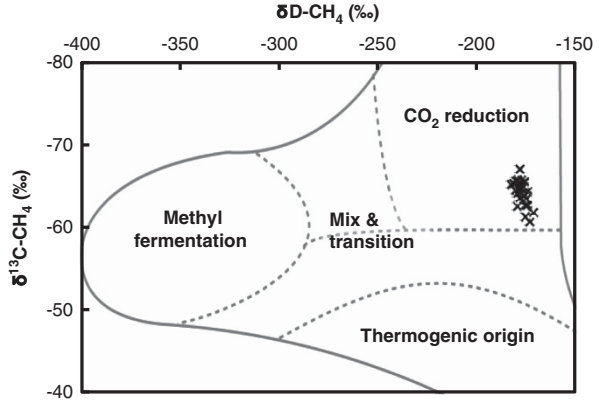

Figure 1 Relationship between δD and δ^{13}C of methane in 24 samples to estimate their origin after Withicar (1999).

(82 mg l^{-1}) wells. A low concentration of Cl^- (<15 000 mg l^{-1}) in the MCI2, AOY1, KNS1 and HBS2 well water may be due to the mixture of meteoric water (Maekawa *et al.*, 2006). Nitrate and nitrite were also nearly depleted, not exceeding 0.12 mg l^{-1}.

The number of microbial cells in the water samples ranged from 10^2–10^4 cells ml^{-1} (Supplementary Table S2 in the Supplemental Material). Those in the AOY2, BRS1, GOT2 and NTK1 wells were not counted because of the high density of suspended solids trapped on the filter together with cells. The microbial cell densities in this gas field are relatively low or comparable with those previously reported in deep aquifers (10^2-10^6 cells ml^{-1}) (Pedersen, 1993).

Methanogenic activity rates in original formation water samples

The methanogenic activity was measured by ^{14}C tracer techniques. Methane production rates through the hydrogenotrophic pathway ranged from 0.45 to 3.35 pmol ml^{-1} per day, which were three orders of magnitude higher, on average, than those via the acetoclastic pathway (Table 1). No correlation was found between the production rates of the two pathways. The acetoclastic methane production rates in GOT1 and MOB7 and the methylotrophic methane production rates in all samples could not be estimated because the concentrations of acetate

Table 1 Methane production rates via the hydrogenotrophic and acetoclastic pathways in the original formation water samples

	Methane production rate	
	Hydrogenotrophic (pmol ml^{-1} per day)	*Acetoclastic (femtomol ml^{-1} per day)*
AOY1	3.2	2.1
AOY2	0.7	0.6
AOY3	1.1	4.0
BHC1	1.4	0.2
BOM3	0.5	0.5
BOM6	1.0	4.7
BRS1	2.0	0.1
GOT1	0.6	ND
GOT2	0.5	0.2
GOT3	1.8	6.1
GOT4	2.7	0.1
GOT5	1.8	1.0
HBS1	1.3	0.1
HBS2	2.2	1.3
HKN1	0.9	0.3
HSY1	0.9	0.6
KDO1	1.7	0.6
KNS1	2.6	3.6
MCI1	0.7	0.2
MCI2	1.1	0.7
MOB4	3.3	0.4
MOB7	2.7	ND
NTK1	1.9	1.1
SRT1	0.4	0.6

Abbreviation: ND, Not determined.

and methanol, respectively, were below the detection limits.

The methane production rates via both hydrogenotrophic and acetoclastic pathways were not correlated with the measured geochemical data, such as, water temperature, Cl^- and SO_4^{2-} concentrations, and total organic carbon. Interestingly, however, when the sampled wells were divided according to the gas–water ratio profile (see Materials and methods) and those in the increasing phase at the time of sampling were selected, the hydrogenotrophic methane production rates of the water samples correlated significantly with the gas–water ratio in the corresponding wells ($r=0.94$, $P<0.001$ by linear regression *t*-test) (Figure 2). This relationship became ambiguous if all wells, except for SRT1, were included.

Archaeal community structure in the original formation water samples

16S rRNA gene amplicon pyrosequencing was used to determine the archaeal community structures within the water samples. The Good's coverage values ranged between 90–99%. The rarefaction curves of samples AOY1, GOT1, GOT2 and GOT4 indicate insufficient sampling (Supplementary Figure S2 in the Supplementary Material). The dominant archaeal groups (> 25% of total) in each sample were assigned to the classes *Methanomicrobia*, *Methanobacteria*, Deep Sea Hydrothermal Vent Group 6 in the order *Halobacteriales* or the order *Thermoplasmatales* (Supplementary Figure S3 in the Supplementary Material). An nonmetric multidimensional scaling biplot revealed no significant correlation between the archaeal community structures of the 24 original water samples and the environmental variables (Supplementary Figure S4 in the Supplementary Material).

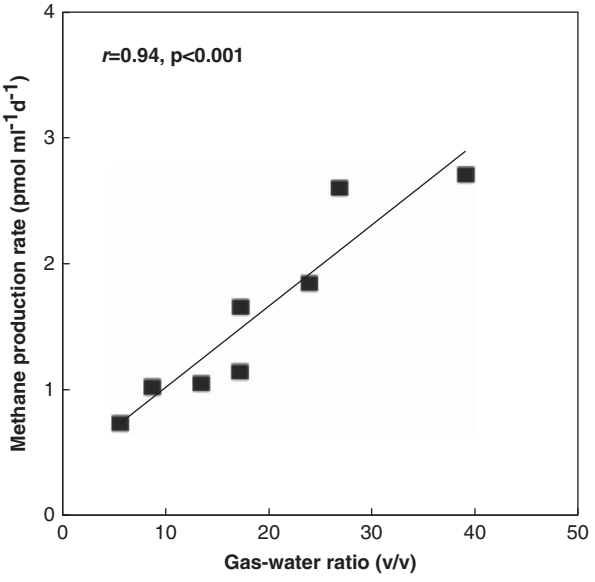

Figure 2 Correlation between the hydrogenotrophic methanogenic activities in the original formation water samples and the natural gas productivity of the corresponding wells. The natural gas productivity is represented by the gas–water ratio (see Methods). A regression line fitting to the data points is also shown.

Diversity of methanogens in the original formation water samples

Among the pyrosequence reads, 66 out of 983 OTUs (97% threshold) were assigned to 19 different genera of methanogens. The relative abundance of methanogens within archaea varied among the samples, ranging 2.4–99.6% (Figure 3). The dominant methanogenic OTUs were classified as either hydrogenotrophic *Methanocalculus* or *Methanobacterium* in almost all samples. By contrast, an abundance of hydrogenotrophic *Methanoculleus* and methylotrophic *Methanosarcina* and *Methanolobus* was found in the BOM6 sample. *Methanosarcina* was also abundant in SRT1. The acetoclastic methanogen assigned to *Methanosaeta* was detected in many samples but accounted for a minor fraction, except in MCI1 in which *Methanosaeta* accounted for 8.1% of the total archaea. Overall, the predominance of hydrogenotrophic over acetoclastic or methylotrophic methanogens was observed in all samples. The taxonomic classification and relative abundance of each methanogenic OTU are summarized in Supplementary Table S3 in the Supplementary Material.

Detection of methanogens by the cultivation experiments

Methanogenic potential was also assessed by the culturing of water samples with methanogenic substrates. During the incubation period of 1 year, methane was detected in 17 of 24 samples amended with H_2/CO_2 but only in 1 and 6 samples amended with acetate and methanol plus trimethylamine, respectively (Table 2). No methane was produced from any water samples amended with 2-bromoethanesulphonic. An average of 87% (v/v) of the maximum theoretical yield of methane was produced in H_2/CO_2-amended cultures. In the H_2/CO_2-amended cultures of AOY1, BOM6, GOT1, HYS1, MCI1, MOB7 and SRT1, the microbial population density increased up to 10^7 cells ml^{-1} after cultivation (Table 2).

A phylogenetic tree based on near full-length 16S rRNA gene sequences of clones showed that all OTUs from the methane-producing cultures were assigned to methanogens, including those predominantly detected in the original water samples (Figure 4). In H_2/CO_2 amended cultures, the sequences were mainly assigned to *Methanobacterium*, *Methanosarcina*, *Methanocalculus* or *Methanoplanus*. In the cultures of HSY1, BOM6 and SRT1 amended with methyl compounds, methylotrophic

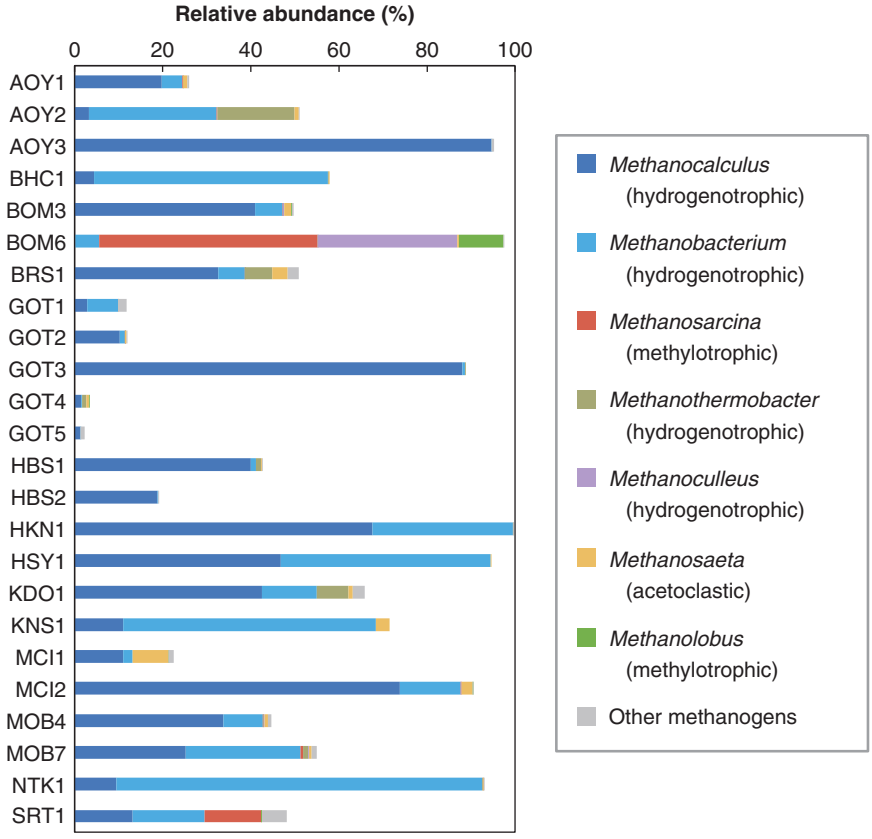

Figure 3 Methanogenic community structure in the original formation waters based on 454-pyrosequencing of 16S rRNA gene amplicons. The relative abundances of the dominant methanogens in the total archaea are shown based on the taxonomic assignments of the methanogenic OTUs.

Methanosarcina and/or *Methanolobus* were predominantly detected. Their predominance in both the original and cultured water samples of BOM6 and SRT1 indicates that methylotrophic methanogenesis occurs in aquifers of these samples and that they may not have been contaminations, as they grew in the formation water (that is, in high salt concentrations). The genera with which the OTUs were clustered together in this phylogenetic tree were identical to what was presumed by Bayesian classification of the partial sequences, showing the validity of the taxonomic assignment based on partial 16S rRNA gene sequences including the pyroreads.

Discussion

A total of 24 formation water samples collected from the sand aquifers of commercial wells in the Minami-Kanto gas field were examined to elucidate the physicochemical impact of well drilling and natural gas production on indigenous methanogens. We first discuss the methanogenesis occurring in the aquifers without taking into account the effects of these natural gas developments.

The radiotracer measurements, as well as 454-pyrosequencing analysis and cultivation experiments, clearly showed the predominance of methanogenesis via the hydrogenotrophic pathway in the aquifers. This result is in good agreement with the empirical isotopic classification of the origin of methane, strongly suggesting that the methane deposited in this gas field formed primarily by carbonate reduction. The predominance of hydrogenotrophic methanogenesis, identified using radiotracers, was also reported in marine gas hydrate-bearing sediments of the Nankai Trough and the Cascadia Margin, where the estimated *in situ* temperatures are below 25 °C (Newberry *et al.*, 2004; Yoshioka *et al.*, 2010). However, in low-temperature oil deposits, molecular analysis revealed the predominance of acetoclastic methanogens (Grabowski *et al.*, 2005; Pham *et al.*, 2009). The consistency in the methanogenic pathway with subseafloor sediments, together with the similar isotopic compositions of methane, further supports the possibility of the Minami-Kanto gas field being an analogue for marine gas hydrate-bearing areas.

The cultivation experiments showed that regardless of the low microbial population density and low methane production rates in the formation water

Table 2 Levels of methane production and microbial cell density after the cultivation of the formation waters with methanogenic substrates

	Methane production[a]			*Number of cells (ml^{-1})*[b]
	H_2/CO_2	*Ace*	*Meth/TMA*	*H_2/CO_2*
AOY1	++++			1.4E+07
AOY2				
AOY3				
BHC1				
BOM3				
BOM6	+++		+	3.3E+07
BRS1	++++			
GOT1	++++		+	2.2E+07
GOT2				
GOT3	++++			
GOT4				
GOT5	++++			
HBS1	++++			
HBS2	++++			
HKN1	++++			
HSY1	++++		++	3.9E+07
KDO1	++++			
KNS1	++++			
MCI1	++++		+	5.6E+07
MCI2	++++	++++	+++	
MOB4				
MOB7	++++			7.9E+06
NTK1	+++			
SRT1	++++		++	8.6E+06

Abbreviations; Ace, acetate; Meth, methanol, TMA, trimethylamine.
[a]The number of + indicates the percentage of maximum theoretical yield of methane. ++++, >75%; +++ >50%; ++, >25%; +, >0%.
[b]The number of cells were counted only in the H_2/CO_2 amended cultures that were also subjected to the clone library analysis.

samples, high-yield methane production along with a significant increase in cell density was observed in many samples after amendment only with H_2/CO_2. According to Gray *et al.* (2009), methane was produced in cultivation of the formation water from North Sea oil/gas deposit amended only with H_2/CO_2, but the yield of methane was much lower than expected unless further N- and *P*-sources and trace metals were added. Because H_2/CO_2 was amended at almost the same level as our study, this contrast clearly indicates the primary limitation of methanogenic substrates rather than other growth factors in the studied aquifers.

Effect of natural gas production on hydrogenotrophic methanogenesis

When we focused on the eight wells with an increasing phase of gas–water ratio profile, there was a strong correlation between the hydrogenotrophic methane production rates of the water samples and the gas–water ratio of the corresponding wells. This correlation can be interpreted as follows: the gas–water ratio, which represents natural gas productivity in a well, increases as gas-associated formation water is produced from many wells at the studied site because excess natural gases accumulated within the mud layers are released into the sand layers (Ueno *et al.*, 1964). As the gas and associated water are produced from the sand aquifers, the *in situ* fluid pressures become lower than those in the mud layers, resulting in the migration of fluids from the mud to sand layers (Figure 5b). Kimura *et al.* (1993) numerically simulated these processes. The gas-associated formation water within the mud layers migrates, together with the excess gases, into the sand layers. Consequently, the amounts of formation water that migrate from the mud to sand layers should be correlated with the current gas–water ratio. As the pressure difference declines, the increase in the gas–water ratio is terminated, followed by a decrease due to the reduction in the driving force for migration of excess gas from the mud layers (Figure 5a). In this phase, the formation water still migrates from the mud layers because the pressure difference is present as long as natural gas and associated water are produced. Accordingly, the direct correlation between the increment of the gas–water ratio and the increase in the amounts of gases and formation water migrating from the mud to sand layers can be established only in the period when a fluid pressure difference between the sand and mud layers is steadily decreasing (that is, when natural gas production in a well continues to increase) (Figure 5a). In other words, in the decreasing or constant phases of the gas–water ratio, the amounts of formation water from the mud layers can no longer be tracked from the gas–water ratio; thus, we excluded those samples from the comparisons.

Given the results of the numerical simulation mentioned above, we interpret the correlation between methanogenic activity and the gas–water ratio as an indication of hydrogenotrophic methanogenesis in the sand aquifers being promoted by the supply of formation water from the adjacent mud layers triggered by natural gas production. The formation water from mud layers is a possible energy source for methanogens in the aquifers where methanogenic substrates are limited. Previous studies have revealed that low-permeability sediments, such as shales, contain organic material or its fermentation products that diffuse into adjacent, more permeable sandstones or aquifers wherein they are consumed for anaerobic respirations (Mcmahon and Chapelle, 1991; Krumholz *et al.*, 1997). Our results further suggest that the flux from mudstones to sand aquifers is enhanced through the decrease in pressure associated with natural gas and water production. In our work and in previous studies, high-porosity rocks allowing microbial activities are interbedded with low-permeability organic-rich rocks. Such rock facies are critically involved in the processes.

Alternatively, the correlation can be explained by the idea that the population density of active

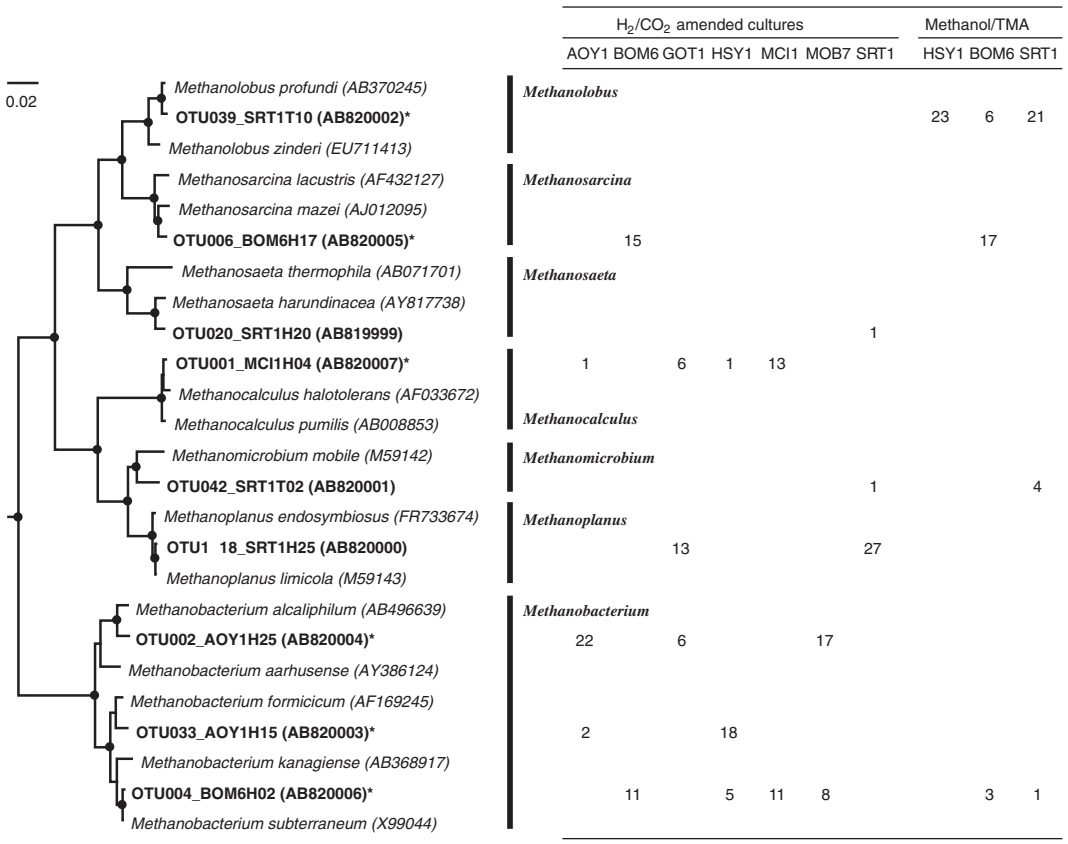

Figure 4 Neighbour-joining tree showing the relationship between representative OTUs of methanogenic culture clones (bold) and validated species of methanogens based on 16S rRNA gene sequences. The number of sequences in each OTU in each culture is listed in the right column. The OTU number corresponds to that listed in Supplementary Table S3 in the Supplementary Materials. The OTUs indicated by asterisks were also detected and abundant in the original formation waters. The dots indicate the bootstrap values above 70% in both the neighbour-joining and maximum likelihood trees. *Methanococcus aeolicus* (DQ195164) was used as an outgroup (data not shown). The scale bar represents 0.02 nucleotide substitutions per position.

methanogens changes as a result of natural gas production, because the methanogenic activity rate depends on the population density of viable methanogenic cells as well as on the substrate concentrations in a sample. It is highly possible that the migration of gas and formation water accompanies the migration of microbial cells including methanogens from mud layers, which are the major sources of biogenic methane in this gas deposit (Ueno *et al.*, 1964). The possible presence of energy sources or active cells in mud layers is supported by the results that the activities of hydrogenotrophic methanogenesis in formation water samples mixed with a piece of mud core were 10–1000-fold higher than those in formation water alone, as assessed by radiotracer experiments (Yoshioka *et al.*, unpublished). As noted in Kimura *et al.* (1993), the amount of the formation water that migrates from the mud layers is very small compared with the gases from the mud layers, which may partially explain the very low increment in the methane production rates compared with the large increase in the gas-water ratio (Figure 2).

Stimulation of methylotrophic methanogenesis by well drilling

The methylotrophic methanogens *Methanosarcina* and *Methanolobus* were found predominantly only in the BOM6 and SRT1 samples whose wells have been in operation for less than 1 and 4 years since well drilling, respectively, and are newer than the other wells. This transient abundance of methylotrophic methanogens associated with well drilling is consistent with our previous results (Mochimaru *et al.*, 2007). A significant proportion of methylotrophic methanogens was found in MOB7 when it had been in operation for 3 years. On the other hand, in MOB4, methylotrophs were detected in minor proportion at the time periods of 10 years since well drilling. In the present study, the water sample of MOB7 analyzed again after 7 years revealed the minority of methylotrophs. It should be, however, noted that the observed difference in methanogenic composition between the studies may have resulted from the difference in molecular tools, for example, PCR primers.

Methanosarcina and *Methanolobus* have been found in coal bed methane reservoirs (Shimizu

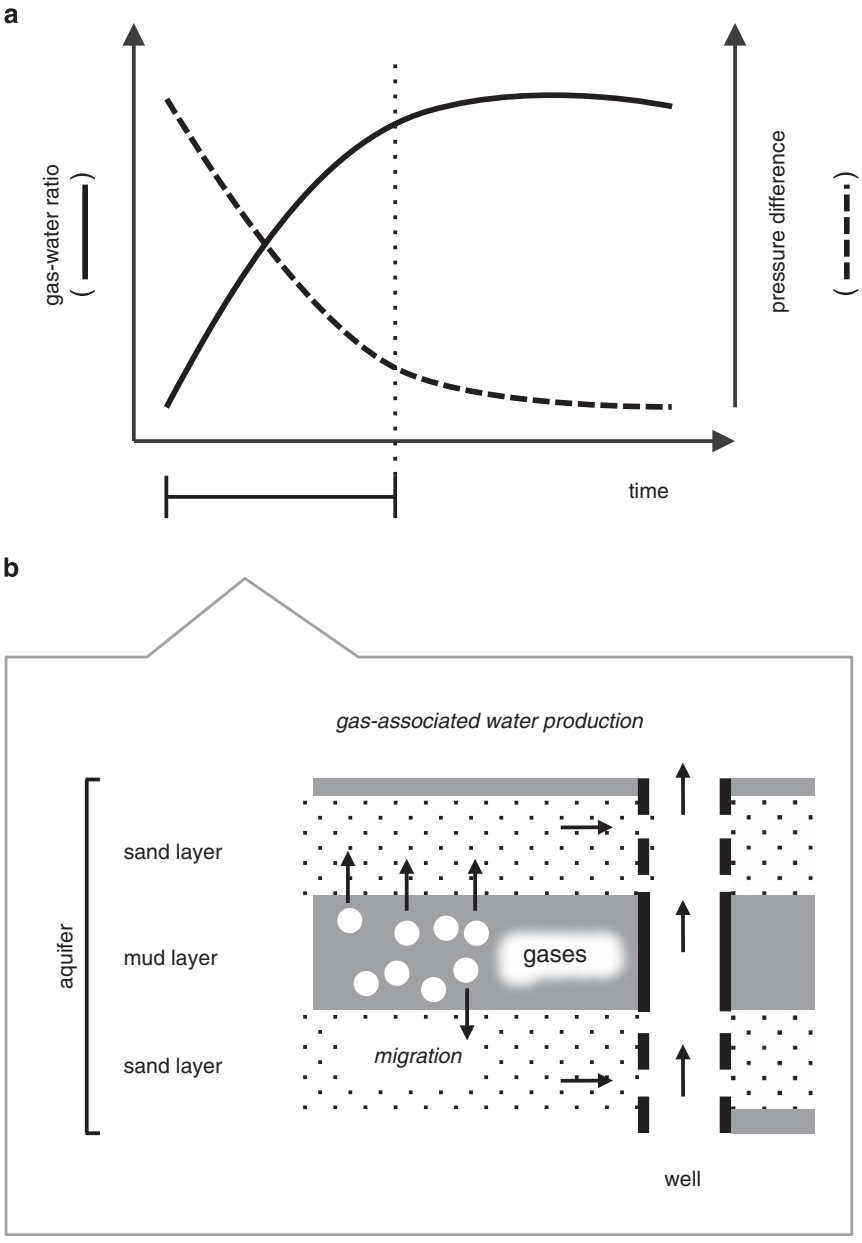

Figure 5 The behaviors of natural gases and gas production in a commercial well in the Minami-Kanto gas field. (**a**) The relationship between natural gas production and the level of fluid pressure difference between the sand and mud layers over time (adapted from Kimura *et al.* (1993)). (**b**) A schematic illustration showing the migration of the gases within the mud to sand layers along with the production of gas-associated formation waters. The bar below the time axes in **a** represents the period when the migration of the gases shown in **b** occurs.

et al., 2007; Strąpoć *et al.*, 2010; Dawson *et al.*, 2012; Guo *et al.*, 2012; Wawrik *et al.*, 2012) and have been thought to participate in the anaerobic degradation of complex organic compounds to methane in the subsurface environments where low-maturity coal was dominant (Strąpoć *et al.*, 2010). As both coal and type III kerogen in mud cores in this gas field are derived from the same type of organic sources (that is, terrestrial higher plants), their chemical properties, such as the C-H-O elemental composition and molecular structure, are known to resemble each other (Vandenbroucke and Largeau, 2007). We therefore speculate that the exposure of unutilized organic compounds in mud layers through well drilling would have temporarily stimulated the methylotrophic methanogens in aquifers.

The stimulation of indigenous methanogenesis in deep subsurface environments by physicochemical impact may not be unique to the Minami-Kanto gas field. Well drilling would affect methanogenesis in worldwide deposits of natural gas, including oil-associated gas, shale gas and coal bed methane,

where active methanogens are present (Meslé *et al.*, 2013). In sedimentary basins consisting of turbidite (alternating layers of sand and mudstones), the massive fracturing of sediments due to earthquakes may facilitate the flux of microbial energy sources from mudstones into sandstones and drive microbial activities. The presence of gas hydrates has been commonly observed across turbidite channels on continental margins, such as the Nankai Trough and the Cascadia Margin (Kvenvolden, 1993), both of which are known to be located in subduction zones where earthquakes have repeatedly occurred. The stimulation of methanogenesis by sediment fracturing due to seismic shaking over geological timescales could be an important factor for the formation of marine gas hydrates. Further studies will be needed to verify this hypothesis.

Conclusions

A number of production wells with different geochemical and geophysical properties present in the vast expanse of the Minami-Kanto gas field offered an opportunity to characterize methanogenesis in deep subsurface environments. Integrated geochemical and molecular analyses of gas-associated formation waters revealed the complete predominance of hydrogenotrophic methanogenesis and the potential for methylotrophic methanogenesis, processes that may be accelerated by anthropogenic physicochemical impacts, such as natural gas production and well drilling. Because the gas field has common characteristics of methanogenesis as well as geological and geochemical similarities to marine gas-hydrate areas, this study would provide a novel insight into the physicochemical impacts on microbial activity not only in other natural gas deposits, but also in sub-seafloor sediments related to the formation of marine gas hydrates.

Conflict of Interest

The authors declare no conflict of interest.

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