Deep-biosphere methane production stimulated by geofluids in the Nankai accretionary complex

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Microbial life inhabiting seafloor sediments plays an important role in Earth’s carbon cycle. However, the impact of geodynamic processes on the distributions and carbon-cycling activities of seafloor life remains poorly constrained. We explore a submarine mud volcano of the Nankai accretionary complex by drilling down to 200 m below the summit. Stable isotopic compositions of water and carbon compounds, including clumped methane isotopologues, suggest that ~90% of methane is microbially produced at 16–30°C and 300 to 900 m below seafloor, corresponding to the basin bottom, where fluids in the accretionary prism are supplied via megascale faults. Radiotracer experiments showed that relatively small microbial populations in deep mud volcano sediments (107–108 cells cm−3) include highly active hydrogenotrophic methanogens and acetogens. Our findings indicate that subduction-associated fluid migration has stimulated microbial activity in the mud reservoir and that mud volcanoes may contribute more substantially to the methane budget than previously estimated.

INTRODUCTION

One of the missions of scientific ocean drilling is to enhance the understanding of the impact of geodynamic processes—such as the subduction of oceanic plates into the Earth’s mantle—on the deep seafloor biosphere. Previous studies on the deep biosphere have demonstrated that microbes in ocean sediments generally experience long-term energy limitation (1, 2). Throughout millions of years of burial, the increasing pressure and temperatures create an ever more challenging environment for sustaining life, unless bioavailable water and energy substrates are supplied from exogenous biotic and/or abiotic sources (3). Submarine mud volcanoes occurring along the margins of convergent plates are surface expressions of natural conduits that transport low-density, deformable sediments from several kilometers below the seafloor up toward the seafloor. These migration pathways may supply subsurface-derived water, gaseous compounds such as methane (CH4), and even microbes to the overlying hydrosphere and atmosphere (4–7). In particular, methane emission from mud volcanoes is a significant (up to ~10%) source of fossil (radiocarbon-free) methane to the atmosphere (6).

Previous geological and geochemical studies at multiple geographic locations suggest that most CH4 erupting from submarine mud volcanoes originates either from destabilization of gas hydrates immediately below seafloor, corresponding to the basin bottom, where fluids in the accretionary prism are supplied via megascale faults. Radiotracer experiments showed that relatively small microbial populations in deep mud volcano sediments (10^7–10^8 cells cm^{-3}) include highly active hydrogenotrophic methanogens and acetogens. Our findings indicate that subduction-associated fluid migration has stimulated microbial activity in the mud reservoir and that mud volcanoes may contribute more substantially to the methane budget than previously estimated.

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beneath the relatively shallow sedimentary layers or from migration of fluids from deeper hydrocarbon accumulations (4, 5). Because of the high-pressure and low-temperature conditions in submarine mud volcano sediments, a large fraction of this migrated CH4 is sequestered in shallow mud volcano sediments as gas hydrates (5). Gas hydrate stability primarily depends on temperature and pressure (water depth). With normal sea-bottom temperatures of 2° to 4°C, gas hydrate can exist if water depths exceed 250 to 400 m (ca. >25 to 40 atm) (8).

Recent studies have shown that variations in dissolved organic matter and CH4 input from deep sediment layers, as well as variations in rates of fluid advection, control microbial community composition and metabolism in surface sediments at mud volcano sites (9–11). For example, at the Haakon Mosby mud volcano in the Norwegian Sea, microbial communities performing anaerobic oxidation of methane (AOM) consume up to ~40% of upward transported CH4 (crobial communities performing anaerobic oxidation of methane (AOM) consume up to ~40% of upward transported CH4 (9). At the Amsterdam mud volcano in the Eastern Mediterranean Sea, acetoclastic methanogenesis was observed in ~60-cm-deep sediments, likely supported by high concentrations of acetate (up to 1878 μM) produced in deeper layers there (10). Similarly, rate measurements and genetic data from an active brine seep mud volcano in the northern Gulf of Mexico revealed a predominance of acetoclastic over hydrogenotrophic methanogenesis (11). Still remaining to be explored, however, were the composition and metabolism of microbial communities at greater depths in mud volcano sediments than could be sampled by traditional near-surface coring surveys.

Carbon and hydrogen isotopic compositions of CH4, considered together with molar ratios of CH4 to higher hydrocarbons (C1/C2+), can help identify CH4 production pathways (12–14). A previous assessment of these chemical signals in more than 200 onshore mud volcanoes estimated that 76% of CH4 released is thermogenic, 4% is microbial, and 20% is a mixture of methane from both origins (15). Mud volcanoes emitting thermogenic CH4 are generally associated with strongly tectonized petroliferous sedimentary basins. In contrast, the factors controlling the presence, distribution, and quantity of microbially generated CH4 in mud volcano fluids are less understood. The sources of CH4 at offshore subduction zones remain largely elusive, mainly because of the lack of opportunities to directly sample deep subseafloor materials and characterize their microbiology and biogeochemistry in situ. In 2009 and 2012, using the drillship Chikyu, we established Site C9004 by drilling into mud volcano no. 5 (KMV#5) in the Kumano forearc basin of the Nankai Trough, Japan (Fig. 1A). While a submarine mud volcano has been drilled once before, during Ocean Drilling Program Leg 160 in the Mediterranean Sea (16), the deep biosphere and carbon cycling of such environments remain underexplored. In the Kumano Basin, 13 mud volcanoes have been observed at anticlines along the landward megasplay faults (Fig. 1, B and C) (17–20). Chemosynthetic macrofaunal communities occur at seep environments on the summits of these features (19). Previous investigations of surface sediments of mud volcanoes in the area inferred that the erupting fluids consist of water derived from dehydration of clay minerals in subducted sediments and basaltic crust, mixed with thermogenic hydrocarbons originating from the old accretionary prism beneath the forearc basin (19, 20). KMV#5 is one of the active mud volcanoes in the area (17, 19). A release of CH4 was observed here in response to a large earthquake in 2004 [moment magnitude (Mw) 7.5] (21). Furthermore, biogenic CH4 production has been implicated as an

Fig. 1. Location of the study sites (KMV#5) and seismic profiles of the Kumano forearc basin. (A) Bathymetric map showing the location of KMV#5 in the Kumano forearc basin and IODP Sites C0002 and C0009. (B) Transect seismic cross-section in the middle part of the Kumano Basin based on the data from Morita et al. (17). (C) P-wave velocity predicted by three-dimensional (3D) tomography inversion during 3D prestack depth migration based on the data from Tsuji et al. (18). The overpressure zone (fluid or gas accumulation zone) is identified as a low-amplitude and low-velocity zone at 400 to 700 mbsf in the forearc basin sequence. The low-velocity zone, which is located above the ridge because of the megasplay fault displacement, suggests that the overpressured fluids are moving upward along the interpreted ancient megasplay faults. The mud volcanoes are located along the northern extension of the megasplay faults.
additional hydrocarbon source at this site (19, 21). KMV#5 was therefore a prime location at which to investigate the relationship between methanogenesis and mud volcanism.

We recovered sediment core samples from Site C9004 down to 200 m below seafloor (mbsf) using a hydraulic piston coring system (HPCS) and a gas-tight hybrid-pressure coring system (Hybrid-PCS) (22). We show here how geological, geochemical, and microbiological analyses on the core material yield constraints on the origins of water and CH₄ in the migrating muds, and provide insights into the presence and activity of microbial life within the Nankai accretionary complex.

RESULTS

Mud volcano sediments

Sediment samples were composed primarily of clay-rich mud, including consolidated to semiconsolidated breccias, which were most likely mixed with fractured sediments by diapiric mud intrusion. After core recovery, small grains of methane hydrates (millimeters to centimeters in diameter) were often observed and collected on the core-cutting area (fig. S1, A and B). X-ray computed tomography (CT) scans of Hybrid-PCS cores also showed that methane hydrates filled pore spaces and veins of sediments (22). The in situ temperature was measured during HPCS coring using the third-generation advanced piston corer temperature tool (APCT-3) at 4, 22, 59, and 121 mbsf, yielding an estimated geothermal gradient of 29 ± 0.3°C km⁻¹ (fig. S2). Linear extrapolation of this geothermal gradient places the lower depth limit of the gas hydrate stability zone (GHSZ) that is, the deepest horizon where pressures and temperatures remain suitable for hydrate formation and stability (19) at an estimated ~590 m below the summit. The summit of KMV#5 is elevated 112 to 160 m above the regional seafloor, so the base of the GHSZ corresponds to 430 to 478 m below the regional seafloor. This is comparable to the depth of the bottom-simulating reflector (BSR) observed in seismic profiles of the sedimentary basin surrounding KMV#5 (411 to 463 mbsf; figs. S1D and S2).

Fluid chemistry

Geochemical analyses of pore water samples show that chlorinity (Cl⁻) decreased sharply from seawater-like values (545 mM) at 0 mbsf to 130 to 150 mM at 4 mbsf and then remained constant downcore (Fig. 2A). Concurrent with the Cl⁻ depletion, the oxygen and hydrogen isotopic compositions of water (δ¹⁸O_H₂O and δD_H₂O) shift toward high (ca. 6‰) and low (ca. −13‰) values relative to seawater (ca. 0‰), respectively (fig. S3, A and B). This trend can be attributed to the addition of water from the dehydration of clay minerals (16, 20) that typically occurs in deeper layers at temperatures ranging from 60° to 160°C (23). Conversely, δD_H₂O and δ¹⁸O_H₂O values of water obtained from methane hydrate melts were higher than those of pore water (Fig. 2, B and C, and fig. S3, A and B). The original δ¹⁸O and δD values of pore water before the formation of methane hydrates are estimated to be +4.3 to +5.3‰ and −9.2 to −16.2‰, respectively (Fig. 2, B and C). These values are based on the averaged δ¹⁸O and δD of hydrate water and known isotopic fractionation factors of oxygen and hydrogen in water between structure I hydrate and liquid water (fig. S1C and Supplementary Methods). Using the measured and estimated original δ¹⁸O and δD values of pore water, we calculated the average hydrate saturation in pore space to be ~22% (maximum, 58%) and ~14% (maximum, 58%), respectively (fig. S4 and Supplementary Methods). The relatively low and constant hydrate saturation from ~5 mbsf down to 120 mbsf is

Fig. 2. Chloride concentration and stable isotopic compositions of pore water in sediments of KMV#5. Vertical profile of Cl⁻ (A), δ¹⁸O (B), and δD (C) of pore water in sediments. Circle and square plots represent pore water sample and water from dissociated gas hydrate fragment, respectively. Dashed line represents the averaged isotopic value of methane hydrates, and blue-filled range denotes original δ¹⁸O and δD values of pore water before the formation of methane hydrates.


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consistent with small hydrate grains homogeneously precipitated in muddy sediments throughout the GHSZ (Fig. S4 and Supplementary Methods). Relatively constant values of Cl\textsuperscript{−}, δ\textsuperscript{18}O\textsubscript{H\textsubscript{2}O, and δD\textsubscript{H\textsubscript{2}O below ~5 mbsf indicate that the pore fluids below ~5 mbsf consist mainly of the original water from dehydration of clay minerals and that the effect of seawater diffusing from the overlying water column is negligible.

**Hydrocarbon gases, dissolved inorganic carbon, and sulfate**
The SO\textsubscript{4}\textsuperscript{2−} concentrations decrease from ~20 mM near the seafloor to less than 1 mM around 4 mbsf (Fig. 3C and table S1). The measured CH\textsubscript{4} concentrations in headspace gas were <1 mM in shallow sediments above 2 mbsf. In the sediments below, CH\textsubscript{4} content ranged from 2 to 26 mM (Fig. 3A and table S1). The ratio of methane to ethane concentration (C\textsubscript{1}/C\textsubscript{2}) in the headspace gas increased from ~120 near the sediment surface to ~500 at 2 mbsf. Below that depth, the C\textsubscript{1}/C\textsubscript{2} ratio ranged from 249 to 1593 (Fig. 4A and table S1). The concentration of DIC increased from 6 mM near the seafloor to ~20 mM at ~2 mbsf and decreased around 30 to 40 mM below the depth (Fig. 3C and table S1). The δ\textsuperscript{13}C value of DIC (δ\textsuperscript{13}C\textsubscript{DIC}) increased steeply from ~20‰ near the seafloor to +40‰ at ~2 mbsf and decreased slightly to +35‰ from 15 to 125 mbsf (Figs. 3D and 4B and table S1). The high δ\textsuperscript{13}C\textsubscript{DIC values at depth are most likely related to isotopic fractionation during microbial CO\textsubscript{2} reduction (26). Relative constancy of the DIC concentration with depth suggests either that CO\textsubscript{2} reduction occurred below the cored depths or that there may be an additional source of CO\textsubscript{2} (perhaps thermogenic) to the migrating fluids (27).

**Gas concentrations in samples recovered under in situ pressure**
Hybrid-PCS samples obtained from 6.5 and 61.5 mbsf were used for the analysis of in situ gas concentrations on the Chikyu (table S3) (22). The CH\textsubscript{4} content in the Hybrid-PCS cores ranged from 1.6 to 11.0 m\textsuperscript{3}CH\textsubscript{4}·m\textsuperscript{−3} (as CH\textsubscript{4} amount per sediment volume at the standard temperature and pressure), corresponding to concentrations of 246 to 1705 mM, respectively (806 mM on average; Fig. 3A). Because these concentrations far exceed solubility (~54 mM at 120 mbsf), most CH\textsubscript{4} should be present in hydrate form in situ. Given these data, methane hydrate saturation in pore space was calculated to be 2.8 to 22.6% (10.4% on average).

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**Fig. 3.** Depth profiles of biogeochemical parameters in sediments of KM V95. (A) Vertical profiles of CH\textsubscript{4} concentrations. (B) δ\textsuperscript{13}C and δ\textsuperscript{D} of methane. (C) Concentrations of SO\textsubscript{4}\textsuperscript{2−}, acetate, and dissolved inorganic carbon (DIC). (D) δ\textsuperscript{13}C of acetate, DIC, and total organic carbon (TOC). (E) H\textsubscript{2} concentrations. δ\textsuperscript{13}CH\textsubscript{3}D temperatures are shown in (B).
Acetate

The concentrations of acetate in the pore water were high in the top 4 m (up to 90 μM at 0.6 mbsf) relative to those deeper than 4 mbsf and decreased to 10 to 20 μM at 20 mbsf. Below 20 mbsf, the concentrations remained at approximately 20 μM (Fig. 3C and table S1). The vertical acetate concentration profile suggests that it is produced largely at shallow depths in situ. The relatively constant acetate concentrations below ~20 mbsf suggest either well-balanced production and consumption or little biological cycling of acetate at depths below 20 mbsf. The δ^{13}C values of acetate (δ^{13}C_{acetate}) increase from −41‰ on the seafloor to −22‰ at 4 mbsf, showing a trend similar to the δ^{13}C_{DIC} profile (Fig. 3D). Below 4 mbsf, the δ^{13}C_{acetate} values (~26‰ to −13‰), which are high compared to the δ^{13}C values of TOC (δ^{13}C_{TOC} = −24.4 ± 0.5‰), suggest the occurrence of fermentative production in the presence of low rates of acetoclastic methanogenesis. This is because the δ^{13}C of acetate derived from fermentation is close to that of TOC, and acetoclastic methanogenesis involves preferential consumption of ^{12}C-acetate, which will enrich ^{13}C in the residual acetate pool (28). Furthermore,
the large isotopic difference between DIC and acetate (ca. 58.8 ± 7‰) indicates aceticogenic CO₂ reduction via the Wood-Ljungdahl pathway as a second possible source of acetate (28). Thermal decomposition of organic matter is the third possible source of acetate. For example, δ¹³C values of acetic acid in oilfield waters of the San Joaquin Basin in California range from +0.6 to −19.3‰ and are generally higher than those of coproduced oils (29). The deviation between the δ¹³C values of acetic acid and oil was reported to be larger than 5‰. The δ¹³Cacetate values higher than δ¹³C_TOC values below 4 mbsf could be attributed to a deep source of acetate.

**Hydrogen**

H₂ concentrations measured onboard were scattered, ranging from 176 to 7760 nM (Fig. 3E and table S1). The gas concentrations of H₂ and hydrocarbon gases in the headspace gas that we report should be taken as minimum estimates because of degassing during core recovery and sampling. Despite degassing, the measured H₂ concentrations are more than two orders of magnitude higher than concentrations of H₂ in pore waters of stratified continental margin sediments (30). A Hybrid-PCS sample at 61.5 mbsf yielded an estimated in situ H₂ concentration of 28.1 µM (Fig. 3E and table S3), which is more than three orders of magnitude higher than in typical continental margin sediments (30) but slightly lower than the 1 to 500 µM (49 µM on average) observed in 1500- to 2500-mbsf sediments at Integrated Ocean Drilling Program (IODP) Site C0020 off the Shimokita Peninsula, Japan (3).

**Cell counts**

The fluorescence image–based analysis of SYBR Green I–stained cells (31) shows that relatively small microbial populations are present throughout the conduit of the intruded mud (Fig. 5A). Cell numbers decrease from 10⁹ cells cm⁻³ at 2.6 mbsf to 10⁸ cells cm⁻³ at 5.2 mbsf and then slightly increase again. Below 15 mbsf, cell numbers range from 10⁷ to 10⁸ cells cm⁻³ (Fig. 5A and table S4). In general, cell abundances in deeper mud volcano sediments are at least three orders of magnitude lower than those observed at similar depths in the surrounding Kumano sedimentary basin (Fig. 5A) and at other locations on continental margins (32). Instead, the cell abundances of deep mud volcano sediments are similar to those observed in the submarine mud volcanoes in the Ryukyu Trench off Tanegashima Island, southern Japan (7), deep sediments associated with lignite coalbeds at ~1.9 to 2.5 km below seafloor (kmbsf) at IODP Site C0020 (3), or the most oligotrophic open ocean sediments of the South Pacific Gyre (1).

**Taxonomic composition of microbial communities**

Among the total (bacterial and archaeal) 16S rRNA gene (16S) sequence reads from all sediment core samples (436,687 reads), almost all 16S sequences detected from deep mud volcano sediments were derived from psychrophilic to mesophilic microbes. The diversity index (Chao-1) of 16S sequence reads showed that the richness of bacterial communities was generally higher than that of archaeal communities, decreased with increasing depth in shallow sediments down to 5.2 mbsf, and was relatively constant in deeper sediments (table S5). Cluster and community network analyses based on the β-diversity also showed that both bacterial and archaeal communities in deeper sediments differed compositionally from those inhabiting shallow sediments above 5.2 mbsf (figs. S5 and S6).

In shallow sediments above 5.2 mbsf, 16S sequences related to Gamma-proteobacteria, Deltaproteobacteria (Desulfbacterales–relatives), and the ANME-1 group were detected predominantly, suggestive of the occurrence of AOM consortia (Fig. 5, B and C). Similarly, numbers of 16S sequences for Acidobacteria, Thaumarchaeota (Nitrospumilus–relatives), and Marine Benthic Group-B [alternatively, Deep-Sea Archaeal Group (DSAG) or “Lokiarchaeota”] decreased with increasing depth. These trends suggest that bacterial and archaeal communities in shallow sediments rely on substrates supplied from the downward diffusing seawater (33).

In deeper sediments, bacterial 16S sequences within Alphaproteobacteria (Sphingomonadales, Rhizobiales), Gammaproteobacteria (Alteromonadales, Pseudomonadales), Betaproteobacteria (Burkholderiales), Chloroflexi, “Atribacteria” (JS1 group), Actinobacteria (OP41 group), and Firmicutes (Bacillales, Clostridiales) were predominantly detected (Fig. 5B). Archaeal 16S sequences were mostly classified to “Bathyarchaeota” (34, 35), previously designated as the Miscellaneous Crenarchaeotic Group (MCG; Fig. 5C). Archaeal 16S related to Methanosarcinacinales dominated sediments at 19.3 mbsf (15,892 reads), where only four and one sequence reads were related to Bathyarchaeota and South African Gold Mine Euryarchaeota Group (SAGMEG), respectively. At 104 mbsf, we detected sequences of the Soil Crenarchaeota Group (SCG), ANME-1, and Methanosarcinales–relatives, comprising 26.9, 71.5, and 1.5% in the total 16S read number (20,714 reads), respectively (Fig. 5C).

### Potential rates of methanogenesis and acetogenesis

¹⁴C–radiotracer incubation analyses showed that the potential activities of homoacetogenesis, hydrogenotrophic methanogenesis, and acetoclastic methanogenesis were 14 to 34,900, 0.6 to 128, and 0.004 to 0.10 pmol cm⁻³ day⁻¹, respectively (Fig. 5D and table S6). These data indicate that both acetogenesis and methanogenesis via CO₂ reduction occur in deep mud volcano sediments, their activities being a few orders of magnitude higher than that of acetoclastic methanogenesis. The potential activities of acetoclastic and hydrogenotrophic methanogenesis are comparable to those measured in the marine sediments at the northern Cascadia margin (36). The activities of methanogenesis and acetogenesis in the shallowest sample are one to two orders of magnitude lower than those below 5 mbsf. The activity of acetogenesis at 13 mbsf is four orders of magnitude higher than that in the shallowest sample. This is consistent with the low activity of methanogenesis and acetogenesis in the sulfide reduction zone in which sulfate reducers outcompete methanogens and acetogens for H₂ (2, 30). Co-occurring homoacetogenesis and hydrogenotrophic methanogenesis are thermodynamically feasible given Gibbs free energy yields under in situ conditions of −84.1 ± 2.7 kJ mol⁻¹ and −86.0 ± 1.4 kJ mol⁻¹, respectively (Fig. 5E). Even if the energy yields were calculated by using the (lower) headspace H₂ concentrations (ranging from 176 to 7760 nM), the resultant Gibbs free energies of −56.1 ± 9.0 kJ mol⁻¹ for homoacetogenesis and −61.4 ± 9.2 kJ mol⁻¹ for hydrogenotrophic methanogenesis still indicate that the co-occurrence of both metabolic reactions is feasible.

### Potential hydrogenase activity

We measured hydrogenase activity in deep mud volcano sediments by incubation with ³H₂, ranging from 0.03 to 2.27 µmol H₂ cm⁻³ day⁻¹ [mean = 0.70 ± 0.75 µmol H₂ cm⁻³ day⁻¹ (n = 6); Fig. 5D and table S6]. Such activities are comparable to those previously observed in sediments at the Equatorial Pacific and the Gulf of Mexico continental slope (37). The relatively high values were observed in shallow sediment samples around the sulfate-methane transition zone (SMTZ; see Discussion). The cell-specific hydrogenase activity estimated by dividing the...
activity value by the respective cell concentration ranged from $1.7 \times 10^{-2}$ to 61 nmol H$_2$ cell$^{-1}$ day$^{-1}$ (table S6).

**Lipid biomarkers**

Reflecting the low cell abundance in deep mud volcano sediments, microbial intact polar lipids (IPLs) were only found in relatively low concentrations (<100 ng g$^{-1}$ of sediment dry weight in most samples; table S7). The IPL contents in deeper mud volcano sediments are at least two orders of magnitude lower than those observed in normal subseafloor sediments at the same depth range (38). Bacterial IPLs were barely detected, and most of the archaeal IPLs were composed of monoglycosydic glycerol-dibiphytanyl-glycerol-tetraethers (GDGTs), possibly because of their high preservation potential (39). Archaeal core lipids representing fossil biomass were generally more abundant than IPLs (39) and consisted of both diethers [or archaeol (AR)] and tetraethers (or GDGTs). Both AR and hydroxylated AR (OH-AR) were detected as major archaeal diether lipids.

H-shaped GDGTs (H-GDGTs) were detected in all samples and were also observed with additional mono-, di-, and trimethylated in the isoprenoidal chain (40). The H-GDGTs are characteristic membrane lipids of thermophilic and hyperthermophilic archaea, including members of the methanogens, Thermococcales and Thermoplasma
tales. H-GDGTs are most likely derived from the relict biomolecules of thermophilic communities (see Supplementary Text). Intact ARs were only found in the deepest sample examined (120 mbsf) and composed of phosphatidylglycerol (PG), monoglycosyl (G), and diglycosyl (2G) headgroups, including a PG-OH-AR. The latter IPL is a signature lipid of the Methanosarcinales (41), and correspondingly, PG-OH-AR is a major archaeal IPL in cold seeps dominated by Methanosarcinales-related ANME-2 (42). The detection of PG-OH-AR and other phosphoglycosylated and glycosylated ARs coincided with the highest concentration of core OH-AR and AR, suggesting the presence of an active methaneogenic population in deeply buried mud volcano sediments.

**Cultivation of methanogens**

We tried to cultivate methanogenic communities from six sediment samples from KMV#5 (0.66, 3.99, 12.10, 12.82, 14.97, and 18.10 mbsf; see Supplementary Methods). After cultivation for ~9 months, the growth of spherical cells was observed in cultures only from 12.82 mbsf, resulting in successful pure isolation of the hydrogenotrophic methanogen strain 1H1 (fig. S7, A and B). Phylogenetic analysis of the 16S rRNA gene showed that strain 1H1 is closely related to *Methanosarcina*...
mazei (fig. S7E and Supplementary Text). The isolate can grow on H2/CO2, acetate, methanol, dimethylamine, and trimethylamine; however, formate, dimethylsulﬁde, ethanol, 1-propanol, 2-propanol, cyclopentanol, 1-butanol, and 2-butanol did not support cell growth. The optimum growth temperature of strain 1H1 was at 40°C in the possible range of 2° to 50°C (fig. S7C). While the isolate can grow under the wide range of NaCl concentrations, it preferentially grows under very low salinity conditions (fig. S7D).

**DISCUSSION**

On the basis of the vertical proﬁles of CH4 and SO42−, we locate the SMTZ, where most of the methane is consumed by microbial AOM coupled to microbial sulfate reduction, between 1 and 3 mbsf. Other geochemical signals are consistent with an SMTZ at this depth. The shift in δ13C value of CH4 (δ13CCH4) from −35%o to −20%o at the seafloor (Fig. 3B) is consistent with preferential consumption of 12C-methane during microbial AOM (14). A conodont drop in the ratio of methane to ethane concentration (C1/C2) in the headspace gas from −500 to 2 mbsf to 119 at the seafloor (table S1) is also consistent with the consumption of CH4 within the upwelling muds at a depth near 2 mbsf. The decrease of CH4 concentration in conjunction with the increase of δ13CCH4 values from the SMTZ to seafloor, and the constant δ13CCH4 value below the SMTZ, indicates that CH4 was derived from larger depths and consumed by AOM near sediment surface. The consumption of CH4 by AOM is indicated by the low C1/C2 ratios and high δ13CCH4 values, with low δ13CDIC values of the data shallower than the SMTZ (Fig. 4, A and B).

The average δ13C (−34.3 ± 2.3%) values of methane below the SMTZ are within the typical range of thermogenic methane (Fig. 4A) (12, 14). However, relatively high C1/C2 ratios (249 to 1593) in the headspace gas below the SMTZ compared to the ordinary C1/C2 ratios of thermogenic gas imply either a predominate of biologically produced CH4 or preferential consumption of thermogenic C2+ hydrocarbons (Fig. 4A) (19). The C1/C2 ratios, which are slightly lower than the typical C1/C2 ratios (>1000) of biogenic CH4, and the δ13C values of ethane ranging from −32 to −26%o indicate a minor contribution of thermogenic hydrocarbons. The C2/C1 ratios are greater than ~1 (table S1), suggesting that C2+ hydrocarbons are predominantly derived from a thermogenic source in which ethane is generally enriched to propane (43). Thermogenic gas here was probably generated from sedimentary organic matter below ~2 kmbsf, where estimated in situ temperatures exceed 80°C (44), and transported with the ascending ﬂuids and muds (see Supplementary Text). When the previously reported data in the Nankai Trough area (fig. S8 and Supplementary Text) and the northern Cascadia margin (27) are plotted, the C1/C2 ratio and the δ13CCH4 at KMV#5 and other mud volcanoes in the Kuman Basin (19) deviate from all possible mixing curves of ordinary biogenic methane with normal δ13CCH4 (~−65%) and C1/C2 (1000) and thermogenic gas (δ13CCH4 ~−40%o; C1/C2 ~8) (Fig. 4A). The trend is rather consistent with a biogenic methane end-member that carries a relatively high δ13C value (~−35%o). Such elevated δ13C values and high C1/C2 (>100) are unusual for biogenic gases and, under some isotope-based classiﬁcation schemes [for example, the study of Whiticar (14)], would be typied as thermogenic methane generated from high-maturity organic-rich shales (45). However, such highly mature shales probably do not occur in the Nankai Trough subduction zone (17, 18). The accretionary prism here is dominated by younger hemipelagic sediments that are quite different from, for example, the old [>100 million years (Ma)] and thick shales found in North America (45) on which the aforementioned interpretive schemes were partially based. As we discuss below, KMV#5 is not the ﬁrst site where biogenic CH4 containing isotopically heavy (13C-enriched) carbon has been observed in marine sediments.

The isotope separation between δ13CCH4 and δ13CDIC, expressed as the isotope separation factor ε (ε = δ13C DIC − δ13CCH4) (14), was 73.9 ± 3.5‰ below the SMTZ (Fig. 4B). We interpret the large ε to indicate hydrogenotrophic methanogenesis (Fig. 4B) (14). The lighter 12C in DIC is preferentially consumed by microorganisms during hydrogenotrophic methanogenesis, leaving the residual DIC pool enriched in 13C (a process sometimes termed "substrate depletion") (14, 26). As more DIC converts to CH4, both the accumulated methane and the remaining DIC pools become increasingly 13C-enriched, with CH4 here approaching values of ca. −34‰. Such enrichment of 13C has been observed in other areas in which biological methanogenesis is the dominant source of CH4, including hydrate-bearing sediment at IODP Site U1329 in the northern Cascadia margin (δ13CCH4, up to −39.5‰; δ13CCO2, up to +25.7‰; C1/C2, ~103 to 105) (Fig. 4, A and B) (27) and Tertiary hemipelagic sediments in the Middle America Trench off Guatemala (δ13CCH4, up to −39.0‰; δ13CCO2, up to +27.6‰; C1/C2, ~102 to 103) (46).

The δDCH4 values of the methane in KMV#5 are very constant at ca. −185‰. The difference between δDCH4 (~126 ± 15‰) and δD of the original pore water (~9.2 to −16.2‰) before methane hydrate precipitation is consistent with observed ranges of δD values for biogenic methane in marine sediments [δDCH4 = δDH2O − 160‰ (or 180‰)] (13, 14). In contrast, thermogenic gases produced from a highly mature marine or humic source rock will typically be −150‰ to −120‰ (13).

On the basis of these relationships between δ13CCH4, δ13CDIC, and C1/C2 (Fig. 4), we infer that the hydrocarbon gas in KMV#5 consists of a mixture of gas of biogenic origin (nearly entirely CH4) with a small amount of thermogenic gas. A two-end-member mixing curve between biogenic gas characterized by δ13CCH4 = −39.5 to −29%o and C1/C2 = 2000 to 6200 and thermogenic gas with δ13CCH4 = −45 to −40%o and C1/C2 = 8 to 11 (table S8 and Supplementary Text) suggests that biogenic CH4 comprises >90% of the methane (Fig. 4A).

Given the in situ methane concentration in the Hybrid-PCS sample at ~806 mM in pore water (~256 mol m−3 in wet sediment; Fig. 4A), we estimate the concentrations of biogenic CH4 and thermogenic CH4 to be ~725 mM (~230 mol m−3), respectively. Although thermogenic CH4 was estimated to account for less than 5% of total CH4, the concentration of thermogenic CH4 amounting to ~81 mM is substantially higher than the CH4 solubility at in situ conditions (44 to 54 mM), suggesting that expulsion of thermogenic CH4 may help drive migration of the ﬂuid and sediment here.

Analyses of methane isotopologues provide additional evidence for a low-temperature, nonthermogenic origin of CH4 in the mud volcano ﬂuids. The ca. 30°C apparent equilibrium temperatures obtained from clumped isotopologue analyses of samples from 6.5 and 61.5 mbsf (Figs. 3B and 4D and table S2) suggest a predominance of methane that has been generated or biogeochemically cycled at relatively low temperatures (<5°C). Similar Δ13CH3D values as well as δ13CCH4 and δDCH4 values between the two samples suggest that CH4 at 6.5 and 61.5 mbsf have the same origin(s). The Δ13CH3D values and D/H fractionation between CH4 and the original pore water plot close to other biogenic gases from deep sedimentary environments (Fig. 4D) (47). Although the δD values of methane are 20‰ higher than expected for equilibrium, the KMV#5 data points are much closer to equilibrated biogenic gases than to previously reported thermogenic gases. These findings
are consistent with generation of methane by microbes at low temperature and low growth rates (47, 48).

We calculate the maximum amount of time required to generate the total biogenic CH₄ to be 1.82 Ma, by dividing the in situ biogenic CH₄ concentration in the wet sediment (maximum, 398 mol m⁻³) by the potential hydrogenotrophic methanogenesis rate (minimum, 0.6 pmol cm⁻² day⁻¹). This estimated duration does not exceed the age of non-fossils in sediments at KMV#5, which are late Early Miocene to early Middle Miocene (13.6 to 18.2 Ma) (17). The potential activity of hydrogenotrophic methanogenesis at KMV#5 is comparable to those measured at the northern Cascadia margin (36). Because incubations used to determine the potential methane production rate are conducted under high H₂ supply, rates of methanogenesis may be even slower in situ than indicated by the incubations. Relatively slow production of biogenic CH₄ is compatible with the near attainment of hydrogen isotopic equilibrium between methane and coexisting water at the temperature indicated by the methane isotopologue data (~30°C) (Fig. 4D) (47, 48).

We further estimated the average temperature at which the biogenic CH₄ was generated based on (i) the measured Δ¹³CH₃D values, (ii) estimated biogenic fraction to total CH₄ (>90%), and (iii) the possible Δ¹³CH₄D temperatures of a thermogenic CH₄ end-member (80° to 220°C) (Supplementary Text). Assuming simple mixing between a single biogenic CH₄ end-member (Δ¹³C, −39.5‰; ΔD, −166‰) and a thermogenic CH₄ end-member (Δ¹³C, −40‰; ΔD, −229‰) (these values are assumed based on the data from Nankai Trough area; see Supplementary Text), we plotted mixing curves between the two end-members, consistent with a Δ¹³CH₄D temperature of 30°C for the CH₄ mixture (fig. S9 and Supplementary Methods). The mixing diagram indicates that the Δ¹³CH₃D of the average biogenic component of CH₄ is <5.9‰ (>16°C in Δ¹³CH₃D apparent equilibrium temperature). The possible range of the minimum Δ¹³CH₄D temperature for the biogenic CH₄ is 13° to 17°C (fig. S9 and Supplementary Methods). On the basis of the in situ temperature profile in KMV#5 and the surrounding sedimentary basin, we suggest that biogenic CH₄ has been produced at 300 to 900 mbsf in the forearc basin, where temperatures range from 16° to 30°C (figs. S2 and S9). The minimum depth of the methanogenic zone was estimated to be ~200 to 400 mbsf, depending on the assumed clumped isotope temperature of the biogenic CH₄ end-member (13° to 17°C) (fig. S9).

A 3D seismic volume over the study area showed that zones of high pore pressure occur at similar depths in the Kumano Basin along the megasplay fault (Fig. 1C) (18). Because the mud volcanoes in the basin are located along this megasplay fault, these elevated pressure zones were thought to be the source of mud volcanoes (Fig. 1C) (18). Such observations are consistent with the age of the sediments of KMV#5, which are late Early Miocene to early Middle Miocene (17), indicating that the mud is originally derived from the bottom of forearc basin and not from the old accretionary prism. Together, our data indicate that biogenic CH₄ production in KMV#5 proceeds slowly in the deeper forearc basin (which sources the intruded mud) and is stimulated to action by a supply of water via the megasplay fault. Note that the pore water in the mud volcano sediments was freshened by the dewatering of clay minerals, which occurred in the old accretionary prism below the forearc basin (fig. S2). Furthermore, a previous study inferred that the fluid in the mud volcanoes was derived from dehydration of sedimentary smectite and basaltic saponite in subducted sediments and basaltic crust (20). Such a deeply sourced low-salinity fluid could conceivably travel upward along the megasplay fault (Fig. 6).

The high concentration of H₂ observed at KMV#5 might be related to the supply of deeply sourced fluid, stimulating methanogenesis and other anaerobic respiratory metabolisms mediated by H₂, such as homoacetogenesis. The first use of Hybrid-PCS demonstrated that the in situ H₂ concentration in deep mud volcano sediments is three orders of magnitude higher than those previously measured in marine sediment samples (30). However, the origin of H₂ is still under debate: In normal marine sediments, microbial H₂ production and consumption are closely coupled via interspecies hydrogen transfer in syntrophic relationships, and H₂ concentrations are kept at a low level to outcompete other hydrogenotrophic microbes that use terminal electron acceptors, which yield less free energy, while at the same time providing the minimal amount of energy needed to sustain the population (30). However, in KMV#5, the high H₂ concentrations suggest that H₂ production and consumption are not coupled tightly, that is, a lack of microorganisms using H₂ or a supply of H₂ exceeding microbial consumption. Considering the total amount of H₂ necessary for the large amount of biogenic methane via hydrogenotrophic CO₂ reduction, as well as relatively low organic content in the mud volcano sediment and old accretionary prism, it is most likely that contributions of low-biomass communities to the vast H₂ production in situ (fermentation) would be much smaller than that of H₂ from abiotic processes. Given the geological setting of the Nankai subduction zone, we suggest that the high concentration of H₂ is possibly derived from coseismic fault friction and/or water-rock interactions in the old accretionary prism, as is the case with deeply derived lithium (Li) in the same mud volcano fluids (49). It has been experimentally demonstrated that fault friction mechanoradically produces a large amount of H₂ (50). Cell-specific hydrogenase activity in the deepest sample (61 nmol H₂ cell⁻¹ day⁻¹) was three orders of magnitude higher than both the shallowest sample (1.7 × 10⁻² nmol H₂ cell⁻¹ day⁻¹ at 1.5 mbsf; table S6) and the previously reported values (37). The generally high cell-specific hydrogenase activity in deep mud volcano sediments may be due to a high dependence of microbial communities upon deeply sourced H₂ as their energy source. The high cell-specific hydrogenase activity, as well as the high potential rates of hydrogenotrophic methanogenesis and homoacetogenesis, suggests that microbial communities in the deep mud volcano biosphere have been stimulated by the geogenic H₂ and water in the Nankai accretionary complex.

On the basis of the high potential rate of homoacetogenesis and the relationship of vertical profiles of Δ¹³Cacetate, Δ³⁷CToC, and Δ³⁷CDIC, we infer a large contribution of acetate production by homooacetogenesis to the acetate pool at depths shallower than the SMTZ. We infer that the acetate is mainly produced at shallow depths in situ on the basis of the vertical concentration profile of acetate (Fig. 3C). The observed higher potential rate of homooacetogenesis than that of hydrogenotrophic methanogens is peculiar, however, because of the slight thermodynamic preference of hydrogenotrophic methanogenesis over homooacetogenesis throughout the cored depths (Fig. 5E). However, the high potential rate of homooacetogenesis observed by our low-temperature incubation (4°C) is consistent with previous studies that incubated anoxic paddy soil or lake sediment and reported homooacetogenesis outcompeting hydrogenotrophic methanogenesis for H₂ at temperatures below 15° to 20°C (51). Considering that the Δ¹³CH₃D temperature data indicate the occurrence of methanogenesis in the deeper forearc basin at a temperature of ~15°C, we speculate that homooacetogenesis predominates in lower temperature horizons, including the cored depths, especially those shallower than ~20 mbsf.

The much lower cell abundances in the deep mud volcano sediments relative to those observed in the Kumano sedimentary basin at the same
depths (down to 120 mbsf) (Fig. 5A) suggest that microbes are derived not from the surrounding sediments through which the mud rises but from the original mud reservoir. The mud reservoir, a deeper and older sedimentary realm than the surrounding basin sediments, is presumably more sparsely populated than the shallow basinal sediments. Our DNA and lipid data revealed that relatively small communities occur even in the deeply derived mud volcano sediments. More than 99.9% of the total 16S sequence reads are classified into taxonomic clusters that predominantly involve psychrophilic to mesophilic microbes, suggesting that most microbes are derived from the original mud reservoir in the forearc sedimentary basin. β-diversity analysis shows that both bacterial and archaeal communities in deep sediment samples are compositionally different from those in the shallow sulfate reduction and AOM zones (figs. S5 and S6). This trend indicates that some commonly distributed bacterial members (for example, Sphingomonadales, Atribacteria, and Chloroflexi) are derived potentially from the mud reservoir and that they contribute to carbon cycling in these sediments (Fig. 5B). Archaeal communities in deep mud volcano samples are predominantly composed of Bathyarchaeota (34, 35) and Methanosarcinales-relatives, which are markedly different from shallow communities dominated by ANME-1, Marine Benthic Group-B, and Nitrosopumilus-relatives (Fig. 5C and figs. S5 and S6). The detection of Methanosarcinales-relatives in the deep mud volcano samples is consistent with the detection of intact PG-OH-AR, which is a signature lipid of the Methanosarcinales-related ANME-2 at 120 mbsf. Recent metagenomic studies of Bathyarchaeota show that two genomes contain divergent homologs of the genes necessary for CH₄ metabolism, including methyl-coenzyme M reductase (34), and for homoacetogenesis.

Fig. 6. Schematic figure illustrating methanogenesis in the deep mud volcano sediments associated with fluid migration via the megasplay fault. The cross-section is based on seismic profiles (see Fig. 1 and fig. S1).
and fermentations (52). The presence of Bathymarchaeota in the mud volcano community suggests that those archaeal members might play some ecological roles in carbon cycling; however, it is currently not known whether the detected Bathymarchaeota are capable of methanogenesis, acetogenesis, or fermentation in the mud reservoir. In addition to those presumably psychrophilic to mesophilic members that have often been observed in cold to warm subseafood sediments, the 16S gene library includes sequences of some unexpected members, which may reflect environmental conditions in the mud reservoir or the deeper old accretionary prism. For example, the occurrence of SCG archaea, which have been detected so far mainly in methanogenic low-salinity terrestrial soils (53, 54) and rarely in deep subseafood sediments, may point to low-salinity conditions in the mud reservoir. This would be consistent with a selection or preferential stimulation of population members that prefer low-salinity conditions for their osmoregulatory functions because of the freshwater supply from clay dehydration. Furthermore, the successful isolation of strain 1H1 [related to M. mazei, which grows at temperatures between 2°C and 50°C with optimum growth at 40°C, and under low NaCl concentrations down to 0 g liter−1 (fig. S7 and Supplementary Text)] strongly supports our notion that hydrogenotrophic methanogenesis occurs in the warm, low-salinity environment, as predicted by the pore water chemistry, clumped methane isotopologue, and seismic survey data (Fig. 6).

It is important to discuss here the carbon budget within KMV#5. The CH4 contents in sediments of KMV#5 averaged 6.4 m3 CH4·m−3 with a maximum value of 11 m3 CH4·m−3 at standard temperature and pressure. Such CH4 contents are comparable to values determined by using gas-tight coring at the Haakon Mosby mud volcano in the Norwegian Sea (average of 15 m3 CH4·m−3, maximum of 34 m3 CH4·m−3 as hydrate-bound CH4) (8). In the active Haakon Mosby mud volcano, the temperature gradient is very high, up to 17°C m−1, because of the high flux of warm fluid through a central conduit toward the seafloor. Thus, gas hydrate occurrence is limited to shallow depths below seafloor (1 to 15 mbsf), and the total mass of hydrate-bound CH4 there was estimated to be 102.5 kt, equal to 2 × 106 m3 (8). On the basis of the data obtained from such active mud volcanoes, the total budget of CH4 hydrates stored in submarine mud volcanoes in the world was previously estimated to be ~1010 to 1012 m3 (5). However, the temperature gradient at KMV#5 (29°C km−1) is notably lower than other active submarine mud volcanoes, resulting in a vast and stable cylindrical GHSZ down to 590 m below the summit. We calculated the methane hydrate saturation in pore space to be 2.8 to 22.6% (10.4% in average) by using the data from Hybrid-PCS samples; this is comparable to Haakon Mosby (up to 21.3%) (8). Furthermore, the methane hydrate saturation in pore space is consistent with estimates based on the values of δ18OH2O and δD H2O below ~5 mbsf (14 to 22% on average), indicating that methane hydrates are most likely homogeneously present in the conduit of the intruded mud throughout the GHSZ.

If the KMV#5 muddy sediments contain 6.4 m3 CH4·m−3 in situ as measured in Hybrid-PCS samples, then the cylindrical conduit (~1000 m in diameter, 590 m in depth down to the lower boundary of GHSZ) should harbor 3.2 × 108 m3 of CH4. This is one order of magnitude larger than previous estimates of the amount of CH4 in other active submarine mud volcanoes (~106 m3) (5, 8). Given the global distribution of submarine mud volcanoes along the plate convergent margin, we infer that mud volcanoes of the type found in the Kumano Basin are quantitatively more important reservoirs of CH4 than previously thought. As there are at least 10 mud volcanoes whose mud volcanic activity is similar to or less than KMV#5 in this area (17, 19), the total CH4 budget in all submarine mud volcanoes of the Kumano Basin is at least ~109 m3, which is comparable to the previous minimum estimate of CH4 in submarine mud volcanoes worldwide (5). The global CH4 budget in submarine mud volcanoes is most likely underestimated; if we assume that the total amount/quantity of methane could apply to the global submarine mud volcanoes, then the total budget of methane might be one order of magnitude higher than the previous estimate (~1011 to 1013 m3). Nevertheless, to accurately characterize the CH4 budget and its biogeochemical significance in submarine mud volcanic systems, further systematic investigations at various geologic and oceanographic settings are necessary.

Our results suggest that the previous assessment of “biogenic” versus “thermogenic” based on C1/C2 and δ13CCH4 is not simply applicable to some environments found in complex plate convergent margins. In terrestrial mud volcanoes, the elevated δ13CCH4 values (~46.5% on average) and mostly high C1/C2 (>500) have been attributed to a thermogenic source, in which a molecular separation during the gas migration causes high C1/C2 ratios (15). Given this interpretation, the fraction of thermogenic gas in the terrestrial mud volcanoes was estimated to be ~76%. In marked contrast, our results suggest that such elevated δ13CCH4 and C1/C2 values are possibly consistent with biogenic gas. If this is the case for other mud volcanoes, then the contribution of biogenic methane might be significantly larger than previously estimated. Because mud volcanoes are a significant source of radiocarbon-free methane to the atmosphere (6), the contribution of subseafood microbial activity to global carbon cycling may be larger than has been assumed.

In conclusion, this study provides a comprehensive view of the mechanism of biogenic CH4 production in the deep mud volcano biosphere. Geogenic reactions caused by heat and/or pressure in the landward old accretionary prism provide water through clay dehydration, hydrogen, and other organic substrates to the overlying forearc sedimentary basin, coincidently stimulating in situ microbial activities such as hydrogenotrophic methanogenesis. As a consequence of such geosphere-biosphere interactions, large amounts of biogenic CH4 are stored as methane hydrates in the mud volcano interior or as free gas in the overpressured sedimentary basin. Similar interactions are possibly globally distributed beneath the ocean along the plate convergent margins, and this type of geobiological system could contribute more substantially to the global carbon budget than previously thought.

**MATERIALS AND METHODS**

**Sample collection by scientific ocean drilling**

All sediment samples used in this study were obtained at Site C9004 on the summit of KMV#5 in the central Kumano Basin of the Nankai Trough [33°67.581′N, 136°56.50885′E; 1986.7-m water depth (Fig. 1A)]. Shallow sediment samples at Site C9004 were collected using a 4-m piston corer by the research vessel Hakuhomaru during the KH06-04 cruise in 2006. Shallow to deep sediment samples were obtained by using HPCS down to 200 m below the summit during the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) Expeditions 903 and 906 of the drilling vessel Chikyuu in 2009 and 2012, respectively. During Expedition 906, in addition to HPCS cores, pressure-conserved sediment cores were recovered by using Hybrid-PCS (22), immediately ice-shocked onboard to prevent temperature increase, and then transferred to the Pressure Core Analysis and Transfer System (PCATS) (Geotek Co. Ltd.) for subsequent gas production tests and geochemical analyses.
During the transfer of the Hybrid-PCS core using PCATS, x-ray CT scanning as well as γ-ray and P-wave velocity measurements were simultaneously conducted. On the basis of the nondestructive physical property data, the core was cut into subsections at the selected point under high-pressure conditions, and then the subsection was transferred to a short aluminum chamber. Gas production tests were conducted using the short high-pressure core samples: Under the controlled depressurization, gas was forced out from the high-pressure chamber, collected by an inverted graduated cylinder in a water column for the volume measurement. A part of the extracted gas was sampled for the compositional analysis. Gas samples for analyses of H$_2$ concentration and clumped isotopes of methane (sampled for the compositional analysis. Gas samples for analyses of H$_2$ concentration and clumped isotopes of methane (Δ$^{13}$CH$_3$D) were collected directly from the depressurized manifold during the production test and then stored in preevacuated glass vials and stainless steel cylinders, respectively.

**Measurement of pore water chemistry and gas compositions**

All pore water sample acquisition and measurements were carried out in the geochemistry laboratory onboard the Chikyu. In short, Cl$^-$ and SO$_4^{2-}$ concentrations in this study were analyzed by titration and ion chromatography, respectively, according to the IODP standard protocols.

Sediment samples for headspace gas analysis were collected using tip-cut plastic syringes immediately after core recovery. The syringe end was tightly capped with a silicone-rubber stopper on the core-cutting area of the Chikyu, transferred to a glass vial in a N$_2$-flushed glove bag, and then capped with a Teflon-coated rubber septum and aluminum seal for subsequent geochemical analyses. Hydrocarbon compositions in headspace gas and Hybrid-PCS gas samples were measured by using gas chromatography (GC)–flame ionization detector and GC–thermal conductivity detector on the Chikyu. H$_2$ concentrations in headspace gas and Hybrid-PCS samples were measured using a GC–trace reduction gas detector (30) immediately after sampling. The total H$_2$ volume in a Hybrid-PCS sample (core 5P-2 at 61.5 mbsf; table S3) was calculated by multiplying the averaged concentration of two subsamples collected during the production test to the total gas volume in the high-pressure chamber. The H$_2$ concentration in pore water was then calculated by dividing the total H$_2$ volume by pore volume.

**Analysis of stable isotopic compositions**

$\delta^{18}$O$_{H_{2}O}$ and $\delta_{H_{2}O}$ were analyzed by laser absorption spectroscopy (Liquid Water Isotope Analyzer, Los Gatos Research), deployed on the Chikyu. DIC concentration and $\delta^{13}$C$_{DIC}$ were measured with a Thermo Finnigan Delta Plus XP isotope ratio mass spectrometer (IRMS) connected to a Flash EA 1112 Automatic Elemental analyzer via a ConFlo III interface (55). The acetate concentration and $\delta^{13}$C$_{acetate}$ were determined by isotope ratio monitoring–liquid chromatography–mass spectrometry (irm-LC-MS) (Thermo Finnigan Delta Plus XP IRMS connected to LC IsoLink) (28). TOC content and $\delta^{13}$C$_{TOC}$ were determined, after removal of carbonate with HCl, by using a Thermo Finnigan Delta Plus XP IRMS instrument connected to a Flash EA 1112 Automatic Elemental analyzer via a ConFlo III interface. The $\delta^{13}$C values of hydrocarbon gases and $\delta^{13}$C$_{CH_{4}}$ were determined via irm-GC-MS (21, 55) (Thermo Finnigan Delta Plus XP IRMS connected to TRACE GC via GC Combustion III) and high-temperature conversion interface, respectively. The $\Delta^{13}$CH$_3$D of methane in Hybrid-PCS gas samples was measured by tunable infrared laser direct absorption spectroscopy (3, 25, 47).

**Cell counts**

Plugs of 1-cm$^2$ sediment were collected from the innermost part of the cores using sterilized syringes (tips cut off) and then immediately fixed with 2% (w/v) paraformaldehyde for ~12 hours at 4°C, washed twice with phosphate-buffered saline (PBS) buffer, and suspended in PBS-ethanol solution (1:1) at a final dilution volume of 10% (v/v). Cell counts were performed by both manual microscopic observation and image-based cell enumeration techniques using SYBR Green I fluorescent dye (31).

**DNA extraction and molecular analysis**

All sediment samples used for molecular analyses were immediately frozen at ~80°C and then stored in deep freezers before use. DNA was extracted from 10 g of the frozen sample using the PowerMax Soil DNA Isolation Kit (MO BIO Laboratories Inc.) according to the manufacturer’s instructions. Despite the generally low cell abundance in deep mud volcano sediments, bacterial and archaeal 16S fragments were directly obtained by polymerase chain reaction (PCR) without any multiple displacement amplification. To identify the taxonomic composition, we sequenced the V1-V2 and V3-V4 hypervariable regions of bacterial and archaeal 16S genes, respectively, using a GS FLX sequencer. In short, PCR amplification for sequencing was performed using domain-specific primers (EU827F and EUB338Rmix for Bacteria, and UNIV530F and ARCO12R for Archaea) with 454 FLX Titanium adapters A and B and a six-base sample identifier tag (56). No PCR products were obtained from 19.3 and 119 mbsf each for bacterial and archaeal 16S, respectively (Fig. 5, B and C). No PCR amplification could be seen in all negative control experiments. Detailed methods for taxonomic classification of the sequence reads, data processing for operational taxonomic unit picking, and β-diversity analyses were described in Supplementary Methods.

**Activity measurements and thermodynamic calculations**

Sediment samples used for activity measurements by incubation with $^{14}$C-labeled substrates were collected from whole-round core sections that were immediately cut after core recovery and stored under anoxic conditions flushed with Ar at 4°C until onshore distribution to vials. A sulfate-free mineral solution, in which Cl$^-$ was adjusted to 86 mM, was added to make slurries. The headspace was flushed by N$_2$, and H$_2$ concentrations were adjusted to 20 μM in water before $^{14}$C-labeled substrates (bicarbonate and acetate, 2 MBq) were added to each vial. After incubation for 130 days at 4°C, the activities associated with hydrogenotrophic and acetoclastic methanogenesis, as well as homooacetogenesis, were measured using modifications of previously described methods (36, 57). The instrument detection limit of the liquid scintillation counting was 0.4 Bq. The method detection limit was determined by the analysis of negative control samples prepared by the autoclave sterilization of sediment samples before the injection of $^{14}$C-labeled substrates. Hydrogenotrophic methanogenesis and acetogenesis from the negative control samples with $^{14}$C-bicarbonate did not exceed the instrument detection limit (0.4 Bq, equal to 0.0002 pmol cm$^{-3}$ day$^{-1}$), and acetoclastic methanogenesis from the negative control with $^{14}$C-labeled acetate was 9 Bq (equal to 0.004 pmol cm$^{-3}$ day$^{-1}$). Sediment samples used for hydrogenase activity measurement were collected from the core-cutting area using sterilized tip-cut plastic syringes immediately after core recovery. Hydrogenase activity was measured as described previously (37). In short, samples were incubated with a tritium gas headspace, and because of the activity of the hydrogenase enzyme, tritiated water will...
be produced and its activity can be quantified by liquid scintillation counting. The Gibbs free energies for homoacetogenesis, hydrogenotrophic methanogenesis, and aceticlastic methanogenesis were calculated on the basis of in situ geochemical and geophysical measurement data [H₂ concentration is 28.1 μM, and CH₄ concentrations are saturated values (44 to 54 mM)], as described previously (2). We also calculated the energy yield by using the H₂ concentration measured by headspace gas, because the in situ H₂ concentration analyzed from only one Hybrid-PCS sample was uncertain.

**Analysis of lipid biomarkers**

Total lipid extracts were obtained from ~20 g of freeze-dried sediments using a modified Bligh and Dyer protocol (38), after adding an internal standard (phosphatidylcholine C₃₀:0). Analysis of IPIs was performed by high-performance LC electrospray ionization MS (HPLC-ESI-MS). Chromatographic separation was achieved on a Waters Acquity BEH C18 column (58) with a Dionex Ultimate 3000RS UHPLC coupled to a maXis quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an ESI source. Detection of lipids was performed in positive ionization mode while scanning a mass-to-charge (m/z) range from 150 to 2000. MS2 scans were obtained in data-dependent mode, and for each MS full scan, up to three MS2 experiments, targeting the most abundant ions, were performed. Active exclusion limits the times a given ion is selected for fragmentation (three times every 0.5 min) and thus allowed us to also obtain MS2 data of less abundant ions. Lipid identification was achieved by monitoring exact masses of possible parent ions (present as either H⁺, NH₄⁺, or Na⁺ adducts) in combination with characteristic fragmentation patterns (59). Lipid quantification was achieved by comparison of parent ion responses relative to known amounts of an internal standard (58).

**Cultivation of mud volcano microbial communities**

Batch-type cultivation of microbial communities in KMV#5 was tested using six sediment samples at 20° and 55°C under the anaerobic condition. Strain 1H1 was successfully isolated from the primary enrichment culture obtained from a sample at 12.82 mbsf. The detailed methods for cultivation conditions including the media used, isolation of strain 1H1, taxonomic identification, and physiological characterizations are described in Supplementary Methods.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/6/eaa04631/DC1

**Supplementary Text**

fig. S1. Photographs and Raman spectra of methane hydrate and a seismic profile of KMV#5.
fig. S2. Thermal gradient based on the temperatures measured in situ at KMV#5 and IODP site C0009.
fig. S5. Average linkage clustering analysis based on the Bray-Curtis dissimilarity distance of 16S rRNA genes.
fig. S9. Mixing curves of δ¹³CCH₄ between biogenic and thermogenic methane end-members consistent with a final clumped isotope temperature of 30°C for the resultant mixture.
fig. S10. Geochemical data from KMV#5 based on 16S rRNA gene sequence analysis.
fig. S11. Diversity indices of microbial communities in sediment core samples from KMV#5.
fig. S12. Concentration of archaeal core and IPLs.
fig. S13. Thermogenic and biogenic end-member values for mixing calculation. References (61–79)

**REFERENCES AND NOTES**


Acknowledgments: We thank all crew, drilling team members, and technical staffs on the deep-sea drilling vessel Chikyu and the research vessel Hakuhomaru for support with core sampling from KMV5S during JAMSTEC Chikyu Expeditions 903, 906, and KH06-04 cruises. We are also grateful to IODP for providing sediment core samples from Site C0002 during Expedition 315. We are grateful to S. Fukunaga, S. Hashimoto, K. Iijima, A. Imajo, H. Machiyama, Y. Nishio, S. Tanaka, H. Tomiyama, and N. Xiao for useful discussions and/or technical assistance. This is a contribution to the Deep Carbon Observatory. Funding: This study was supported, in part, by the Japan Society for the Promotion of Science (JSPS) Strategic Fund for Strengthening Leading-Edge Research and Development (to JAMSTEC and F.I.), the JSPS Funding Program for Next Generation World-Leading Researchers (GR102 to F.I.), the Grant-in-Aid for Science Research (nos. 23681007, 26287128, and 17H01871 to A.I.; no. 26251041 to F.I.; no. 17H06105 to N.Y.), and the East Asia and Pacific Summer Institutes program through the NSF and JSPS (no. 1308171 to D.H.C.). Lipid analyses by M.Y.Y. and shipboard work by F.S. were financed by the European Research Council under the European Union’s Seventh Framework Programme—“Ideas” Specific Programme (no. 247153 to K-U.H.). Author contributions: A.I. and F.I. designed the study. F.I. led the project as the chief scientist of JAMSTEC Chikyu Expeditions 903 and 906. Y.K. coordinated Expeditions 903 and 906 as the expedition project manager. A.I., F.I., R.R.A., T.H., Y.M., T. Toki, G.L.A., J.A., D.H.C., T.F., Y.I., H.U., J.K., H.K., K.-I.N., Y.N., M.N., H.R., S.S., F.S., A.T., W.T., T. Terada, H.T., and Y.T.Y. collected and analyzed the sediment core samples and data as shipboard scientists. S.H., S.K., Y.O., S.O., K.T., D.T.W., M.Y.Y., K-U.H., M.I., M.A.L., S.M., V.J.O., T. Tuji, U.T., and N.Y. analyzed the samples and data as shore-based scientists. A.I. and F.I. cowrote the manuscript. All authors discussed the results and commented on the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The 16S rRNA gene sequences in this study were all deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence databases under accession no. DRA001034. The 16S rRNA gene sequence of strain 1H1 was also available under accession no. LC170394. The additional data related to this paper may be requested from the authors.

Submitted 24 July 2017
Accepted 1 May 2018
Published 13 June 2018
10.1126/sciadv.aao4631


All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The 16S rRNA gene sequences in this study were all deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence databases under accession no. DRA001034. The 16S rRNA gene sequence of strain 1H1 was also available under accession no. LC170394. The additional data related to this paper may be requested from the authors.
Deep-biosphere methane production stimulated by geofluids in the Nankai accretionary complex


Sci Adv 4 (6), eaao4631.
DOI: 10.1126/sciadv.aao4631