The Potential for CH₄ Production by Syntrophic Microbial Communities in Diverse Deep Aquifers Associated with an Accretionary Prism and its Overlying Sedimentary Layers

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Accretionary prisms are thick masses of sedimentary material scraped from the oceanic crust and piled up at convergent plate boundaries. This material, which was originally deposited on a subducting ocean plate, is accreted on a non-subducting continental plate during Paleogene periods and is traceable for 1,800 km in parallel, including Alaska and Washington in the United States, New Zealand, Chile, Peru, Indonesia, Taiwan, and Japan (Kano et al., 1991; Reed et al., 2002; Hervé et al., 2013; Lee et al., 2017).

The Shimanto Belt, an accretionary prism found in southwest Japan, was mainly formed during the Cretaceous and Paleogene periods and is traceable for 1,800 km in parallel with the Nankai Trough and Ryukyu Trench (Kano et al., 1991; Taira et al., 1992). The Shimanto Belt originated from ancient marine sediment deposited on the Philippine Sea Plate (Kano et al., 1991). This sediment is approximately 10 km thick and contains layers of water-bearing permeable sandstone and water-impermeable shale that are rich in complex organic matter (Maki et al., 1980; Taira et al., 1982). Rainwater and seawater recharge through faults or fracture zones formed by earthquakes that occur at the plate boundaries, and these waters then collect in the deep aquifers of accretionary prisms in which they become anoxic over time (Sakata et al., 2012; Baito et al., 2015). Deep aquifers contain large amounts of groundwater with dissolved and free phase natural gas, mainly methane (CH₄) (Kimura et al., 2010; Matsushita et al., 2016; Matsushita et al., 2018). The origin of CH₄ in natural gas reserves in subsurface sedimentary deposits is biogenic (formed by methanogenic archaea) or thermogenic (formed by the thermal degradation of organic matter in sedimentary layers). Previous studies investigated CH₄ production processes in the Shimanto Belt’s deep aquifers, which are affected by rainwater and seawater flowing down from surface environments, and reported that the syntrophic biodegradation of organic matter by hydrogen (H₂)-producing fermentative bacteria and H₂-utilizing methanogens, as well as a thermogenic reaction, contributes to CH₄ production in these deep aquifers (Kimura et al., 2010; Matsushita et al., 2016; Imachi, 2017; Matsushita et al., 2018; Tamaki, 2019). On the other hand, Matsushita et al. (2016; 2018) demonstrated that groundwater in the deep aquifers associated with the Shimanto Belt had different chemical characteristics from site to site, based on measurements of temperature, salinity, chemical components, and oxygen and hydrogen stable isotope ratios. These findings suggest that the subsurface environments of the accretionary prisms are not constant and are affected by the geological and geochemical features of each region.

The anaerobic deep aquifers associated with accretionary

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prisms contain large amounts of \( \text{CH}_4 \) (Kimura et al., 2010; Sakata et al., 2012). \( \text{CH}_4 \) is a potential greenhouse gas and important energy resource. However, all of the research on \( \text{CH}_4 \) production processes conducted to date has targeted deep aquifers that are affected by rainwater and seawater derived from surface environments. Accretionary prisms and their overlying sedimentary layers that contain deep aquifers are influenced by magmatic \( \text{CO}_2 \) derived from the deep subsurface environments of active volcanoes or by ancient seawater containing high concentrations of iodine and bromine (Kato, 1985). Mayumi et al. (2013) previously reported that a high concentration of \( \text{CO}_2 \) may change microbial \( \text{CH}_4 \) production pathways in subsurface oil reservoirs. Additionally, iodine and bromine are generally known to exhibit antimicrobial activity (Odlaug, 1981). These factors may affect the microbial activity, community structure, and \( \text{CH}_4 \) production pathway in deep aquifers associated with accretionary prisms; however, there is currently no experimental evidence to support this.

We herein investigated the deep aquifers associated with the accretionary prism and its overlying sedimentary layers in the southeastern part of Kyushu Island, Japan, near active volcanoes. To characterize each deep aquifer, we analyzed groundwater and natural gas samples with chemical and isotopic techniques. The aim of the present study was to clarify whether \( \text{CH}_4 \) production by syntrophic communities of \( \text{H}_2 \)-producing fermentative bacteria and \( \text{H}_2 \)-utilizing methanogens occurs universally in deep aquifers associated with accretionary prisms. Therefore, we investigated microbial community structures and their potential for \( \text{CH}_4 \) production using microbial cell counts, a 16S rRNA gene analysis, and anaerobic culture methods. An understanding of the underlying mechanisms and potential for \( \text{CH}_4 \) production in deep aquifers associated with accretionary prisms, which are distributed worldwide and at which geodynamic phenomena, such as huge earthquakes and fault formation, occur, may provide novel insights into greenhouse gas emissions and global warming.

Materials and Methods

Study sites

The present study was conducted in the southeastern part of Kyushu Island, Japan, near some of Japan’s most active volcanoes (e.g., Kirishima Volcano and Sakurajima Volcano). The accretionary prism known as the Nichinan Group (mainly Paleogene) in the Shimanto Belt is distributed across this region (Fig. 1). The Nichinan Group is composed principally of sandstone, siltstone, conglomerate, and basalt (Kato, 1985). Sedimentary layers that unconformably overlay the Nichinan Group are referred to as the Miyazaki Group (mainly Neogene). The Miyazaki Group is mainly composed of sandstone, mudstone, and conglomerate that were deposited in a forearc basin (Suzuki, 1987; Oda et al., 2011).

Sampling of groundwater and natural gas

Anoxic groundwater and natural gas samples were collected from deep aquifers associated with the Nichinan and Miyazaki Groups through six deep wells: GRY, OYD, MR2, MR4, KGO, and KG5 (Fig. 1B and Table S1). These wells were drilled down to 810 to 1,301 m and constructed from tight steel-casing pipes including strainers (Table 1) (Kato et al., 2011; Sakata et al., 2012). Groundwater at these wells is anoxically drawn up to ground level by a water pump. In the present study, to prevent contamination by air and water remaining inside the pipe, 200 to 700 tons of groundwater was pumped before sampling. Groundwater samples were collected under anoxic conditions into autoclaved serum bottles and polycarbonate bottles using a sterile silicone tube. The concentrations of dissolved natural gas were so high that...
gas exsolved at ground level. Natural gas samples were collected into autoclaved 100-mL serum bottles under water. Serum bottles were tightly sealed with sterile butyl-rubber stoppers and aluminum crimps to prevent contamination by air. Collected samples were transferred to the laboratory on ice and then stored at 4°C until later use.

Physicochemical and stable isotope analyses
The temperature, pH, oxygen-reduction potential (ORP), and electrical conductivity (EC) of groundwater samples were measured at the outflows of the wells. Temperature was measured with a CT-460WR thermometer (Custom). pH was measured with an HI-9813 sulfide ion detector tube (Gastec). Dissolved organic carbon (DOC) in the groundwater, filtered through a pre- combusted Whatman GF/F glass microfiber filter (GE Healthcare), was measured on a TOC-V total organic carbon analyzer (Shimadzu).

H₂, N₂, O₂, CO₂, and CH₄ concentrations in natural gas were measured with a GC-2014 gas chromatograph (GC) (Shimadzu) equipped with a thermal conductivity detector (TCD) and packed column (ShinCarbon ST, 6.0 m x 3.0 mm i.d.; Shinwa Chemical Industries). The GC conditions used were as follows: injector temperature, 170°C; column oven temperature, 150°C; detector temperature, 170°C. Argon was used as a carrier gas at a constant flow rate of 50 mL min⁻¹. CH₄, C₂H₆, and C₃H₈ concentrations were measured with a GC-2014 GC equipped with a flame ionization detector (FID) and packed column (Sunpak-A, 2.0 m x 0.3 mm i.d.; Shinwa Chemical Industries). The GC conditions used were as follows: injector temperature, 100°C; column oven temperature, 65°C; detector temperature, 100°C. N₂ was used as a carrier gas at a constant flow rate of 50 mL min⁻¹. Samples were analyzed in triplicate. The confidence limits of the measurement were 0.01 vol.% for H₂, N₂, O₂, CO₂, and CH₄, and 0.001 vol.% for C₂H₆ and C₃H₈.

The stable hydrogen and oxygen isotope ratios of groundwater samples (D/H and ¹⁸O/¹⁶O) were measured with a DLT-100 liquid water isotope analyzer (Los Gatos Research) following the procedures described by Dawson et al. (2015). The stable carbon isotope ratio (δ¹³C/δ¹₂C) of dissolved inorganic carbon (DIC; consisting mainly of HCO₃⁻) was measured with a Trace GC Ultra GC (Thermo Fisher Scientific) connected to a Delta plus XL IRMS (Thermo Fisher Scientific) according to the method described by Miyajima et al. (1995). The δ¹²C/δ¹³C of CH₄ in natural gas was measured with a Flash EA1112 elemental analyzer (Thermo Fisher Scientific) connected to a Delta V Advantage ConFlo IV system (Thermo Fisher Scientific) (Miyajima et al., 1995). All isotope ratios are reported relative to international standards: Vienna Standard Mean Ocean Water for δD and δ¹⁸O, and Vienna Pee Dee Belemnite for δ¹³C.

Microbial cell counting and next-generation sequencing (NGS) analysis of 16S rRNA genes
Groundwater samples for microscopic observations were filtered using polycarbonate filters (pore size, 0.2 μm; diameter, 25 mm) (Millipore). Total microbial cells trapped on the filters were stained with SYBR Green I (Thermo Fisher Scientific) (Yanagawa et al., 2016). A LIVE/DEAD BacLight bacterial viability kit (Thermo Fisher Scientific) was used to obtain the ratio of live to total microbial cells (Vezzulli et al., 2015). Stained cells were observed under a BX51 epifluorescence microscope equipped with a U-MBIB3 fluorescence filter (Olympus), and more than 20 microscopic fields (approximately 20 cells in each field) were counted for each sample. Cells were counted within 48 h of groundwater sampling.

To analyze the archaeal and bacterial communities in the groundwater, 10 L of groundwater samples was aseptically filtered using Sterivex-GV filter units (pore size, 0.22 μm) (Millipore). Bulk DNA was extracted from trapped cells using an ISOIL for beads beating kit (Nippon Gene). The V3–V4 region of the 16S rRNA gene was amplified using a primer set, Pro341F and Pro805R for prokaryotes (Takahashi et al., 2014). PCR products were purified through a MultiScreen PCR filter plate (Millipore) and analyzed using an Agilent DNA 1000 kit on an Agilent 2100 Bioanalyzer system (Agilent Technologies) to detect primer dimers and obtain the average molecular weight of each product. The Illumina sequencing library was generated according to the method described by Takahashi et al. (2014). Sequencing was conducted using a 600-cycle paired-end MiSeq reagent kit v3 (Illumina) on an Illumina MiSeq platform. After sequencing was complete, an image analysis, base calling, and error estimations were performed using Illumina Real-Time Analysis software version 1.17.28. Bioinformatic processing was performed using a combination of Quantitative Insights Into Microbial Ecology (QIME) version 1.9.1 and USEARCH version 6.1 (Caporaso et al., 2010; Edgar, 2010). Only reads that had quality value scores of ≥20 for more than 98% of the sequence were extracted for further analyses. Potential chimeras were removed using USEARCH version 6.1. Quality-filtered reads were assigned to operational taxonomic units (OTUs) at a 97% similarity level, and the Good's coverage, Chao 1, and Shannon indices were then calculated with QIIME version 1.9.1. Taxonomy was assigned using the Ribosomal Database Project (RDP) MultiClassifier version 2.11 with a confidence level of 80% (Wang et al., 2007). Sequences were deposited under GenBank/ENA/DDBJ accession numbers DRA004443 and DRA005244.

Measurement of potential microbial CH₄ production
Thirty milliliters of each groundwater sample was anoxically injected into an autoclaved 70-mL serum bottle that was then
Physicochemical signatures of groundwater and natural gas

Anoxic groundwater and natural gas samples were collected from deep aquifers through six deep wells. The groundwater temperature measured at the outflows of the wells ranged between 24.9 and 50.0°C (Table 1). The pH of groundwater ranged between 7.1 and 7.4. The ORP was measured as an indicator of anoxic conditions in the deep aquifers and ranged between −231 and −141 mV. The EC, an indicator of salinity, ranged between 488 and 5,000 mS m⁻¹. These parameters were measured several times between May 2014 and May 2019, and no temporal variations were detected. The concentration ranges of various chemical components were measured, including Br⁻ and I⁻ (0.04 to 1.38 mM and 0.05 to 0.95 mM, respectively; both were particularly high in samples from GRY), HCO₃⁻ (1.5 to 46 mM), and DOC (0.05 to 0.83 mM) (Table S2). PO₄³⁻, NO₃⁻, SO₄²⁻, S²⁻, acetate, and formate were found at trace amounts or were below detection limits.

CH₄ was the predominant component of natural gas (Table 1). The other principal components were CO₂ and N₂. In addition to CH₄, natural gas collected from KGO contained a particularly large amount of CO₂ (34.8 vol.%). C₂H₆ and C₃H₈ were found at trace amounts. The hydrocarbon gas composition C₅/C₆/C₇ of natural gas samples ranged between 571 and 11,211. H₂, O₂, and C₆H₆ concentrations were found at trace amounts or were below detection limits.

Stable isotopic signatures of groundwater and natural gas

The δD and δ¹⁸O of groundwater samples ranged between −33.9 and −5.8‰ and between −5.1 and 1.9‰, respectively (Fig. 2 and Table S3). To estimate the origins of groundwater in the deep aquifers, we plotted δD and δ¹⁸O values with respect to the global meteoric water line (Craig, 1961). Groundwater sampled from KG5 was plotted closer to the local surface water (Kato et al., 2011). The groundwater from OYD, MR2, MR4, and KGO fell in the upper right region of the local surface water. The groundwater from GRY was plotted closer to normal seawater and ancient seawater, as reported previously by Maekawa et al. (2006).

The δ¹³C of DIC (consisting mainly of HCO₃⁻) in groundwater (δ¹³CDIC) ranged between 0.16 and 8.95‰ (Fig. 3A and Table S3). The δ¹³C of CH₄ in natural gas (δ¹³CCH₄) ranged between −57.8 and −39.6‰. To estimate the origin of CH₄ in natural gas, we assessed carbon isotope fractionation.

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tion ($\alpha_1$) between $\delta^{13}$C$_{\text{DIC}}$ and $\delta^{13}$C$_{\text{CH}_4}$. $\alpha_1$ values were 1.041 to 1.065 (Fig. 3A and Table S3). In a plot of stable isotopic values on a $\delta^{13}$C$_{\text{DIC}}$ versus $\delta^{13}$C$_{\text{CH}_4}$ diagram (Smith and Pallasser, 1996), the sample from GRY fell within the region of biogenic origin ($\alpha_1$=1.06–1.08). In contrast, all other sample values fell within the boundary between biogenic and thermogenic origins ($\alpha_1$=1.04–1.06), indicating that natural gas samples contained CH$_4$ of mixed biogenic and thermogenic origins. We also plotted the observed isotopic values in a $\delta^{13}$C$_{\text{CH}_4}$ versus hydrocarbon gas composition C$_i$/C$_{\text{CH}_4}$ diagram according to Bernard et al. (1978).

In this diagram, all sample values fell within the boundary between biogenic and thermogenic origins (Fig. 3B).

**Abundance and diversity of microbial communities in groundwater**

The abundance of total microbial cells stained with SYBR Green I ranged between $8.2\times10^4$ and $1.0\times10^6$ cells mL$^{-1}$ (Table 2). Live/dead staining revealed that the density of live microbial cells ranged between $1.4\times10^4$ and $1.4\times10^6$ cells mL$^{-1}$ (Fig. S1). The percent cell viability (% live microbial cells/total microbial cells) ranged between 7.5 and 38.9% (Table 2).

To elucidate microbial community structures in anoxic groundwater derived from deep aquifers, we performed a NGS analysis targeting the archaean and bacterial 16S rRNA genes. We obtained 12,678 to 54,714 reads and 234 to 804 OTUs (Table S4). Coverage reached >98.3%. The Chao1 and Shannon indices were 245 to 1,542 and 4.74 to 6.84, respectively.

Archaeal 16S rRNA genes, which accounted for between 1.0 and 23.9% of the total reads obtained from each sample (Fig. 4A), revealed the predominance of methanogenic archaean belonging to the order Methanobacteria (Fig. 4B). We also detected minor amounts of Methanomicrobiales, Methanocellales, and Methanomassiliicoccales. These methanogenic archaean are generally known to use H$_2$ and CO$_2$ to produce CH$_4$, while some are capable of methylotrophic methanogenesis (Sakai et al., 2008; Zhu et al., 2011; Dridi et al., 2012; Sakai et al., 2012). In groundwater samples from OYD, MR2, and KG5, we also identified archaean 16S rRNA genes that are closely related to those of Methanosarcinales, an order of methanogenic archaean that uses acetate, methanol, or H$_2$ and CO$_2$ as a methanogenic substrate (Kamagata et al., 1992).

The analysis of bacterial 16S rRNA genes revealed the presence of bacterial groups that belong to the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Chlorobi (Fig. 4C). At the order level, 6–40% of bacterial 16S rRNA genes obtained from all sites, except KGO, were closely related to Rhizobiales, an order of Alphaproteobacteria. The bacterial 16S rRNA genes closely related to the orders Lactobacillales, Clostridiales, and Bacteroidales were also detected from all sites. The bacterial groups of the orders Coriobacteriales and Ignavibacteriales were identified mainly in OYD and KGO, respectively.

**Potential for microbial CH$_4$ production**

To assess the potential for CH$_4$ production by methano-
cultures after incubations of longer than 60 d.

On the other hand, the high potential for CH$_4$ production by microbial communities was confirmed in cultures using YPG medium-amended groundwater (Fig. 5 and S2). In cultures using groundwater from OYD, MR4, KGO, and KG5, the production of H$_2$ and CO$_2$ was observed within 3 d. In cultures using groundwater from GRY and MR2, H$_2$ and CO$_2$ were detected after 7 d. After the production of H$_2$ and CO$_2$, the concentration of H$_2$ decreased to below the detection limit in all cultures. CH$_4$ production was observed after H$_2$ levels began to fall. In cultures using groundwater from GRY, MR2, MR4, KGO, and KG5, CH$_4$ production was observed within 10 d. In cultures using groundwater from OYD, CH$_4$ was detected after 17 d. In a killed control using the groundwater samples amended with YPG medium, abiotic H$_2$ and CH$_4$ production was not observed during an incubation of more than 120 d. Similarly, H$_2$ and CH$_4$ production was not observed in cultures using groundwater without organic substrates.

To identify microorganisms that generated biogases (i.e., H$_2$, CO$_2$, and CH$_4$), we constructed archaeal and bacterial 16S rRNA gene clone libraries. The 16S rRNA gene analysis suggested that H$_2$-utilizing methanogenic archaea and H$_2$-producing fermentative bacteria were predominant in the microbial population (Table S5) and were related to the archaeal orders Methanobacteriales and Methanomicrobiales (Fig. S3) and the bacterial orders Bacillales, Bacteroidales, Clostridiales, Tissierellales, and Ignavibacteriales, respectively (Fig. S4) (Parshina et al., 2003; Watanapokasin et al., 2009). Additionally, bacterial 16S rRNA genes closely related to the order Desulfovibrionales, which is known to form syntrophic microbial communities with H$_2$-utilizing methanogens, were also confirmed in the clone libraries associated with GRY and OYD (Morris et al., 2013).

**Discussion**

To characterize the subsurface environments and identify the potential for CH$_4$ production in anoxic deep aquifers associated with the Shimanto Belt and its overlying sedimentary layers in the southeastern part of Kyushu Island, Japan, we herein revealed the geochemical and microbiological features of deep aquifer-derived anoxic groundwater and natural gas samples. Groundwater from the KG5 site had the lowest EC value (488 mS m$^{-1}$), which was approximately 10% that of normal seawater (Table 1), and had similar δD and δ$^{18}$O values to those of the local surface water (Fig. 2). Collectively, these characteristics suggest that the KG5 deep aquifer was affected by rainwater that flowed down from surface environments. In contrast, groundwater from GRY had almost the same EC value as normal seawater and ancient seawater (Table 1). The δD and δ$^{18}$O values of groundwater sampled from the GRY site were also plotted closer to those of normal seawater and ancient seawater (Fig. 2). Additionally, the groundwater from GRY contained higher levels of Br$^-$ and I$^-$ than normal seawater (Table S2) (Millero et al., 2008). These chemical features are consistent with those of ancient seawater, which is comprised of groundwater that originated from seawater and was main-
tained for a long period in a low-temperature deep aquifer (Katayama et al., 2015). Therefore, the GRY deep aquifer was likely influenced by ancient seawater. This result differs from that previously reported in deep aquifers found in other regions of the Shimanto Belt that are affected by rainwater and normal seawater from surface environments (Matsushita et al., 2016; Matsushita et al., 2018).

The EC values of groundwater from OYD, MR2, MR4, and KGO were between approximately 20 and 44% that of normal seawater, suggesting that the deep aquifers at these sites hold groundwater derived from a mixture of rainwater and seawater that flowed down from the surface and the seafloor, respectively (Table 1). However, the δD and δ18O values of OYD, MR2, MR4, and KGO fell to the right of a line joining the plots of surface water and seawater, suggesting large δ18O enrichment (Fig. 2). The high δ18O values suggest that the groundwater in these deep aquifers was affected by water-rock interactions in thermal subsurface environments (Bowers and Taylor, 1985). Alternatively, these isotopic characteristics may be the result of groundwater evaporation due to geothermal heat (Connelly et al., 1990).

CH₄ was the predominant component of all natural gas samples with the exception of KGO, which also contained particularly high amounts of CO₂ (34.8 vol.%) and CH₄ (62.9 vol.%) (Table 1). Kato et al. (2011) reported that CO₂ in natural gas from the KGO site is of magmatic origin based on its δ13C value. The accumulation of magmatic CO₂ in natural gas has frequently been found in tectonically active oil- and gas-bearing basins (e.g., Dai et al., 1999). The highest groundwater temperatures recorded in the present study were also from the KGO site (50°C). Thus, the deep aquifer at the KGO site may be affected by magmatic CO₂ and geothermal groundwater that rose from deep subsurface environments of active volcanoes, e.g., the Kirishima Volcano and Sakurajima Volcano (Fig. 1). These results are distinct from previous findings on the deep aquifers of the Shimanto Belt, which is found in the mid-western part of Shizuoka Prefecture and the southern part of Okinawa Island, Japan (Matsushita et al., 2016; Matsushita et al., 2018).

The results of chemical and isotopic analyses of groundwater and natural gas samples suggested that the environmental features of the deep aquifers markedly differed among the sites studied herein. In contrast, stable carbon isotope analyses and microbiological assays suggested the presence of CH₄ production by a syntrophic community of H₂-producing fermentative bacteria and H₂-utilizing methanogenic archaea in the deep aquifers. The δ13C(CH₄) versus δ13C(CO₂) diagram and δ13C(CH₄) versus C3/C1(C2+3C) diagram both indicated that all natural gas samples contained CH₄ of biogenic origin or a mixture of CH₄ of biogenic and thermogenic origins (Fig. 3). The NGS analysis of target archaeal 16S rRNA genes revealed the predominance of H₂-utilizing methanogenic archaea belonging to the orders Methanobacteriales, Methanomicrobiales, and Methanomassiliicoccales in the groundwater samples (Fig. 4B). The NGS analysis of target bacterial 16S rRNA genes indicated the presence of bacteria belonging to the orders Lactobacillales, Clostridiales, Bacteroidales, Coriobacteriales, and Ignavibacteriales (Fig. 4C). These bacterial groups have the ability to degrade organic matter to H₂ and CO₂ by fermentation (Benno et al., 1983; Kandler et al., 1983; Liu et al., 2008; Podosokorskaya et al., 2013). The presence of Rhizobiales in groundwater samples was also confirmed. Although Rhizobiales is generally known to comprise aerobic diazotrophs, some species that belong to this order may grow by fermentation and are present in deep subsurface environments (Kodama and Watanabe, 2011;
Miettinen et al., 2015; Dutta et al., 2018). The presence of methanogenic archaea and fermentative bacteria identified in the present study has also been reported in deep aquifers of other regions associated with the Shimanto Belt (Matsushita et al., 2016; Matsushita et al., 2018). Additionally, these microorganisms have frequently been found in subsurface gas and oil reservoirs, coal deposits, and subsurface floor sediment cores in which microbial hydrogenotrophic CH4 production has been confirmed (Colwell et al., 2004; Meslé et al., 2013; Katayama et al., 2015; Hirai et al., 2017). These results suggest that a syntrophic community of H2-producing fermentative bacteria and H2-utilizing methanogenic archaea contributes to CH4 production in the deep aquifers tested. The microbial CH4 production process may be flexible to environmental differences by changing the microbial community structure while maintaining similar metabolic functions.

The microbial cell densities observed in the present study were similar to or higher than those previously reported in deep aquifers of the accretionary prism and other geothermal aquifers (Table 2) (Katayama et al., 2015; Matsushita et al., 2016; Matsushita et al., 2018). These results also suggest the presence of microbial activity in the deep aquifers. Additionally, this is the first study to measure live cell density as well as total cell density in the groundwater of the Shimanto Belt. Groundwater from GRY showed relatively high live cell density (Table 2). Since CH4 from GRY was suggested to be of biogenic origin by the δ13CIC versus δ13CDIC diagram (Fig. 3A), the impact of high concentrations of Br− and I− on microbial CH4 production appears to be small. In contrast, the lowest live cell density was observed in KGO. This may be due to magmatic CO2 and geothermal groundwater from deep subsurface environments. The factors that influence microbial viability in deep aquifers may be more complex, such as the residence time of groundwater and the degradation rate of dead cells.

Although the potential for CH4 production was not confirmed in cultures using H2/CO2-amended groundwater, this may have been due to the growth inhibition of H2-utilizing methanogenic archaea caused by high concentrations of H2 and CO2 (Sakai et al., 2009). On the other hand, the addition of organic substrates to groundwater samples demonstrated a high potential for H2 and CO2 production by H2-producing fermentative bacteria as well as rapid H2 consumption and significant CH4 production by H2-utilizing methanogenic archaea (Fig. 5 and S2). Since we used high concentrations of organic substrates, the microbial activity observed in the cultures does not reflect the activity level in the actual subsurface environment. Changes in microbial community structures between natural groundwater and culture samples, particularly bacterial communities, are attributed to the strong selective pressure caused by using YPG medium (Fig. 4 and Table S5). On the other hand, the present results showed high live cell densities and a high potential for microbial CH4 production. Thus, previous and ongoing microbial CH4 production appears to contribute to the significant CH4 reserves in the deep aquifers. A previous study reported that the average contents of total organic carbon in the sedimentary layers of the Nichinan Group and Miyazaki Group were 0.86 and 0.58%, respectively (Maki et al., 1980). These contents are consistent with those of total organic carbon in the sediments of other accretionary prisms containing biogenic CH4 (Davie and Buffett, 2003; Oba et al., 2015). Therefore, this organic matter appears to support the activity of microbial communities that generate CH4 in the deep aquifers. Further clarification of the composition of this organic matter is of fundamental importance for future studies.

The NGS analysis of archaeal 16S rRNA genes in natural groundwater samples revealed the presence of Methanosarcinales, which generally uses acetate or methanol for CH4 production (Fig. 4B). Additionally, Methanobacteriales and Methanomassiliicoccales identified in the present study grow by methylotrophic methanogenesis. On the other hand, CH4 production was not confirmed in cultures using groundwater amended with acetate, methanol, and formate. These results suggest that the potential for CH4 production using acetate, methanol, and formate in the deep aquifers is lower than that of H2-utilizing methanogenesis. This is consistent with the low concentrations of these methanogenic substrates in the groundwater (Table S2). However, we cannot exclude the possibility that CH4 production from these methanogenic pathways occurs in the deep aquifers because the culture conditions used in the present study were significantly different from the actual subsurface environments and may not have been suitable for these methanogenic archaea.

Conclusion

In the present study, we demonstrated the potential for CH4 production by a syntrophic microbial community of H2-producing fermentative bacteria and H2-utilizing methanogens in deep aquifers that have widely varying geological and geochemical features. The production process of CH4 is similar to that previously reported in deep aquifers found in other regions of the Shimanto Belt that are affected by rainwater and normal seawater from surface environments (Matsushita et al., 2016; Matsushita et al., 2018). Since accretionary prisms are derived from ancient marine sediments scraped from the subducting ocean plate, they are rich in complex organic matter (Berner and Koch, 1993; Kaneko et al., 2010). The results of the present study support a model in which the biodegradation of organic matter in ancient sediments contributes to the generation of CH4 reserves in deep aquifers associated with accretionary prisms and their overlying sedimentary layers, which are distributed globally and have diverse geological and geochemical features.

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