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Characteristics of Microbial Community Structure at the Seafloor Surface of the Nankai Trough

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Abstract

Phylogenetic analysis of bacteria and archaea on the seafloor surface of the Nankai Trough was conducted. DNA was extracted from a total of 14 samples (2 samples from 1 methane seep area and 12 samples from 12 general seafloor areas) and analyzed, targeting the 16S rRNA gene. As a result of the phylogenetic analysis of bacteria, 1 clone was found to have 96% homology with the 16S rRNA gene sequence of *Methylomicrobium alcaliphilum*, a halophilic methane-oxidizing bacterium, in a sample taken from the methane seep area. However, overall, no characteristic pattern was observed in the bacterial community structure between the methane seep area and the general seafloor. In contrast, in archaea, the genus *Methanosarcina* was predominantly detected in the samples from the methane seep area. Among them, many sequences that were closely related to anaerobic methane-oxidizing archaea, which perform anaerobic methane oxidation, were detected.

Keywords: Methane seep, ANME, methane oxidation, MOB.

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INTRODUCTION

In the sea around Japan, it is known that there is a sand–mud alternating layer structure in the deep seafloor at a depth of 1,000–2,000 m, and methane hydrate is known to exist under the sand layer[1,2]. In the Nankai Trough, located off the southwest coast of Japan, the geological phenomenon is confirmed which related to the presence of methane hydrate under the seafloor, and observations using bottom-simulating reflectors showing the hydrate layer confirm this[2,3]. Methane hydrate is a clathrate hydrate; it is an ice-like solid substance formed only at low temperature and high pressure when numerous water molecules surround methane molecules. If we develop a technology that enables us to extract methane from methane hydrate, it would become a secure Japan's original natural gas resources independent of import and expect development as a domestic energy resource.

In the future, methane gas may leak into seawater when recovering it from the methane hydrate layer in the deep seafloor because of the influence of ground deformation. Leakage of methane gas is a concern not only because of the loss of domestic energy source but also because of the effect that leakage could have on the ocean floor ecosystem. Therefore, the development of technology that prevents damage by detecting methane leakage at the bottom of the sea is considered to be an important issue in obtaining public acceptance.

Methane leakage can be defined as "a state in which methane exceeding a certain concentration is maintained for a certain period." To investigate the effects of methane leakage on an ecological system, a group of *Calyptogena* on the southern slope of the Daini-Tenryu Knoll has been studied[4,5]. *Calyptogena*, known as deep-sea cold-seep clam, has been observed in cold-seep or hydrothermal vent and one of the representative organism in the cold-seep communitites[6]. It is presumed that methane leakage has some effect on the microbial community structures of an ecosystem because it causes a change in the surrounding environment.

Under aerobic condition, microbial oxidation of methane is well studied and aerobic methane-oxidizing bacteria (MOB), such as the genera *Methylobacter*, *Methylococcus*, and *Methylosinus*, are involved in the metabolism of methane[7,8]. Under the anaerobic condition, anaerobic oxidation of methane (AOM) is occurred. AOM has been reported that it is syntrophic reaction of sulfate-reducing bacteria and anaerobic methanotrophs called anaerobic methanotrophic (ANME) archaea[9-12]. These methane metabolism-related microbial communities can grow using methane as a electron donor, if the methane concentration in the surrounding environment rises due to methane leakage from the seafloor, and they can become a dominant microorganism group in that environment. Incidentally, regarding aerobic MOB, there are only few cases where they have been isolated and cultured from the deep seafloor[13,14], but there are many cases where their existence has been confirmed at the genetic level[15-18]. The monitoring of the microorganisms that are characteristic of the methane seep area and the use of them as indicators can therefore help determine the levels of methane leakage.

PCR can be used to detect and analyze unknown microorganisms that have not been isolated previously, by performing phylogenic analysis of target genes[18,19].

Therefore, in this study microbial community in the surface layer of the ocean floor was analyzed based on 16S rRNA gene sequence. In this paper, samples of submarine mud surface were collected in the methane seep and general seafloor areas (control) from the surface layer sediments of the Nankai Trough. We then investigated the composition and characteristics of the microbial ecosystems to determine whether differences exist between methane seepage and control areas.

MATERIALS AND METHODS

Sampling sites and methods

In October-November 2004, sediments on the sea floor from 13 different locations were collected during the voyage of *Bosei-maru*, owned by Tokai University. The sampling sites were targeted at the Nankai Trough area located to the east of the Kumano-nada (Table 1). Here, the seafloor surface layer sample (S1) from the methane seep area was set as the target area where methane was present, and the other 12 locations were investigated as nonmethane-existence areas. S1 is the point where the colony

of *Calyptogena* was observed[19], and the remaining were general seafloor sediments.

In the marine sediment of S1, surface mud (approximately 20 cm) of the seafloor was collected, together with the group of *Calyptogena*, using a grab sampler (Kinoshita-type sampler, equipped with a seafloor camera). The surface of S1 sample was gray, but the central part of the sediment was black. The black part was designated as "reductive sample, S1k," and the remaining part was designated as "oxidative sample, S1s." These 2 samples were investigated. The remaining 12 samples (MC01, MC02, MC03, MC04, MC05, MC06, PC01, PC03, PC04, PC09, PC10, PC11) were general seafloor samples taken as controls for comparison with samples from the methane seep area. For MC01, MC02, MC03, MC04, MC05, and MC06, the surface layer part (approximately 30 cm) of the seabed was sampled in an undisturbed manner using a multiple corer. Columnar surface sediments (from the seafloor surface to approximately 5 m below) were collected for PC01, PC03, PC04, PC09, PC10, and PC11 using a piston corer (pipe length: 5 m, with an Ewing-type pilot corer).

Among the marine sediments collected using the multiple corer or piston corer, approximately 10 ml of pore water was extracted from about 200 g of sediments, using hydraulic pressure, for chemical analysis. A portion (approximately 2 ml) of the extracted pore water was collected in a 2-ml vial bottle containing mercuric chloride and amidosulfuric acid, and the bottles were tightly sealed for the analysis of gas components. For dissolved methane analysis, surface mud sample of S1 site and sea water samples just above the seafloor (ca. 10 cm) of the other site were used.

Dissolved methane concentration

The dissolved methane concentration was measured by the purge method because the concentration was predicted to be too low in the pore water sample[20]. The measurement of the methane concentration in the sediment sample was performed according to the method of Tsunogai et al.[21], using a continuous-flow isotope ratio mass spectrometer by the headspace method.

Phylogenetic analysis

DNA was extracted from 1.5–2.0 g of the ocean sediment using an Ultra Clean Soil DNA Kit (Mo Bio Laboratories) and ISOIL (Nippon Gene) and mixed in equal amounts. This DNA solution was used for PCR to amplify the 16S rRNA gene of bacteria and archaea. For the PCR, thermal cycler GeneAmp 9600 (Perkin Elmer) and Ampli Taq Gold (Applied Biosystems) were used. The PCR reaction was performed under the same conditions for both archaea and bacteria at 94°C for 10 minutes followed by 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes. After 35 cycles, the samples were held at 72°C for 10 minutes. For primer pairs, 8F and 1492R were used for the amplification of the bacterial 16S

Table 1 Locations of sample collection sites

Sample name	Latitude	Longitude	Water depth (m)	note
S1	34°04.594'N	137°47.265'E	611	Methane seep
MC01	33°55.770'N	137°18.902'E	984	
MC02	33°49.005'N	136°30.353'E	2,053	
MC03	33°59.983'N	137°10.225'E	1,852	
MC04	34°08.740'N	137°29.415'E	1,359	
MC05	34°12.230'N	137°27.615'E	1,258	
MC06	34°16.557'N	137°57.921'E	734	
PC01	33°57.649'N	137°15.829'E	1,440	
PC03	33°49.164'N	136°30.056'E	2,053	
PC04	33°50.734'N	136°29.658'E	1,886	
PC09	34°13.346'N	137°40.265'E	1,166	
PC10	34°16.587'N	137°44.841'E	922	
PC11	34°13.145'N	137°42.702'E	1,076	

rRNA gene[22], and ARC344F and 1492R were used for the amplification of the archaeal 16S rRNA gene[23].

After confirming the fragment size by agarose gel electrophoresis, the obtained PCR product was excised from the gel, extracted, and purified using a Gel Extraction Kit (QIAGEN, Hilden, Germany). Thereafter, it was cloned into the TA Cloning vector pCR 2.1 and transfected into an One Shot TOP10 competent cell (Invitrogen, Carlsbad, San Diego, CA). The sequencing was performed with 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The obtained sequence was checked for homology with nucleotide sequences on the NCBI databases, and after alignment with the ClustalW program[24], a phylogenetic tree was constructed by the neighbor-joining method.

To compare the microbial community structure in each sample, principal component analysis (PCA) was performed. For the analysis, we used the detection rate of the phylum and class level classification of each sample; STAMP software[25] was used for calculations based on default setting of Euclidean distance.

The 16S rRNA gene sequence data obtained in this study and used for phylogenetic analysis were deposited in the GenBank/EMBL/DDBJ databases under accession numbers LC382285 to LC382364.

RESULTS

Dissolved methane concentration

The methane concentration in the sediment sample of the general seafloor was approximately 0.25µmol/L, which is the background level in the seafloor of this survey area. Because the methane seep area was inhabited by a group of *Calyptogena*, the corer could not penetrate and the sediment sample was collected using a grab-type sampler. Therefore, the dissolved methane concentration was not measured.

Phylogenic analysis of bacterial 16S rRNA gene sequence

In total, 1,038 bacterial clones were sequenced for the 16S rRNA gene. Most of the clones obtained from each sample were classified into the phyla Proteobacteria or Bacteroidetes, which are Cytophaga and Flavobacterium (Fig. 1). Although the ratio of the dominant clone was different for each site, γ-Proteobacteria, δ-Proteobacteria, and Bacteroidetes were detected

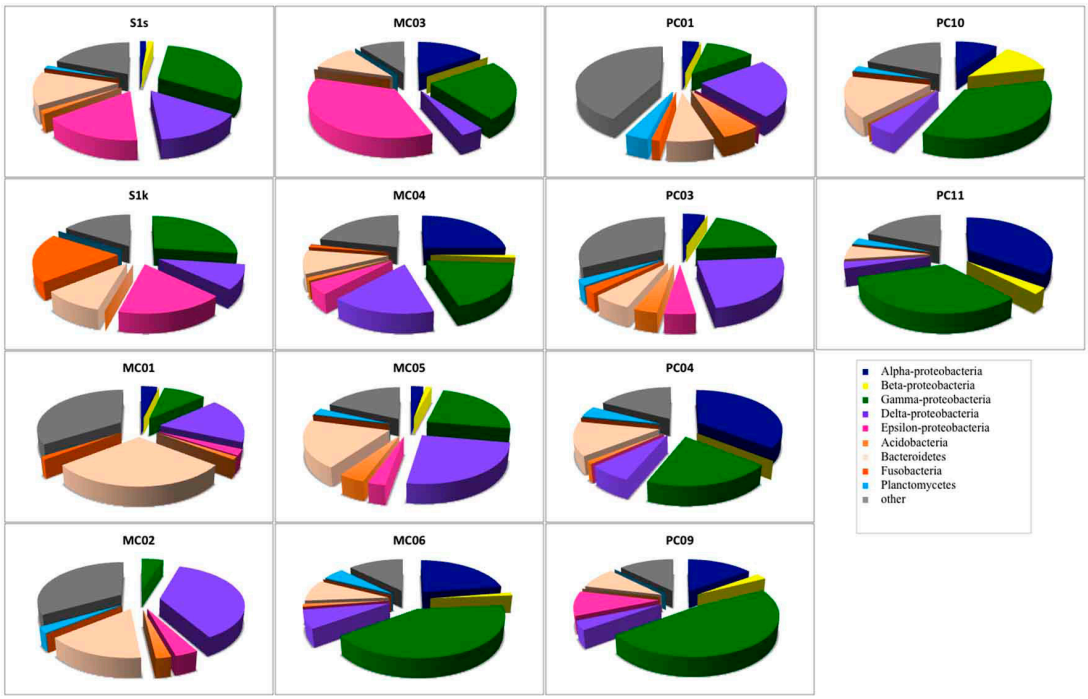

Fig. 1. Bacterial community compositions of marine sediments based on 16S rRNA gene sequence analysis

at all sites. The proportion of γ-Proteobacteria, which belongs to type I of aerobic methane-oxidizing bacteria, was overwhelmingly 47.5% in PC09, followed by PC10 (35.7%) and PC11 (32.4%). In addition, δ-Proteobacteria, to which many sulfate-reducing bacteria belong, was detected at all sites. The largest percentage was 37.2% for MC02, followed by 25.0% for MC05. ε-Proteobacteria, to which many sulfur-oxidizing bacteria belong, were detected in PC03, and the proportion accounted for 35.9%. Although α-Proteobacteria were not detected at some sites, a relatively large proportion was detected in PC04 and PC11 (35.6% and 35.1%, respectively).

Analysis of each site showed that there were many γ-Proteobacteria (approximately 30%) in S1s and S1k that were from the methane seep area.

The groups that most dominated in the mud sample of the general seafloor were as follows; MC01: Bacteroidetes (29.3%), MC02: δ Proteobacteria (37.0%), MC03: ε-Proteobacteria

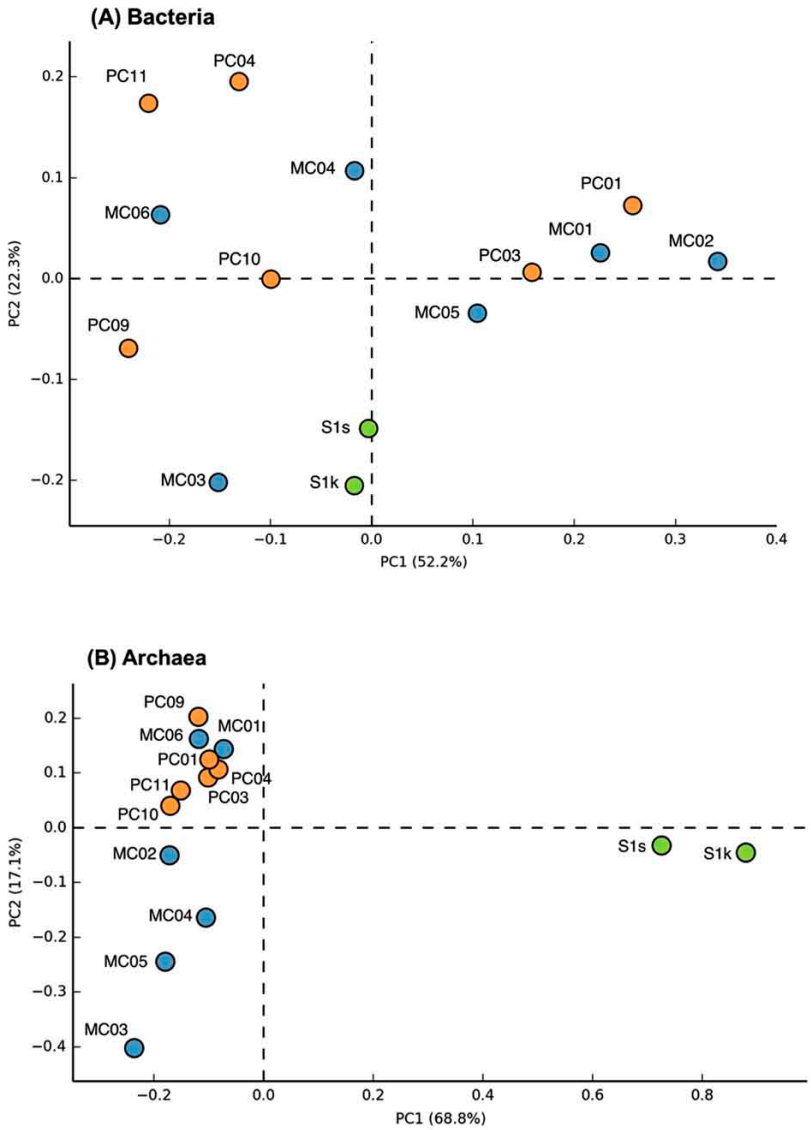

Fig. 2. Principal component analysis plots of bacterial (A) and archaeal (B) communities of marine sediments.

(35.9%), MC04: α-Proteobacteria (25%), MC05: δ-Proteobacteria (25.0%), MC06: γ-Proteobacteria (41.3%), PC01: δ-Proteobacteria (23.8%), PC03: δ-Proteobacteria (23.8%), PC04: α-Proteobacteria (35.6%), PC09: γ-Proteobacteria (47.5%), PC10: γ-Proteobacteria (35.7%), PC11: α-Proteobacteria (35.1%). Thus, the microflora exhibited different compositions for each site, and when compared with the 16S rRNA gene sequence of bacteria as an index, a significant pattern, capable of discriminating between the methane seep and general seafloor areas, was not observed. The same result was obtained by PCA based on the detection ratio of each bacterial group. However, it was found that the 2 samples in the methane seep area were similar to each other (Fig. 2A).

Nearly half of the sequenced clones demonstrated low homology (<95%) with strains and genes from the database and were derived from microorganisms that were completely unknown at the genus level. In almost all the samples, no distinction was made between whether the sample was from the methane seep or general seafloor areas. Clones with high homology with clones collected from the methane seep area[26,27] in the sea around Japan and other oceanic gas hydrate[28,29] and hydrocarbon seep areas[30] were obtained (≥97%). In addition, clones related to methane oxidation (closely related to the methanotrophic community) were obtained from 8 samples of S1s, S1k, MC01, MC02, MC03, MC04, PC01, and PC03. However, in our study, clones corresponding to the known methanotrophic bacteria were not obtained, and only 1 clone with 96% homology with *Methylomicrobium alcaliphilum*[31], a halophilic methane-oxidizing bacterium, was found in a sample from the methane seep area.

Phylogenic analysis of archaea 16S rRNA gene sequence

In the archaea 16S rRNA gene, 1, 105 clones were sequenced. As a result of the analysis of archaea, a remarkable difference was observed between the methane seep and general seafloor areas (Figs. 2B and 3). From the S1s sample, 76 clones were sequenced, 84% of which were closely related to the genus *Methanosarcina*. From the S1k sample, the 80 clones obtained were classified into 2 phylogenies, *Methanosarcina* (79 clones) and DSAG (1 clone). Thus, the genus

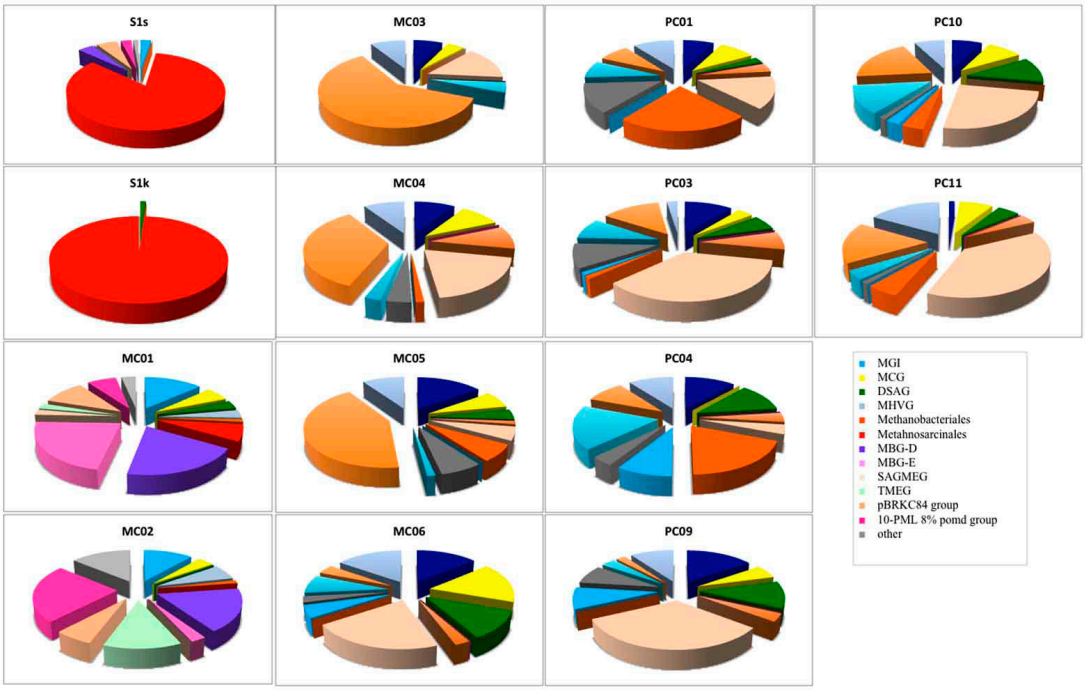

Fig. 3. Archaeal community compositions of marine sediments based on 16S rRNA gene sequence analysis

Methanosarcina overwhelmingly dominated in the methane seep area, whereas there were no sites dominated by the genus *Methanosarcina* in the other 12 general seafloor samples.

In the 12 general seafloor samples, it was found that each site has a unique composition at the genus level. When analyzing the dominant group at each site, it was found that MC01 is

Fig. 4. Phylogenetic analysis of archaeal 16S rRNA gene sequences obtained from S1 site sample. The scale bar represents the number of nucleotide changes per sequences position.

dominated by the marine benthic group-E (MBGE) and marine benthic group-D (MBGD) (22% and 20%, respectively). The clones obtained in MC02, MC03, MC04, and MC05 were dominated by the 10-PML 8% pond group (24%, 60%, 35% and 42%, respectively), which is composed of clones obtained from saline soil and solar saltern with homology as low as 90%. MC06 was dominated by MBGD (22%), PC01 was dominated by MBGE (23%), and PC03 was dominated by MBGD (35%). In PC04, MBGE accounted for 19%, and the pBRKC 84 group[32] (formed by clones with homology as low as 90% with clones obtained from hypersaline microbial mats) accounted for 18%. PC09 was dominated by MBGD (33%). In PC10 and PC11, MBGD dominated (25% and 38%, respectively), followed by 10-PML 8% pond group (19% and 17%, respectively).

Similar to the bacterial results, many archaea clones obtained in this study were not matched with any strain or gene clone from the database (<95%) and were derived from microorganisms that are completely unknown at the genus level. Similar to the bacterial results, in all samples from the general seafloor area, clones with high homology with clones collected from the methane seep area in the sea around Japan and other oceanic gas hydrate and hydrocarbon seep areas were obtained (≥97%). Clones showing high homology (≥97%) with clones involved in methane oxidation (closely related to the methanotrophic community) were obtained from general seafloor samples other than PC01 and PC10.

To further analyze the genus *Methanosarcina* obtained from S1, the phylogenetic relationship was analyzed by the sequence of the 16S rRNA gene sequence classified in the genus *Methanosarcina*. Consequently, it was found that 52 of 64 genus *Methanosarcina* clones obtained from S1s are closely related to ANME-2, and some clones were closely related to ANME-3 belonging to the genus *Methanococcoides*. In contrast, among 79 clones of the genus *Methanosarcina* obtained from S1k, 51 were closely related to ANME-2 and 27 were closely related to ANME-3 (Fig. 4).

DISCUSSION

Although methane concentration in the ocean is <0.5 nmol/l[33], in some special ocean areas, it exceeds 5 nmol/l[34]. Areas in the sea have been identified where methane is generated by microorganisms from sludge sediments (e.g., in Tokyo Bay, where organic matter has become sludge) and where methane is released by thermal generation from cold-water seep areas and hydrothermal polymetallic ores of the open sea. For example, it has been reported that methane concentration is 340 Ͻmol/kg in pore water in sediments of cold-water seep areas (depth, 1,100 m), which contain methane hydrates, of the Ryuyo Canyon in the eastern Nankai Trough area[35]. In the present study, the methane concentration in the pore water could not be measured in the cold-water seep area (S1 site, Table 1) of the Daini Tenryu Knoll, selected in the survey cruise of the eastern Nankai Trough area in 2004. However, the fact that unusual organisms inhabit the methane seep area, such as populations of *Calyptogena*, demonstrates that an ecosystem that is clearly different from other low-methane-concentration seafloors exists.

A group of microorganisms called methanotrophs is known to be involved in methane conversion and are related to methane oxidation in an aerobic environment[6,7]. Based on this, we can predict that aerobic MOB will be detected in the S1 sample that is collected from a site where methane seeps from the sea floor; however, known aerobic methane-oxidizing bacteria were not detected. This is because 16S rRNA is a gene common to all microorganisms, it was considered that it is not appropriate to target bacterial 16S rRNA as a detection method focusing on aerobic MOB only.

Interestingly, compared with the sequence analysis results of other studies, we found clones showing high homology (e”97%) with sequences presumed to be involved in methane oxidation (members of the methanotrophic community) in the samples, not only from the methane seep area (S1) but also from the general seafloor area in the Nankai Trough[10, 36]. The microorganism possessing these sequences yet to be isolated and cultured, thus it is unknown whether these microorganisms can utilize methane in nature. However, it has been suggested that microorganisms capable of utilizing methane are widely distributed in the surface layer of the ocean floor. Thus, it can be considered that there exists a microbial community universally

involved in methane oxidation in the mud of the seafloor, and if methane is supplied from the bottom of the sea, a potential methane utilization community may be constructed.

Many studies have found that ecological systems exist in deep sea mud volcanoes and methane seep areas; these ecological systems are supported by chemolithotrophs that depends on methane and sulfuric acid, and anaerobic methane oxidation occurs in places where methane and sulfuric acid coexist. Therefore, the process of anaerobic methane oxidation is thought to be associated with ANME archaea and sulfate-reducing bacteria (SRB)[9,10,37,38], and a report has stated that ANME-2 has a close relationship with SRB genera, *Desulfosarcina* and *Desulfococcus*[9,39,40]. In addition, in the present study, because clones closely related to ANME-2 were detected in the samples from methane seep areas, we focused on clones of δ-Proteobacteria, to which many SRB belong. However, clones corresponding to or closely related to the known genera *Desulfosarcina* and *Desulfococcus* were not detected.

Microbial community analysis, based on archaeal 16S rRNA gene sequence, showed a remarkably different microbial community structure between samples from the methane seep and general seafloor areas (Fig. 2B). In the S1 methane seep area, the genus *Methanosarcina* dominated overwhelmingly, and the diversity of the microbial flora was extremely low. This result was similar to other reported results of methane-rich sediment samples[12,41,42]. Furthermore, the S1 methane seep area community contained many clones closely related (97% to 99%) to the ANME group that perform anaerobic methane oxidation. ANME has also been detected in several other methane seep areas investigated in previous studies[43].

In culture experiments conducted by Girguis et al., anaerobic methane oxidation activity and sequences of the ANME-2 group were detected after culturing with methane[44]. Therefore, their results support the speculation obtained from the results of our analysis. In general submarine environments that consist of various microbial community structures, dominance of the *Methanosarcina* seen in sample S1 is believed to be the result of a change in the microbial flora along with the environmental change of the methane seep area. From this, it was considered that a sequence of the ANME group could be one of the characteristic microbial indices on the seafloor where methane exists.

In our analysis, sequences closely related to ANME-2 and ANME-3 were detected in large amounts in the S1 site, which is a methane seep area. It has been reported that ANME-1, another group involved in anaerobic methane oxidation, are detected and are present specifically in methane seep areas[8]. Although it was previously confirmed that the detection of ANME-1 with the primer pair used in our study is possible, ANME-1 was not detected at all in our analysis. Moreover, it has been proven that ANME-1 exists in the deep ocean floor[45]. Because the water depth of the S1 site analyzed in our study was approximately 600 m, there is a possibility that it is not the habitat of ANME-1.

In the marine mud where methane hydrate is present, it is known that only moisture from saltwater is taken up by methane hydrate, and the salt concentration increases in the surrounding area. In the phylogenetic analysis of archaea, although the homology was low, many clones were obtained that form the same branches as the sequences obtained from areas with extremely high salt concentrations. In addition, clones closely related to halophilic methane-oxidizing bacteria were obtained in the phylogenetic analysis of bacteria. It is interesting to observe the relationship between these results and methane hydrate.

In conclusion, generally, it is highly likely that a unique microbial flora is formed on the seafloor where methane is released, and this analysis suggests that it is not an exception in the Nankai Trough. By targeting genes of microorganisms that rapidly respond to methane leakage, it is possible to detect methane leakage on the seafloor.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

T.H. and S.S. designed research; T.H. and S.S. performed sample collection; N.O., T.H. and M.H. analyzed data; N.O., M.H. and T.H. wrote the manuscript.

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DATA AVAILABILITY

Sequence data obtained in this study were available in GenBank/EMBL/DDBJ databases at accession numbers LC382285 to LC382364.

ETHICS STATEMENT

This article does not contain any studies involving human participants and animals performed by any of the authors.

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