Characterization of archaeal and bacterial communities thriving in methane-seeping on-land mud volcanoes, Niigata, Japan

Nori Miyake1 · Ryo Ishimaru1 · Goro Komatsu1,2 · Takafumi Matsui1

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Abstract
Submarine mud volcanoes (MVs) have attracted significant interest in the scientific community for obtaining clues on the subsurface biosphere. On-land MVs, which are much less focused in this context, are equally important, and they may even provide insights also for astrobiology of extraterrestrial mud volcanism. Hereby, we characterized microbial communities of two active methane-seeping on-land MVs, Murono and Kamou, in central Japan. 16S rRNA gene profiling of those sites recovered the dominant archaeal sequences affiliated with methanogens. Anaerobic methanotrophs (ANME), with the subgroups ANME-1b and ANME-3, were recovered only from the Murono site albeit a greatly reduced relative abundance in the community compared to those of typical submarine MVs. The bacterial sequences affiliated to Caldatribacteriota JS1 were recovered from both sites; on the other hand, sulfate-reducing bacteria (SRB) of Desulfobulbaceae was recovered only from the Murono site. The major difference of on-land MVs from submarine MVs is that the high concentrations of sulfate are not always introduced to the subsurface from above. In addition, the XRD analysis of Murono shows the absence of sulfate-, sulfur-related mineral. Therefore, we hypothesize one scenario of ANME-1b and ANME-3 thriving at the depth of the Murono site independently from SRB, which is similar to the situations reported in some other methane-seeping sites with a sulfate-depleted condition. We note that previous investigations speculate that the erupted materials from Murono and Kamou originate from the Miocene marine strata. The fact that SRB (Desulfobulbaceae) capable of associating with ANME-3 was recovered from the Murono site presents an alternative scenario: the old sea-related juvenile water somehow worked as the source of additional sulfur-related components for the SRB-ANME syntrophic consortium forming at a deeper zone of the site. However, the reason for the differences between Murono and Kamou is still unknown, and this requires further investigation.

Keywords On-land mud volcano · ANME

Introduction
Mud intrusion and extrusion on Earth are well-known phenomena whereby fluid-rich (e.g., water, methane), fine-grained sediments ascend within a lithologic succession mainly because of their buoyancy (Kopf 2002). The resulting accumulation of the mud could form a conical or shield-like edifice, mud volcano, as large as a few kilometers in width and a few hundred meters in height (Aliyev et al. 2009).

Mud volcanoes (MVs) are an important “window” into the unknown biosphere of underlying strata because both a low-competence parent bed (clay-rich layer) and some rock fragments are transported to the surface and bring together any microbes that are thriving under those extreme conditions (Mazzini and Etiope 2017). Therefore, molecular investigation of those extremophiles is part of a new frontier in understanding the limit to the terrestrial deep biosphere. MV-related extremophiles are known to exist in a community and form a complex metabolic network using fluid-rich minerals (e.g., Ijiri et al. 2018; Ma et al. 2021). Hence those microbial activities are known to be related to a concentration of natural gas (e.g., methane) seepage. We carried out fieldwork in October 2018 to investigate two of
the most active on-land MVs in central Japan (Etiope et al. 2011), followed by a recent characterization of their archaeal and bacterial communities by using the 16S rRNA amplicon sequencing approach for the first time at these MVs.

MV vents occur in various geological settings but they are predominantly observed at convergent plate margins (Kopf 2002). In recent years, MVs on seafloor beds have been preferentially studied and a complex configuration of microbial communities in the submarine sedimentary biosphere is gradually revealed (e.g., Ijiri et al. 2018; Lee et al. 2018a) . A microbial metabolism, such as sulfur oxidation, sulfate reduction, and anaerobic methane oxidation (AOM) and methanogenesis, forms a complex redox sequence at the subsurface of the sediments (Lin et al. 2018; Bhattarai et al. 2019). Furthermore, many investigated archaeal communities in seafloor-bed MVs are mostly dominated by methane consumers, known as anaerobic methanotrophs (ANMEs), which can catalyze AOM with their symbiotic sulfate-reducing bacteria at shallow layer (Orphan et al. 2001; Michaelis et al. 2002; Knittel et al. 2005; Lõskeann et al. 2007; Pach Adriani et al. 2011; Ijiri et al. 2018; Lee et al. 2018a; Bhattarai et al. 2019; Gründger et al. 2019; Iasakov et al. 2022). On the contrary, active MVs located across terrestrial lands are less investigated, and their configurations of microbial communities are still not well known. Since the origins of mud fluids and compositions and environments of on-land MVs could be different from those of deep seafloor-bed MVs, the impact of the differences on the microbial community structure and potential activity in each location must be separately investigated. A few previous investigations of on-land MVs identified the presence of several archaeal taxa including ANMEs (Alain et al. 2006; Cheng et al. 2012; Wrede et al. 2012; Huang et al. 2016; Tu et al. 2017; Lin et al. 2018; Mardanov et al. 2020; Ma et al. 2021; Merkel et al. 2021). On submarine MVs, ANMEs were found to be abundant in the sulfate-methane transition zone (SMTZ), where diffused sulfate \((\text{SO}_4^{2-})\) from the ocean above and seeped methane from beneath MVs coexist at shallow layer, but for on-land MVs this relationship is not well understood.

Investigation of microbial communities associated with land-based MVs is also important for implications on astrobiology of Mars (Komatsu et al. 2014; Hosein et al. 2014). Features interpreted to be on-land MVs occur widely in the northern plains of Mars and other localities (e.g., Oehler and Allen 2010; Komatsu et al. 2011, 2016). Therefore, searching and understanding of extant life or biomarkers in the on-land MV-derived fluids and sediments can bring important information to possible future astrobiological missions to the planet (Komatsu and Ori, 2000; Komatsu et al. 2014).

**Geological setting**

This paper describes two of the most active on-land MVs situated at the Murono and Kamou Districts in the Tokamachi, Niigata Prefecture, Japan (Etiope et al. 2011) (Fig. 1). The Late Miocene Sugawa Formation consists primarily of dark-color massive mudstones and accompanying alternating mudstones-sandstones, and it is highly folded and widely distributed in the Tokamachi area (Fig. 1a). The MVs occur in the areas of the exposed Sugawa Formation (Shinya and Tanaka 2005). The two vents that we have investigated are located at Murono (Fig. 1b, c; 37°7′15.16″N, 138°33′27.76″E, 320 m a.s.l.) and Kamou (Fig. 1d, e; 37°7′58.45″N, 138°34′30.24″E, 313 m a.s.l.), and they do not have clearly visible edifice structures. The Murono and Kamou sites are about 2 km apart. Further detailed descriptions of those MVs are provided elsewhere (e.g., Shinya and Tanaka 2005, 2009; Etiope et al. 2011). The erupted mud contains slightly alkaline (pH 7.5) groundwater with very high salinity and very high electrical conductivity (Murono 14 mS/cm, Kamou 13 mS/cm) compared with the local surface water (Shinya and Tanaka 2005 in Japanese). The oxygen and hydrogen isotopic ratio of groundwater and vitrinite reflectance of coal fragments show the estimated origin of those mud to be at the depth range of 3400 to 4000 m (Shinya and Tanaka 2009 in Japanese).

The total methane emission from Murono and Kamou MV areas were estimated to be at least 20 and 3.7 ton/year, respectively, of which more than one half was from invisible seepage surrounding the MVs (Etiope et al. 2011). The subsurface of those MVs are known to contain petroleum biodegradation; however, no biological analysis has been performed at these localities until today. In this paper, we characterized the archaeal and bacterial communities within mud-filling and methane-seeping vents of Murono and Kamou MVs.

**Results**

**Geochemical characterization**

The active on-land mud volcanoes (MV) we have investigated, Murono and Kamou, are spouting mud together with seepage of natural gas (e.g., methane), groundwater, and oil. Our temperature measurements of those vents at the Murono and Kamou sites (Fig. 1) revealed 15 °C at 40-cm depth with 23 °C ambient temperature, and 17 °C at 40-cm depth with 23 °C ambient temperature, respectively. Those spouting
muds are thought to be originated from the depth range of 3400 to 4000 m and the temperature at that range is estimated to be about 120 °C (Aoyagi and Kazama 1980; Shinya and Tanaka 2009 in Japanese). The mud fluids from Murono and Kamou had pH value of 7.88 and pH 7.65, respectively.

XRD analysis revealed identifiable mineral components from the spouting mud samples collected from each site (Fig. 2A). Overall, there is no difference between the two samples, and the XRD results show that the erupted mud at each site has almost the same mineral assemblages which include quartz, Na-smectite, chlorite, halite, and probably biotite. Halite may result from the deposition of the mud water as it was drying, which suggests that the pore water is classified as a NaCl-type water.

We conducted a measurement of methane concentration over the MV vents at 0.5-s intervals (Fig. 2B). As shown, the methane column density above the vents of both sites increased and decreased repeatedly. The peaks are associated with eruption of gas bubbles on the surface of mud, supporting that the measured methane gas is originated from the MVs. The average values of methane seepage were 2483.68 ppm-m in Murono and 827.05 ppm-m in Kamou. Their column densities were 2–3 orders of magnitude higher than those in the backgrounds. The average background values were 88.97 ppm-m in Murono and 12.58 ppm-m in Kamou.

**Molecular characterization of a microbial community**

In order to understand the nature of microbial population in each site, the cells from the mud samples were stained and counted under the fluorescence microscopy. However, many mud particles formed large aggregates, hiding the stained targets behind. Therefore, we extracted the whole DNA-content from 250 ml of each sample bottle and quantified as shown in Table 1. The mean value of DNA-content of soil bacteria is known to be 1.6 to 2.4 fg cell\(^{-1}\) (Bakken and Olsen 1989) whereas that of seawater bacteria is 2.5 fg cell\(^{-1}\) (Button and Robertson 2001). Since the source of Tokamachi mud volcanoes is possibly correlated with sea-related juvenile water, which will be explained later, we used the mean value of DNA-content as 2.5 fg cell\(^{-1}\) to calculate the expected total number of microbial cells in each sample. The results show that the numbers of cells in “depth” of MV are much lower than those in “control” (about 69-fold in Murono and twofold in Kamou). We note that this calculation estimates the minimum number of cells, and the real concentrations of microorganisms may be higher.

The expected total number of cells in 1 ml of mud samples in each site was back calculated. The mean value of 2.5 fg DNA-content cell\(^{-1}\) was used for the calculation.

A total of 568,714 high-quality 16S rRNA sequences (328,280 reads from V4 region, 109,808 reads from archaeal-specific V3V4 region, and 130,626 reads from...
bacterial-specific V3V4 region) were used for microbial community comparison. A total of 5305 amplicon sequence variants (ASVs) (3113 from V4 region reads, 257 from archaeal-specific V3V4 region reads, and 1935 from bacterial-specific V3V4 region reads) were obtained based on the 99% similarity threshold against Silva database v138.

The results of the taxonomic classification of the ASVs are summarized in Supplementary Table S1.

The relative abundances of ASVs obtained from 16S rRNA (V4) sequencing were compared for the depth and the control of the Murono and Kamou sites as shown in Fig. 3 (Accession Nos. DRX251432, DRX251433, DRX251434.

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**Fig. 3** Pie charts representing the relative abundance of ASVs obtained from 16S rRNA (V4 region) amplicon sequencing of (A) “depth” of Murono MV, (B) “control” of Murono MV, (C) depth of Kamou MV, and (D) control of Kamou MV. Outer ring represents the kingdom and inner ring represents the phylum as according to the taxonomic classifications of Silva database (138). The abundant taxa (defined as ASV size ≥ 1% abundance) are shown in percentage of their ASV shares. The taxa with ASV size less than 1% are gathered and shown as ASV < 1% due to the limited graphical field of view.
and DRX251435). Figure 3 compares ASV abundance in the kingdom and the phylum level of those which have their ASV size greater than 1%. ASVs which have their size less than 1% are gathered in one section due to the limited graphical field of view. All those ASVs and their taxonomy are listed in Supplementary Table S1. The relative abundances of archaeal ASVs (include ASV <1%) show that the depth contains more archaeal organisms (Murono 16.7%; Kamou 14.0%) than those in the control (Murono 1.2%; Kamou 1.6%), suggesting the significant role of archaeal communities in the deep terrestrial MVs. The archaeal ASVs in the depth of the Murono site are dominated by the phylum Altiarchaeota (7.9%) and Halobacterota (7.9%), whereas the depth of the Kamou site is dominated by Euryarchaeota (5.1%) and Halobacterota (8.5%). The relative abundances of bacterial ASVs in the depth and the control of both sites are dominated by the phylum Proteobacteria (29.2 ~ 34.0%), followed by Desulfovibrio (10.9%) in the depth of the Murono site, Firmicutes in the depth of the Kamou site (12.0%), Acidobacteriota (15.8%) in the control of the Murono site, and Actinobacteriota (19.3%) in the control of the Kamou site. The deeper characterizations of archaeal and bacterial communities were conducted separately using their specific universal primers for the 16S rRNA amplicon sequencing analysis.

The relative abundances of ASVs obtained from archaeal-specific 16S rRNA (V3V4) sequencing were compared for the depth and the control of the Murono and Kamou sites in the phylum and the class level as shown in Fig. 4. The figure shows the relative abundances of those ASVs which have their size greater than 1% due to the limited graphical field of view. All the ASVs’ taxonomy including their size less than 1% are listed in Supplementary Table S1. The ASVs obtained from the depth of the Murono site (Accession No. DRX221641) represent 4 phyla, namely, Altiarchaeota (3.4%), Crenarchaeota (5.9%), Halobacterota (84.2%), and Thermoplasmatota (4.3%), whereas from the depth of the Kamou site (Accession No. DRX221643) represent only 2 phyla, namely, Euryarchaeota (36.0%) and Halobacterota (62.9%). The ASVs obtained from the control of both sites (Murono Accession No. DRX221642; Kamou Accession No. DRX221644) represent the same 4 phyla, namely, Crenarchaeota (Murono 33.6%; Kamou 46.0%), Euryarchaeota (Murono 7.6%; Kamou 13.6%), Halobacterota (Murono 53.2%; Kamou 39.2%), and Thermoplasmatota (Murono 5.5%; Kamou 11.1%).

As Fig. 4 shows, both Murono and Kamou sites have a high abundance of the phylum Halobacterota, which differ in the relative abundances of belonged taxa. Only the depth of both sites have the Methanomicrobia genera Methanolinea (Murono 4.0%; Kamou 9.8%), Methanoregula (Murono 4.3%; Kamou 30.2%), Rice Cluster II group (Murono 11.2%; Kamou 0.6%), and the Methanosarcina genus Methanosaeta (Murono 8.4%; Kamou 22.0%), whereas the control of both sites are predominated by the Methanocellia genera Methanocella (Murono 34.1%; Kamou 19.4%), Rice Cluster I (Murono 4.8%; Kamou 1.2%), and the Methanosarcina genus Methanosarcina (Murono 14.3%; Kamou 18.5%). The Methanosarcina genus Methanomicrococcus (46.3%) is observed only in the depth of the Murono site. The genus Methanobacterium, which attributes to the phylum Euryarchaeota, is observed only in the depth of Kamou site (35.7%) and the control of both sites (Murono 7.6%; Kamou 13.6%). Those genera mentioned above are the most active methanogens known to produce an extensive amount of methane on Earth. The genera Methanolinea, Methanoregula, Methanomicrococcus, and Methanobacterium are hydrogenotrophs that can metabolize molecular hydrogen as a source of energy (e.g., Sprenger et al. 2000; Kitamura et al. 2011; Yashiro et al. 2011; Sakai et al. 2012), while Methanoseta metabolizes acetate as its sole source of energy (e.g., Berger et al. 2012).

The subgroups of anaerobic methanotroph (ANME) are retrieved only from the depth of the Murono sites. They are the ANME-1 genus ANME-1b (1.8%) and the Methanosarcina genus ANME-3 (5.7%). Both are dominantly found in many cold seeps, seafloor-bed, and on-land MVs (e.g., Pachiodaki et al. 2011; Ijiri et al. 2018; Merkel et al. 2021; Iasakov et al. 2022). To note, another well-discussed subgroup of anaerobic methanotrophs, the Methanosarcina genus ANME-2a-2b, is found with a very low abundance at our studied site (Murono 0.01%). The Altiarchaeia genus Candidatus Altiarchaeum (3.4%) which was commonly found in deep anoxic groundwater (e.g., Probst and Moissl-Eichinger 2015) and the class Thermoplasmata (2.2%), originally found in anoxic marine and subsaline sediments (e.g., Stocek and Epstein 2003; Ferrer et al. 2011), are also found only from the depth of the Murono site.

Other than those referred methanogens above, only the control of both sites are dominated by the class Nitrososphaerlia (Murono 32.4%; Kamou 45.6%), which suggests that this is the row model of the microbial communities in the surrounding environments.

The relative abundances of ASVs obtained from bacterial-specific 16S rRNA (V3V4) sequencing were compared for the depth and the control of the Murono and Kamou sites as shown in Fig. 5. The figure shows the relative abundances of those ASVs which have their size greater than 1% due to the limited graphical field of view. All the ASVs’ taxonomy including their size less than 1% are listed in Supplementary Table S1. The bacterial ASVs obtained from the depth of both sites (Murono Accession No. DRX221637; Kamou Accession No. DRX221639) and the control of both sites (Murono Accession No. DRX221638; Kamou Accession No. DRX221640) represent the same 4 phyla, namely, Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria.
The depths of both sites represent the extra phyla of Caldatribacteriota, Desulfobacterota, Chloroflexi (Murono only), and TA06 (Kamou only). The control of both sites represent the extra phyla of Acidobacteriota, Chloroflexi, Myxococcota, and Planctomycetota, and only the control of the Kamou site has Patescibacteria and WPS-2.

As Fig. 5 shows, the relative abundances of bacterial ASVs form a complex community. Briefly, the dominant ASVs observed from the depth of both sites include the Caldatribacteriota class JS1 (Murono 4.9%; Kamou 12.0%), the Bacteroidia genus Lutibacter (Murono 7.8%; Kamou 9.2%), the Desulfotomaculia genus SCADC1-2-3 (Murono 2.0%; Kamou 1.3%), and the Methanobacteriota class Thermoplasmata (Murono 5.5%; Kamou 1.3%).

The abundant taxa (defined as ASV size ≥ 1% abundance) are shown in percentage of their ASV shares. The taxa with ASV size less than 1% are excluded due to the limited graphical field of view.

Fig. 4 Pie charts representing the relative abundance of ASVs obtained from archaeal-specific 16S rRNA (V3V4 region) amplicon sequencing of (A) “depth” of Murono MV, (B) “control” of Murono MV, (C) depth of Kamou MV, and (D) control of Kamou MV. Outer ring represents the phylum and inner ring represents the class according to the taxonomic classifications of Silva database (138).
Kamou 17.7%), the Alphaproteobacteria order Rhodobacterales (Murono 2.1%; Kamou 4.9%), and the Gammaproteobacteria genus *Rhodoferax* (Murono 29.8%; Kamou 22.1%) from. The dominant ASVs observed only from the depth of the Murono site include the Desulfobulbia uncultured Genus, Desulfobulbaceae (7.9%), the Desulfuromonadidae genus *Rhodothermobacter* (1.1%), the Coriobacteriidae genus *OPB41* (5.2%), Thermoleophilia uncultured Gaiellales (1.6%), the Anaerolineae genus *SJA-15* (2.5%), and the Gammaproteobacteria genus *Dechloromonas* (9.0%). In contrast, the

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**Fig. 5** Pie charts representing the relative abundance of ASVs obtained from bacterial-specific 16S rRNA (V3V4 region) amplicon sequencing of (A) “depth” of Murono MV, (B) “control” of Murono MV, (C) depth of Kamou MV, and (D) control of Kamou MV. Outer ring represents the phylum and inner ring represents the class as according to the taxonomic classifications of Silva database (138). The abundant taxa (defined as ASV size ≥ 1% abundance) are shown in percentage of their ASV shares. The taxa with ASV size less than 1% are excluded due to the limited graphical field of view.
dominant ASVs observed only from the depth of the Kamou site include the phylum TA06 (2.9%), the Actinobacteria genus Demequina (2.3%), the Bacteroidia genus Vicingus (1.5%), the Desulfuromonadidae genus Sva1033 (2.2%), and finally the Gammaproteobacteria genus Dechlorobacter (1.4%), genus Sulfitalea (3.5%), and order Immundisolibacterales (2.3%).

It is noteworthy that the Caldatribacteriota (formerly known as Atribacteria) class JS1, observed from both depth sites, is a heterotrophic anaerobic bacterium, which is predominantly found in anoxic subseafloor sediments, especially in organic-rich or gas hydrate-containing sediments. It can perform syntrophic acetate oxidation in consortium with hydrogen or formate scavenging methanogens (e.g., Hoshino et al. 2017; Lee et al. 2018b). The depth sites also contain the taxa belonging to the phylum Desulfovibacterota, which includes most of the known sulfate- and Fe(III)-reducing bacteria. The Murono site has sulfate-reducing bacterium (SRB) of Desulfovibulbaceae (e.g., Taylor and Parkes 1983; Orphan et al. 2001) and Fe(III)-reducing bacterium of Geothermobacter (Kashefi et al. 2003; Wunder et al. 2021), and the Kamou site has Fe(III)-reducing bacterium of Sva1033 (Greene et al. 2009; Wunder et al. 2021). Other well-known SRBs are found to be either absent or in a very low abundance, such as Desulfoarcina (Murono 0.04%).

The control on both Murono and Kamou sites are dominated by the class Acidobacteria (Murono 15.5%; Kamou 5.2%), the class Acidimicrobiia (Murono 6.0%; Kamou 2.1%), the Actinobacteria genera Mycobacterium (Murono 1.9%; Kamou 6.0%) and Acidothermus (Murono 13.0%; Kamou 14.2%), the Thermoleophilia genus Conexibacter (Murono 3.7%; Kamou 4.0%), the Bacteroidia order Chitinophagales (Murono 1.2%; Kamou 3.6%), the class ktedonobacteria (Murono 1.1%; Kamou 1.6%), the class Clostridia (Murono 1.3%; Kamou 3.4%), the class Polyan gia (Murono 2.8%; Kamou 2.5%), the class Planctomycetes (Murono 2.8%; Kamou 1.6%), the Alphaproteobacteria order Rhizobiales (Murono 22.9%; Kamou 15.6%), and the Gammaproteobacteria order Gammaproteobacteria Incertae sedis (Murono 2.7%; Kamou 3.3%). Only the control of Murono site has the class TK10 (1.5%) and the class Myxococccota (1.1%), whereas the control of Kamou site has the class Bacilli (2.0%), the class Saccharimonadida (2.4%), the Gammaproteobacteria order Xanthomonadales (6.3%), and the class WPS-02 (1.4%).

Discussion

Microbial communities of deep-seafloor mud volcanoes (MVs) have preferentially been studied in recent years; however, those of active on-land MVs are still rarely investigated. Hereby, we investigated two of the most active on-land MVs in central Japan, namely, Murono and Kamou sites, and characterized their microbial communities including their archaeal communities for the first time. The relative abundances of archaeal ASVs obtained from 16S rRNA (V4 region) amplicon sequencing in both sites revealed the presence of higher share of archaeal ASVs in 30-cm depth than that of control soils (Fig. 3), suggesting the possible significant roles of archaeal community beneath these MVs. Furthermore, we managed to detect archaeal and bacterial taxa from both sites, allowing us to understand the architecture of those microbial communities present in the terrestrial subsurface biosphere.

Our molecular analysis shows a high abundance of methanogens from the depth of Murono and Kamou sites (Methanolinea, Methanoregula, Rice Cluster II group, Methanoseta, Methanomicroccus, and Methanobacterium) and they are known to carry out methanogenesis (e.g., Sprenger et al. 2000; Kitamura et al. 2011; Yashiro et al. 2011; Sakai et al. 2012; Berger et al. 2012), which produces methane as a metabolic byproduct. Our methane gas measurement from those vents showed the average values of 2483.68 ppm·m from the Murono site and 827.05 ppm·m from the Kamou site, and they are 2–3 orders of magnitude higher than those in the backgrounds. Carbon isotopic analyses of the gas obtained from the same sites by other groups (Etiope et al. 2011; Kakizaki et al. 2018 in Japanese) indicated enrichment of 13C in CO2 and propane, suggesting a possible biodegradation of hydrocarbons taking place in the deep subsurface, which might lead to an enrichment of carbon source for those methanogens.

Interestingly, the Murono site is affiliated with anaerobic methanotrophic archaea (ANME) of the groups ANME-1 and ANME-3 and a very low abundance of ANME-2. ANME-1 is known to form a syntrophic consortium with sulfate-reducing bacteria (SRB), such as members of the family Desulfobacteraceae and Desulfobulbaceae, to mediate anaerobic oxidation of methane (AOM) coupled with sulfate reduction (Michaelis et al. 2002; Ijiri et al. 2018; Merkel et al. 2021) but its phylogenetic and metabolic diversity is still under investigation as we speak. AOM mediated by ANME is an enzymatic reversal of methanogenesis of which process is still poorly understood.

From the previous investigations, it is known that the seafloor-bed MVs are usually dominated by ANMEs; for example, ANME-1 have been found to dominate at shallow...
layer of hypersaline sediments (Lloyd et al. 2006; Yakimov et al. 2007), and ANME-1 are also detected predominantly at the shallow layer of 5.2 mbsf and the deeper layer of 104 mbsf in Kumano Basin (Ijiri et al. 2018). ANME-2 (56.3%) are dominantly detected at the top of the surface, ANME-1 (54.6%) at 10 cmbsf, and ANME-3 (70.8%) are found only at the depth of around 30 cmbsf at the Amsterdam mud volcano in the eastern Mediterranean Sea (Pachiadaki et al. 2011). At Canadian Beaufort Sea, ANME-2 (3.7–14.9%) at 0.4–0.6 mbsf and ANME-3 (2.5–14.1%) at 0–0.2 mbsf are detected (Lee et al. 2018a). The modern ocean contains a high concentration of sulfate, which gives an easily accessible energy source for the SRB and ANME consortium at the sulfate-methane transition zone (SMTZ) (e.g., Ijiri et al. 2018; Iasakov et al. 2022). However, the major difference between on-land MVs and submarine MVs is that the high concentrations of sulfate are not always available for the on-land MVs. Tu et al. (2017) demonstrated that the sulfate-depleted on-land MV at Taiwan consisted of ANME-2 clades (9–36%) which possibly mediate AOM in association with ion/manganese reducer, instead of SRB.

In this study, the Murono site exhibits ANMEs (ANME-1b 1.8%; ANME-3 5.7%) and SRB (Desulfobulbaceae 7.9%) in relatively low abundance proportional to the whole community. This is possibly due to the scarce amount of sulfate available at the subsurface of this site. The microbial oxidation of sulfur-bearing minerals, such as pyrite and greigite from the ascended mud, can give an additional sulfur source to enhance the sulfate concentration in anoxic sediments of subsurface (Cheng et al. 2012; Lin et al. 2018), but the XRD analysis of the mud from the Murono site has shown presence of halite, quartz, mica, smectite, and chloride but no sulfur-related mineral (Fig. 2A). The recent studies reported that ANME-1b occupied the sulfate-depleted layers below the SMTZ in methane-seep sediments at the Eastern Japan Sea Offshore Joetsu (Yanagawa et al. 2011) and the Baltic Sea methane pockmark (Iasakov et al. 2022). They explained this by ANME-1b’s higher energy efficiency during AOM and/or due to their superior ability to switch to methanogenic metabolism in the absence of sulfate and form a separated niche different from inside the SMTZ (Yanagawa et al. 2011, 2014), which indicates that ANME-1b from the Murono site might be taking a similar metabolic pathway. In the case of ANME-3, it apparently has the capacity to metabolize AOM without forming any consortium with SRB, probably due to direct electron transfer to sulfate (Bhattarai et al. 2017; Merkel et al. 2021). ANME-3 is suggested to effectively use high methane concentrations even without sulfate (Pachiadaki et al. 2011). These reports support our finding of ANME-3 in scarce sulfate environment of the Murono site.

The above discussion presents a scenario of ANME-1b and ANME-3 thriving at the depth of the Murono site independently from SRBs. Nevertheless, the Murono site exhibits SRB of the Desulfobulbaceae which is known to associate with ANME-3 (Lösekann et al. 2007; Li et al. 2020); therefore there can be an alternative scenario of ANME-3 and the family Desulfobulbaceae forming a syntrophic consortium at the depth. Since the erupted materials from the Murono and Kamou sites probably originate from the depth range of 3400 to 4000 m where the Miocene marine strata exist (Shinya and Tanaka 2009 in Japanese), indicating a possibility of the old sea-related juvenile water being the source of additional sulfur-related components for the SRB of the Desulfobulbaceae at the Murono MV. Furthermore, this can hypothesize the origin of ANME to be uniquely deeper than the depth range other investigated on-land MVs have suggested. Having said that, the reason for the differences in microbial communities between Murono and Kamou is still unknown, and this requires further investigation.

Murono and Kamou MVs are also associated with Calhydrateriobacteria (formerly known as Atribacteria), which plays heterotrophic roles using various substrates in anoxic sediments. Although they have not been found to be directly linked to AOM yet, it was suggested that they can mediate AOM possibly using humic acids as electron shuttles in some cold-seep environments (Saxton et al. 2016). They may also be indirectly responsible for methane production through the production of acetate or CO₂ (Nobu et al. 2016). Acetate is the end-product of propionate oxidation by one of species in the family Desulfobulbaceae as well (Widdel and Pfennig 1982), and it is also known to be utilized by methane-producing archaea, for example, Methanosarcina thermophile (Ferry and Maupin-Furlow 1996). Such methanogens and SRB are often correlated with methane production in submarine MVs (Lazer et al. 2011); therefore the acetate can be a key biomarker.

Overall, this study revealed the nature of the microbial communities thriving at the active on-land MVs in the Niigata Prefecture, Japan, enabling comparison with submarine and other on-land MVs. ANMEs found at the Murono site can live and operate independently from SRB as other submarine and on-land MVs have shown; on the other hand, the Murono MV is unique to have a sea-related juvenile water from the beneath, which gives the possibility of ANME-3 and SRB forming a syntrophic consortium at the deeper zone of the site. Other on-land MVs formed over sediment deposited near the seashore might possibly have the same condition. The recent research trend focuses on niche separation of ANME clusters at different depths of cold seeps and submarine MVs. Similar study of on-land MVs is still limited despite that investigation of on-land MVs has a great advantage in accessibility and easier sampling compared to those of submarine MVs. Conducting research of the deep biosphere through on-land MVs has a high potential for understanding its communities and activities and warrants further study effort.
Materials and methods

Field measurements

Temperature

The temperature of both Murono and Kamou MVs were measured outside (ambient temperature) and inside the vent (40-cm depth) using a thermocouple thermometer.

XRD analysis

The simple elutriation technique was applied to separate small clay minerals from other minerals. After shaking the sample cases, the measurement sample was collected by a syringe at the top and bottom and dropped into slide glasses. The slide glasses were dried in the oven at 70 °C. The XRD patterns were recorded by using the RINT UltimaIV X-ray diffractometer (Rigaku) in NIMS (National Institute for Materials Science) using Cu-Kα radiation (40 kV, 30 mA) with a 0.5° divergence slit, a 0.5° scattering slit, a 10-mm length-limiting slit, and a 0.3-mm receiving slit at a scan rate of 4° per minute at 0.02° steps in 2θ = 1.5–65°. For identification of the mineral species from the measured diffraction patterns, American Mineralogist Crystal Structure Database and X-Search (Digital Data Management) were used.

Methane analysis

Methane gas was detected using a handheld gas detector (Laser Methane Detector SA3C05A from Tokyo Gas Engineering Co., Ltd., in Japan). Remote measurement of gas concentration was conducted with an infrared absorption spectroscopy. A 1.65-μm laser beam, which is absorbed by methane gas, was directed to the target vent and the reflected beam was received by the detector. Absorption was measured and calculated into a column density of methane. The distance between the detector and the target was kept at 1 m for every measurement.

Sampling

Mud samples were collected using sterile 1-m-long syringe samplers (Buerkle™ HDPE ViscoDispo disposable sampler). By wearing sterile nitrile gloves, a sampler rod was plunged into a mud-filling vent (where methane gas is seeping out) and the mud at approximately 30 cm in depth (named depth) were sucked, which were then transferred into a 250-ml wide-mouth HDPE bottle. The surrounding mud sample (1 cm in depth) was also collected at about 1 m away from the vent for control (named control). Those sample bottles were immediately frozen at the sites in the container filled with dry ice, and they were later transferred to the freezer (−80 °C) at our laboratory and kept until molecular analysis.

Molecular analysis

Fluorescence microscopy

DNA in the mud sample was stained with SYBR Green I (Fisher; ex. 494 nm/em. 520 nm) for its identification using fluorescence microscopy through ®Nikon GFP filter (ex. 488 nm/em. 507 nm).

Amplicon sequencing

The frozen samples were crashed and for extraction of DNAs using the MPure Bacterial DNA Extraction Kit (MP Bio). The extracted DNAs were quantified by Synergy H1 (Bio Tek) and Quantifluor dsDNA System (Promega). The genomic library was then created by using various primers. 16S rRNA genes were amplified using the primer 341F (5′-CCTACGGGAGGCACAG-3′) and modified 806R (5′-GACTACHVGGGTATCTAAATCC-3′) for the bacterial V3V4 region (Muyzer et al. 1993; Caporaso et al. 2011), the primer modified ARC344F (5′-ACGGGGYGCAGCCGCGGA-3′) and ARC806R (5′-GGACTACVGGGTATCTAAATCC-3′) for the archaeal V3V4 region (Raskin et al. 1994; Takai and Horikoshi 2000), and the primer 515f MIX (5′-GTGCCACGCMGCCGCGGTAA-3′) and 806r MIX (5′-GGACTACHVGGGTATCTAAATCC-3′) for the bacterial and archaeal V4 regions (Caporaso et al. 2011). Two-step tailed PCR was carried out as described previously (Takahashi et al. 2014). The PCR fragments were quantified using Synergy H1 (BioTek) and Quantifluor dsDNA System (Promega). The sequencing was then carried out by Illumina MiSeq Reagent Kit v3 (2 × 300 bp), and the obtained sequence data that match with the primer were filtered using fastx barcode splitter tool of the Fastx tool kit (ver. 0.0.14); then those short (< 40) and less quality (average score below 20) reads were discarded using sickle (ver. 1.33). After the pair-end reads were joint using FLASH (ver. 1.2.11), QIIME2 (ver.2022.2) dada2 plugin was used to delete the chimera sequences and any other noises. By using the feature-classifier plugin (Bokulich et al. 2018a, b; Robinson et al. 2020) , the amplicon sequence variants (ASVs) were compared with 99% global identity threshold against Silva database (ver. 138.1) (https://www.arb-silva.de) and constructed their taxonomic classifications (Supplementary Table S1). Nucleotide sequence data reported are available in the DDBJ sequenced Read Archive under the accession numbers DRX221637 to DRX221644 and DRX251432 to DRX251435.
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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Nori Miyake, Ryo Ishimaru, and Goro Komatsu. The first draft of the manuscript was written by Nori Miyake and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability Nucleotide sequence data reported in this paper are available in the DDBJ sequenced Read Archive under the accession numbers DRX221637 to DRX221644 and DRX251432 to DRX251435. Please contact author for any other data requests.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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