Laguna (2022) 29: 133-143 ISSN 2185-2995

Methanogenic archaeal community structure in estuary sediments of the Onga River, northern Kyushu Island, Japan

Wipoo Prasitwuttisak[1], Shingo Araki[1] and Katsunori Yanagawa[1*]

Abstract: Methane, which contributes to global warming, is one of the microbial end-products of anaerobic organic matter remineralization in anoxic sedimentary environments. Estuary sediment with high primary production is known as the most active methane source because the enhanced microbial activity ultimately releases metabolic products such as methane and nutrients to the overlying water. To date, microbial methane production was thought to be phylogenetically limited to the archaeal phylum *Euryarchaeota*. However, advances in metagenomics-based studies have revealed that unsuspected lineages of archaea may contain key enzymes for methanogenesis, which expanded our view of methane metabolism. In this study, brackish water sediments of the Onga River, northern Kyushu, Japan were examined to assess the diversity of methanogenic archaea, through comparative phylogenetic analysis of 16S rRNA genes and functional genes necessary for methane production. Members of *Methanosarcinales* were only detected in the 16S rRNA gene amplicons, while the taxonomic classification of the functional genes showed the existence of diverse archaeal lineages, particularly hydrogenotrophic *Methanomicrobiales*. Furthermore, yet-uncultivated methanogens such as Verstraetearchaeota and Methanofastidiosales were also detected, indicating the importance of hydrogen-dependent methane generation from methanol, methylated amines, and dimethyl sulfide. Estuary methane production might be conducted by more phylogenetically widespread archaea than previously thought. Therefore, clone library analysis for the functional genes showed the potential to assess the diversity of methanogens not identified by the 16S rRNA gene amplicon analysis, which may improve our understanding of methane origins in estuary systems.

Key words: estuary sediment, methane, methanogenic archaea, *mcrA*

Introduction

Methane is one of the main contributors to greenhouse effect because it has a warming potential 25 times greater than carbon dioxide. Natural sources of methane include anaerobic environments in the hydrosphere, such as wetlands, lakes, paddy fields, and oceans. Intestinal systems of ruminants and wood-feeding insects are

[1] Faculty of Environmental Engineering, The University of Kitakyushu, Kitakyushu 808-0135, Japan.

[*] Corresponding Author

受付日：2022 年 6 月 3 日，受理日：2022 年 8 月 5 日，WEB 掲載日：2022 年 9 月 12 日

also known to supply large amount of methane. In those habitats, methanogenic archaea can convert several substrates including hydrogen, formate, acetate, methylated compounds, and methanol into methane (Conrad, 2009). Approximately 70% of the global supply of methane originates from microbial activity, which is presumably equal to as high as one billion tons (Reeburgh, 2007). Methane cannot permanently persist in the atmosphere as it is lost through photochemical reactions with OH radicals, with a persistence time of up to 10 years. This is very short compared to the 5-200 years for carbon dioxide, which raises concerns about the short-term impacts of methane. However, methane has been steadily increasing at about 1% per year since the Industrial Revolution (Schaefer et al., 2016). Therefore, it is a major challenge to study the suppression of methane in a long-term time scale framework, and to comprehensively understand the methane sources, emission mechanisms, and fluxes.

The largest reservoir of methane exists beneath the seafloor (Kvenvolden, 1988; Yanagawa et al., 2012). Therefore, methane released from the seafloor to the hydrosphere and the atmosphere should be carefully monitored. However, its contribution is relatively small because of biological process in sediment, called anaerobic oxidation of methane. In this reaction, anaerobic methane-oxidizing archaea and sulfate-reducing bacteria syntrophically oxidize methane to carbon dioxide (Borowski et al., 1996; Knittel and Boetius, 2009). It has been estimated that anaerobic oxidation of methane consumes 90% of the methane released from marine sediments (Valentine and Reeburgh, 2000). Furthermore, competition between methanogenic archaea and sulfate-reducing bacteria is indirectly involved in the suppression of methane flux. Methanogenic archaea compete with sulfate-reducing bacteria for hydrogen and acetate, and sulfate-reducing bacteria tend to dominate due to their higher substrate affinity (King, 1984). Therefore, methanogenic archaea are not detected in typical sulfate-bearing shallow sediments.

Estuaries and coastal areas are distinctive environments where the enhanced supply of organic matter due to active biological production causes promotion of methane production. These environments are susceptible to eutrophication because of the direct input of organic matter and nutrients from domestic wastewater (Egger et al., 2016). In addition, the brackish water of an estuary is located at the downstream end of a river, which delivers large amounts of suspended solids to the estuary. It is estimated that 95% of the suspended solid is deposited at an estuary during high and low tides when the water flow is slower. In particular, Japanese rivers tend to erode and form steep gradients because Japan corresponds to a relatively high rainfall region in the world. These suspended sediments are composed of clay minerals and organic matter. The organic portion is decomposed by microbial aerobic respiration, which reduces dissolved oxygen in the surface sediment environment. Finally, sulfate reduction and methanogenesis proceed as the last step of organic matter decomposition. For these reasons, although estuaries cover only 0.4% of the total ocean area, they could be major sources of methane emissions, accounting for about 75% of the methane released from the entire ocean (Bange et al., 1994).

Recently, next-generation sequencing (NGS) has been widely applied in the field of environmental microbiology to obtain a large number of gene sequences. This method allows us to explore microbial communities and reveal complex interactions among many microbial populations. Most of the NGS-based amplicon analysis targets the 16S rRNA genes. Other highly conserved genes such as *rpoB*, *gyrB*, and *recA* are also useful in determining taxonomic classification (Rajendhran and Gunasekaran, 2011). To detect methane-producing archaea, the *mcrA* gene encoding the alpha subunit of the methyl-coenzyme M reductase that catalyzes the last step of methanogenesis, is frequently used in molecular ecological analysis (Hales et al., 1996). The molecular biological evidence has shown that the archaea involved in methane production span seven orders: *Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanomassiliicoccales*, *Methanomicrobiales*, *Methanopyrales*, and *Methanosarcinales*. However, recent advances in microbiology have revealed that several uncultured archaeal lineages contain genes involved in methanogenesis, suggesting that archaea engaged in methanogenesis are phylogenetically more widespread than previously thought. For instance, methane production potential of "*Candidatus* Bathyarchaeota" (formerly known as the Miscellaneous Crenarchaeota Group, MCG) were recently identified

based on genome-centric metagenomics (Evans et al., 2015). On the other hand, conventional PCR-dependent methods have successfully detected the *mcrA* gene, presumably from the *Ca*. Bathyarchaeota, in the hydrothermal sediments of Yellowstone National Park (McKay et al., 2017). The construction of a PCR clone library focusing on a specific functional gene remains a useful tool for exploring microbial populations, while it is sometimes not suitable for searching unknown methanogenic lineages. In this study, we focused on the methanogenic archaea in brackish environment by comparing the 16S rRNA gene amplicon analysis with the functional gene analysis based on the clone library construction.

Materials and Methods

Sample collection and preparation

Samples were collected in the estuary of the Onga River, northern Kyushu (33°53'24.2'' N, 130°40'26.5'' E). The Onga River is the most important source of freshwater flowing into the Hibiki Sea, with a flow rate of about 944×10^6 m^3 yr^{-1}. On August 16, 2018, surface sediments were collected using a cylindrical corer (Wildco, Yulee, FL, USA) from the estuary of the Onga River. The temperature and salinity at the sampling site were 24.9°C and 1.2%, respectively. Sediment layers were sampled at depths of 0-1, 1-2, 2-3, and 3-4 cm. Samples for molecular biological analysis were kept at -80°C until further processing.

Quantification of microbial gene abundance

To estimate the microbial biomass in the samples, gene quantification by real-time quantitative polymerase chain reactions (qPCR) was performed based on previously described methods (Yanagawa et al., 2019). Prokaryotic DNA in sediment samples was extracted using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Prokaryotic 16S rRNA gene numbers were quantified using the Taqman probe-based qPCR method with the universal primer-probe set (Uni340F/Uni806R/Uni516F probe), the archaea-specific primer-probe set (Arch349F/Arch806R/Arch516F probe, Takai and Horikoshi, 2000), and the innuDry qPCR MasterMix Probe (Analytik Jena AG, Germany). The amplification was conducted in a 20-μL reaction volume with initial denaturation at 98 °C for 2 min, 50 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C (for universal 16S rRNA gene) or 52 °C (for archaeal 16S rRNA gene) for 45 s, and elongation at 72 °C for 30 s. The *mcrA* gene, a functional key gene for methanogenesis, was quantified using SYBR Green-based qPCR assay with the specific primers (ME3MF and ME2r', Nunoura et al., 2008) and MightyAmp for Real-Time PCR (TaKaRa Bio) under the following amplification conditions: initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C for 30 s, and elongation at 68 °C for 1 min. All targeted gene abundances were normalized to the ROX reference dye and quantified in triplicate using a real-time PCR system qTOWER^3 G Touch (Analytik Jena).

Microbial community composition analysis based on 16S rRNA gene and *mcrA* gene

To determine the microbial community composition, the hypervariable V3–V4 region of the 16S rRNA gene was PCR-amplified from the extracted DNA using MightyAmp DNA Polymerase Ver.3 (Takara Bio) and the universal primers (515F/806R, Caporaso et al., 2011). The amplification protocol comprised initial denaturation at 98 °C for 5 min, then 35 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 30 s, and a final extension at 68 °C for 5 min. The amplified products were purified, indexed, and sequenced using the Illumina MiSeq platform. The acquired data were processed using QIIME2 (Bolyen et al., 2019). The *mcrA* gene was amplified using a specific primer set as described in real-time quantitative PCR. After gel extraction and purification, the PCR products were used to generate a clone library as previously described (Yanagawa et al., 2019). Molecular phylogenetic tree of the *mcrA* gene was constructed using ARB software (Ludwig et al., 2004). Molecular phylogeny and compositional ratio were determined by referring to known sequences in the database at the 95% dissimilatory cut-off levels. The *mcrA* gene sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers, LC720689–LC720720.

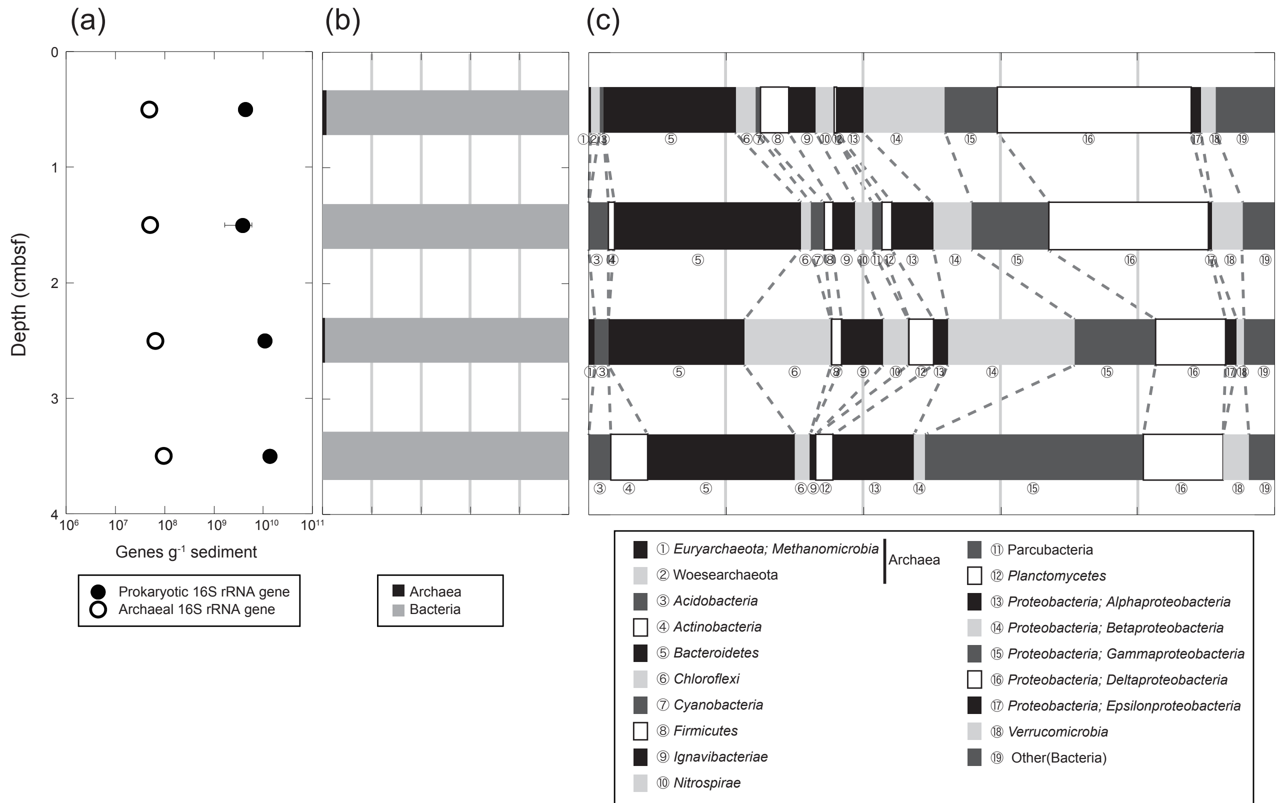

Fig. 1. (a) Number of microbial 16S rRNA genes in the estuary sediments quantified by qPCR. The number of genes g^{-1} sediment was plotted logarithmically in the depth direction. Error bars in the plots indicate the standard deviation in triplicate assays. In some cases, the error bars are hidden within the symbols. (b) Microbial community composition at the domain level based on 16S rRNA gene amplicon analysis. (c) Microbial community composition at the phylum and class level.

Results and Discussions

Microbial gene abundance

Microbial gene distribution against sediment depth was quantified by qPCR. Prokaryotic 16S rRNA gene numbers ranged from about 4.2×10^9 to 1.3×10^{10} genes g^{-1} sediment (Fig. 1a). The archaeal gene numbers ranged from 5.0×10^7 to 9.6×10^7 genes g^{-1} sediment. The highest archaeal gene abundance was found at a depth of 3-4 cm among the analytical samples. The ratio of archaeal 16S rRNA genes to prokaryotic 16S rRNA genes was 0.6-1.3%, indicating archaea were low in abundance at all depths. These values are generally consistent with relative abundances obtained in other estuarine ecosystems around the world (Liu et al., 2018).

Microbial community structures

Microbial community composition in each sediment sample was determined by the 16S rRNA gene amplicon sequencing. In the present study, a total of 12,171 reads were obtained from the sediment samples with an average length of 464 bp. Bacterial communities were dominant in the estuary sediments, at over 98.5%, and archaea accounted for less than 1.4% (Fig. 1b). In comparison, this ratio was consistent with the result obtained from qPCR analysis (0.6-1.3%).

Taxonomic classification at the phylum level showed that *Bacteroidetes* and *Proteobacteria* were dominant among the bacterial population (Fig. 1c). The dominant members in the *Proteobacteria* were *Gammaproteobacteria* and *Deltaproteobacteria*. The *Methanomicrobia*, methanogenic archaea belonging to the *Euryarchaeota*, were detected with a proportion

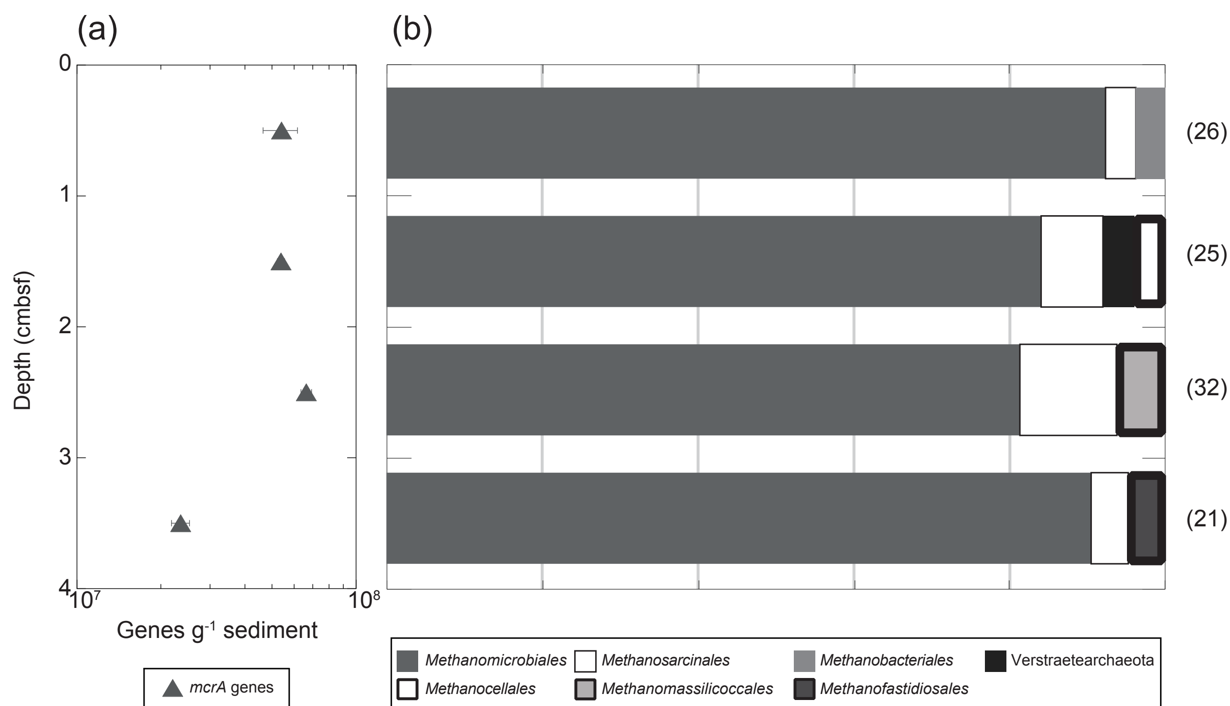

Fig. 2. (a) Vertical distribution of the *mcrA* gene abundance. The number of genes g^{-1} sediment was quantified by qPCR and plotted logarithmically in the depth direction. Error bars in the plots indicate the standard deviation in triplicate assays. In some cases, the error bars are hidden within the symbols. (b) Phylogenetic composition of the *mcrA* gene based on the phylogenetic affiliation shown in Fig. 3. The number of *mcrA* clones is indicated in the brackets.

of 0.4-0.7%. These archaea were taxonomically classified to the family *Methanosaetaceae* in the order *Methanosarcinales*. In addition, *Ca*. Woesearchaeota (formerly called DEVEG-6) was detected at the top layer of sediment with a ratio of 1% (Fig. 1c), as shown in the other oxygen-limited environments (Liu et al., 2018). The deltaproteobacterial sulfate-reducing bacteria, affiliated with *Desulfobacterales*, *Desulfuromonadales*, and *Desulfarculales*, were dominant throughout the sediment core. Next-generation sequencing has proved to be a useful tool used for a comparison of the microbial community among multiple samples and detection of rare populations that are probably difficult to identify by conventional community composition analysis based on the clone library method.

Phylogenetic diversity based on *mcrA* gene

Quantitative real-time PCR showed that the *mcrA* gene ranged from 2.4×10^7 to 6.6×10^7 genes g^{-1} of the surface sediments at depth of 0-3 cm (Fig. 2a). This indicated that large amounts of the *mcrA* gene were distributed in the surface layer of the sediments that are easily exposed to oxygen. In organic-rich sediments, aerobic bacteria actively consume molecular oxygen, resulting in the formation of anaerobic environments and the production of methane as the final product of organic matter decomposition. The nucleotide sequences of the amplified *mcrA* gene were determined using a clone library method. Phylogenetic composition of the *mcrA* gene sequences obtained in this study is shown in Fig. 2b, and the molecular phylogenetic classification on the phylogenetic tree is shown in Fig. 3. At all depths of sediment, most of the obtained *mcrA* genes were classified into the order *Methanomicrobiales*, accounting for 81.3-92.3% of the total sequences. *Methanosarcinales*-derived *mcrA* genes were the second most abundant, accounting for 3.8-12.5%. The *Methanosarcinales mcrA* contained genes closely related to the family *Methanosaetaceae*,

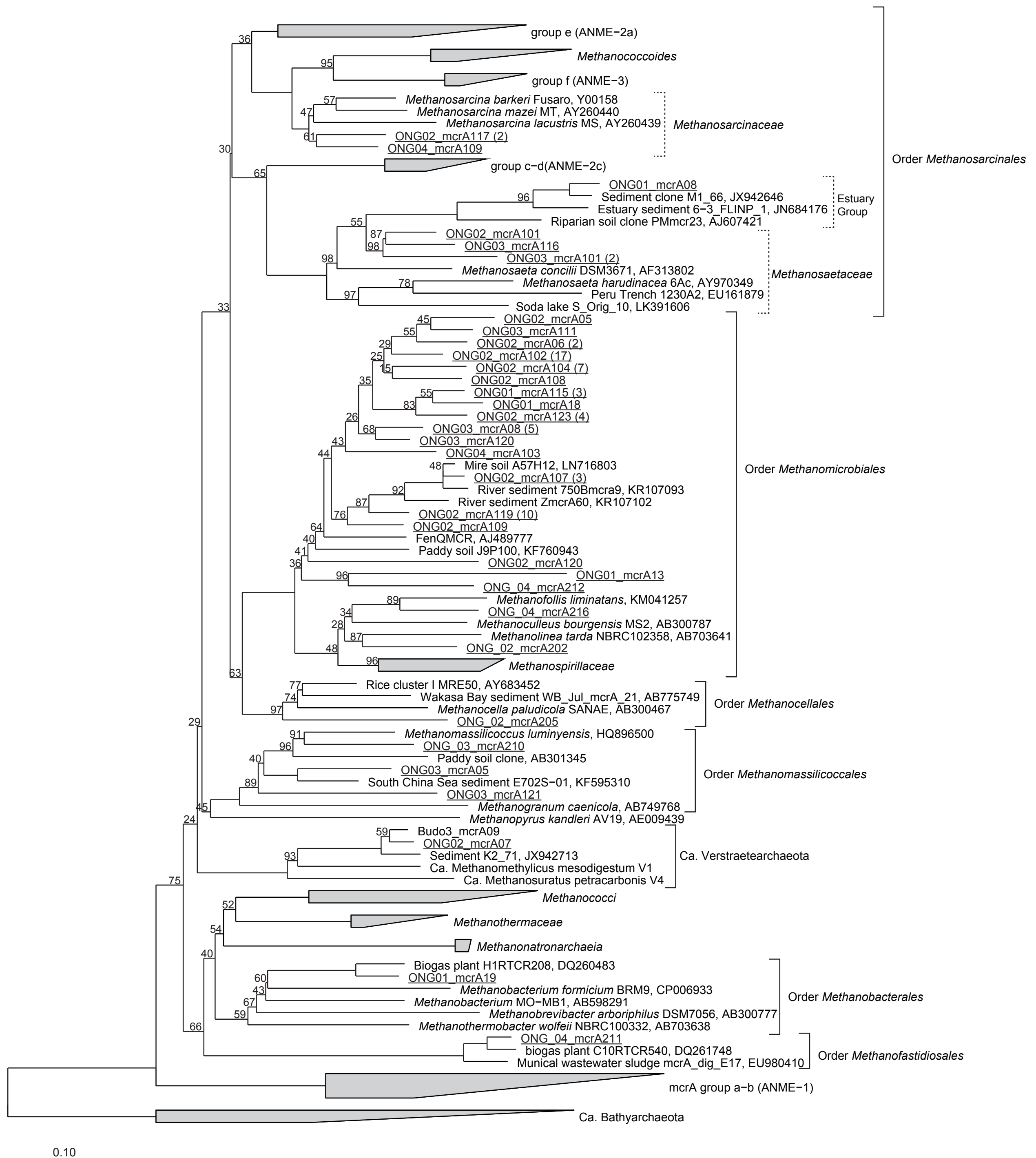

Fig. 3. Phylogenetic tree of the *mcrA* gene sequences detected in the estuary sediments. Only one representative of sequence group >95% identical is shown in this tree. The parenthetic numbers show the total number of phylotypes. The sequences obtained in this study are shown in the underline. Scale bar indicates the number of substitutions per site.

which were also detected as minority by 16S rRNA gene amplicon analysis (3.8%). At a depth of 0-1 cm, members of *Methanocellales* and uncultured methanogenic archaea of *Ca*. Verstraetearchaeota (previously known as Terrestrial Miscellaneous Crenarchaeota Group, TMCG) were present in about 4% of the total sequences. In addition, *Methanomassiliicoccales* (6.2%) and *Methanofastidiosales* (4.7%) were detected at a depth of 2-3 cm and 3-4 cm, respectively. This suggests that a highly diverse methanogenic archaea community was not recovered by the 16S rRNA gene amplicon analysis in this study. It should be noted that the *mcrA* sequence ONG01_*mcrA*08 obtained from the top-most sediment (0-1 cm) showed a close relationship to only three sequences. The closest relatives were reported from estuarine sediments in China and Germany (accession number JX942636 and JN684176, respectively) with an about 92.1% similarity. The other sequences showed remarkably low similarity (less than 82%) and formed their own cluster (Estuary Group on the phylogenetic tree in Fig. 3). For the *mcrA* gene sequence, it is suggested that the average similarity was 88.9% at a genus level and was 83.5% at a family level (Steinberg and Regan, 2008). Therefore, the Estuary Group likely represents a distinct lineage at the family level.

Potential methane cycle in brackish water sediments

In this study, the *Methanosaetaceae* were detected based on 16S rRNA gene amplicon analysis while the *mcrA* gene cloning analysis recovered more diverse sequences spanning 7 phylum- or order-level lineages such as *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, *Methanomassiliicoccales*, *Methanobacterales*, *Methanofastidiosales*, and *Ca*. Verstraetearchaeota. This discrepancy may be due to the low coverage of the 16S rRNA gene-specific PCR primers for the known methanogen sequences in the study site. Members of the *Methanomicrobiales* were dominant based on the *mcrA* gene cloning. Most of them are known to be hydrogenotrophic methanogens, which produces methane from H_2 and CO_2 (Liu and Whitman, 2008). The second most dominant group was *Methanosarcinales*, including *Methanosarcinaceae* and *Methanosaetaceae*. They were widely detected in major sources of methane emissions such as rice paddies, permafrost, and peat bogs (Oren, 2014a). They are capable of acetoclastic methanogenesis, which is thought to be an important factor for increasing atmospheric methane. Members of *Methanosarcinales* also use other substrates such as CO_2/H_2, formate, and methyl compounds (methanol and methylamine). The *Methanosaetaceae* were detected based on both the 16S rRNA gene amplicons and *mcrA* gene clone libraries. They have been known to utilize only acetate as a methanogenic substrate (Liu and Whitmann, 2008). *Methanobacteralaes*-related *mcrA* genes were detected at a depth of 0-1 cm. Most of them have been known to use CO_2/H_2 and formate (Oren, 2014b). The *mcrA* genes from *Methanocellales* and *Ca*. Verstraetearchaeota was found at a depth of 1-2 cm. All the isolated species of *Methanocellales* are known to reduce CO_2 with H_2 (Sakai et al., 2014). *Ca*. Verstraetearchaeota were frequently detected in anaerobic environments with high methane flux. Recently, the metagenomic analysis revealed that they had potential to produce methane from methanol, methanethiol, and methylamine (Vanwonterghem et al., 2016). The *Methanomassiliicoccales*, methanogenic archaea in the phylum *Thermoplasmata* (Iino et al., 2013), was detected at a depth of 2-3 cm. They have been found in animal intestines, wetlands, and sewage treatment plants. *Methanomassiliicoccales* are known to generate methane from methanol, methylamine, and dimethyl sulfide by using H_2 as an electron donor dependently (Lang et al., 2015). The *Methanofastidiosales*, a lineage previously referred as WSA2 or Arc I (Chouari et al., 2005), were observed at a depth of 3-4 cm. This group has been reported in freshwater, marine sediments, contaminated groundwater, and bioreactors. Recently, the metagenomic analysis showed that they reduced methylated thiol for methane generation since none of the essential genes in CO_2 reduction and acetoclastic methanogenesis pathway were detected (Nobu et al., 2016).

Our results suggested that surface sediments in brackish water could be a suitable site for methanogenesis. It is well known that sulfate-reducing bacteria and methanogenic archaea compete for acetate and hydrogen in sulfate-containing environments, such as the seafloor, and sulfate-reducing bacteria can utilize them at lower concentrations than methanogens according to thermodynamic calculations. Thus, methanogenesis is less likely to proceed in sulfate-replete zones (Lueders et

al., 2001). Nonetheless, several studies have shown that methane was detected in surface sulfate-rich sediments (Dale et al., 2008; Knab et al., 2008; Sela-Adler et al., 2017). This is attributed to active methanogenesis using specific substrates not used by sulfate-reducing bacteria, such as methanol, methylamine, methanethiol, and dimethyl sulfide (Oremland and Polcin, 1982). Since the present study site is a sulfate-rich environment supplied by seawater, the methanogenic archaea detected in our samples likely utilized those non-competitive substrates. In fact, methanogenic archaea with the ability to use methyl compounds have been detected based on the *mcrA* gene analysis. The methylotrophic methanogens can be divided into two groups based on the presence of cytochromes. *Methanosarcinales* oxidizes methyl groups to CO_2 via cytochrome, whereas *Methanomassiliicoccales* does not use cytochromes but depend on hydrogen (Thauer et al., 2008). In addition, *Ca*. Bathyarchaeota is thought to use hydrogen to produce methane from methanol, methylamine, and methyl sulfide (Evans et al., 2015). In this study, the *Methanomassiliicoccales*, *Verstraetearchaeota*, and *Methanofastidiosales* were expected to exhibit similar hydrogen-dependent metabolism. We suggest that these lineages perform methylotrophic methanogenesis and supply methane to the surface sediment in the study site.

In the marine environment, anaerobic oxidation of methane (AOM) is a well-known biodegradation process using sulfate as an electron acceptor. The 16S rRNA gene amplicon analysis and the *mcrA* gene clone library method used in this study can detect the anaerobic methanotrophic archaea as well as methanogens (Yanagawa et al., 2019), but they were not detected in our samples. Hence, AOM may be less likely to proceed in this brackish water environment where dilution of seawater by freshwater inflow is variable. If AOM does not occur, the methane produced in the surface sediments will directly escape into the upper water and the atmosphere. Alternatively, coupling reactions of AOM and denitrification have been reported from river sediments and intertidal zones (Shen et al., 2019; Wang et al., 2019). Currently, mechanisms of the methane consumption in brackish water environments are not deeply understood.

Microbial community composition analysis using next-generation sequencing has become a major technique for environmental microbiology. This approach has made it possible to detect minor microbial populations in environmental sample. However, when targeting rare populations, less than a few percent, multiple methods should be applied for cautious verification. For example, new approaches for exploring functional genes in the pool of nucleotide sequences obtained by metagenomic analysis have allowed researchers to construct genome bins (Speth and Orphan, 2018). For detection of methanogenic archaea, PCR amplification targeting the *mcrA* gene is still an effective method, as applied in this study and previous research (Wilkins et al., 2015). It should be noted that PCR-dependent methods do not always detect all target populations due to primer biases. Since the discovery of methanogenic potential besides *Euryarchaeota*, i.e., *Ca*. Bathyarchaeota and *Ca*. Verstraetearchaeota, primer selection should be reconsidered to detect the potential methanogenic archaea. The present study showed that diverse methanogenic archaeal components were recovered by applying the frequently used *mcrA* primers.

Conclusions

In this study, we analyzed the microbial community compositions in brackish water sediments. Next-generation sequencing of the 16S rRNA gene and classical molecular cloning targeting the *mcrA* gene were used to examine the potential of biogenic methane production. The 16S rRNA gene amplicon analysis detected only the *Methanosaetaceae*, whereas the *mcrA* gene cloning analysis recovered more diverse sequences spanning 7 phylum- or order-level lineages, including a new candidate family-level lineage. Although the 16S rRNA gene-based sequencing is often regarded as the definitive method for microbial community analysis, this study suggested that diversity of minor populations should be assessed in combination with conventional cloning methods based on functional genes.

Acknowledgements

This work was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Number 17K18808 and 20K05404) and the Sumitomo Foundation.

References

Bange, H. W., Bartell, U. H., Rapsomanikis, S., & Andreae, M. O. (1994) Methane in the Baltic and North Seas and a reassessment of the marine emissions of methane. Global Biogeochem Cycles, 8: 465.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol, 37: 852-857.

Borowski, W. S., Paull, C. K., & Ussler, W., III. (1996) Marine pore-water sulfate profiles indicate in situ methane flux from underlying gas hydrate. Geology, 24: 655-658.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA, 108: 4516.

Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., & Sghir, A. (2005) Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ Microbiol, 7: 1104-1115.

Conrad, R. (2009) The global methane cycle: recent advances in understanding the microbial processes involved. Environ Microbiol Rep, 1: 285-292.

Dale, A. W., Regnier, P., Knab, N. J., Jørgensen, B. B., & Van Cappellen, P. (2008) Anaerobic oxidation of methane (AOM) in marine sediments from the Skagerrak (Denmark): II. Reaction-transport modeling. Geochim Cosmochim Acta, 72: 2880-2894.

Egger, M., Lenstra, W., Jong, D., Meysman, F. J. R., Sapart, C. J., van der Veen, C., et al. (2016) Rapid sediment accumulation results in high methane effluxes from coastal sediments. PLOS ONE, 11: e0161609.

Evans, P. N., Parks, D. H., Chadwick, G. L., Robbins, S. J., Orphan, V. J., Golding, S. D., & Tyson, G. W. (2015) Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. Science, 350: 434.

Hales, B. A., Edwards, C., Ritchie, D. A., Hall, G., Pickup, R. W., & Saunders, J. R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. Appl Environ Microbiol, 62, 668-675.

Iino, T., Tamaki, H., Tamazawa, S., Ueno, Y., Ohkuma, M., Suzuki, K.-i., et al. (2013) *Candidatus* Methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of *Methanomassiliicoccaceae* fam. nov. and *Methanomassiliicoccales* ord. nov., for a methanogenic lineage of the class *Thermoplasmata*. Microbes Environ, 28: 244-250.

King, G. M. (1984) Utilization of hydrogen, acetate, and “noncompetitive” substrates by methanogenic bacteria in marine sediments. Geomicrobiol J, 3: 275-306.

Knab, N. J., Cragg, B. A., Borowski, C., Parkes, R. J., Pancost, R., & Jørgensen, B. B. (2008) Anaerobic oxidation of methane (AOM) in marine sediments from the Skagerrak (Denmark): I. Geochemical and microbiological analyses. Geochim Cosmochim Acta, 72: 2868-2879.

Knittel, K., & Boetius, A. (2009) Anaerobic oxidation of methane: progress with an unknown process. Annu Rev Microbiol, 63: 311-334.

Kvenvolden, K. A. (1988) Methane hydrate — A major reservoir of carbon in the shallow geosphere? Chem Geol, 71: 41-51.

Lang, K., Schuldes, J., Klingl, A., Poehlein, A., Daniel, R., Brune, A., & Elliot, M. A. (2015) New mode of energy metabolism in the seventh order of methanogens as revealed by comparative genome analysis of “*Candidatus* Methanoplasma termitum”. Appl Environ Microbiol, 81: 1338-1352.

Liu, Y., & Whitman, W. B. (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann N Y Acad Sci, 1125: 171-189.

Liu, X., Pan, J., Liu, Y., Li, M., & Gu, J.-D. (2018) Diversity and distribution of Archaea in global estuarine ecosystems. Sci Total Environ, 637-638: 349-358.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Res, 32: 1363-1371.

Lueders, T., Chin, K.-J., Conrad, R., & Friedrich, M. (2001) Molecular analyses of methyl-coenzyme M reductase α-subunit (*mcrA*) genes in rice field soil

and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. Environ Microbiol, 3: 194-204.

McKay, L. J., Hatzenpichler, R., Inskeep, W. P., & Fields, M. W. (2017) Occurrence and expression of novel methyl-coenzyme M reductase gene (*mcrA*) variants in hot spring sediments. Sci Rep, 7: 7252.

Nobu, M. K., Narihiro, T., Kuroda, K., Mei, R., & Liu, W.-T. (2016) Chasing the elusive Euryarchaeota class WSA2: genomes reveal a uniquely fastidious methyl-reducing methanogen. ISME J, 10: 2478-2487.

Nunoura, T., Oida, H., Miyazaki, J., Miyashita, A., Imachi, H., & Takai, K. (2008) Quantification of *mcrA* by fluorescent PCR in methanogenic and methanotrophic microbial communities. FEMS Microbiol Ecol, 64: 240-247.

Oremland, R. S., & Polcin, S. (1982) Methanogenesis and sulfate reduction: Competitive and noncompetitive substrates in estuarine sediments. Appl Environ Microbiol, 44: 1270-1276.

Oren, A. (2014a) The Family *Methanosarcinaceae*. In The prokaryotes: Other major lineages of bacteria and the archaea, (eds.) Rosenberg, E., Delong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. pp. 259-281. Springer Berlin Heidelberg, Berlin, Heidelberg.

Oren, A. (2014b) The Family *Methanobacteriaceae*. In The prokaryotes: Other major lineages of bacteria and the archaea, (eds.) Rosenberg, E., Delong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. pp. 165-193. Springer Berlin Heidelberg, Berlin, Heidelberg.

Rajendhran, J., & Gunasekaran, P. (2011) Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. Microbiol Res, 166: 99-110.

Reeburgh, W. S. (2007) Oceanic methane biogeochemistry. Chem Rev, 107: 486-513.

Sakai, S., Conrad, R., & Imachi, H. (2014) The Family *Methanocellaceae*. In The prokaryotes: Other major lineages of bacteria and the archaea, (eds.) Rosenberg, E., Delong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. pp. 209-214. Springer Berlin Heidelberg, Berlin, Heidelberg.

Schaefer, H., Fletcher, S. E. M., Veidt, C., Lassey, K. R., Brailsford, G. W., Bromley, T. M., et al. (2016) A 21st-century shift from fossil-fuel to biogenic methane emissions indicated by $^{13}CH_4$. Science, 352: 80-84.

Sela-Adler, M., Ronen, Z., Herut, B., Antler, G., Vigderovich, H., Eckert, W., & Sivan, O. (2017) Co-existence of methanogenesis and sulfate reduction with common substrates in sulfate-rich estuarine sediments. Front Microbiol, 8:

Shen, L.-d., Ouyang, L., Zhu, Y., & Trimmer, M. (2019) Active pathways of anaerobic methane oxidation across contrasting riverbeds. ISME J, 13: 752-766.

Speth, D. R., & Orphan, V. J. (2018) Metabolic marker gene mining provides insight in global *mcrA* diversity and, coupled with targeted genome reconstruction, sheds further light on metabolic potential of the *Methanomassiliicoccales*. PeerJ, 6: e5614.

Steinberg, L. M., & Regan, J. M. (2008) Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. Appl Environ Microbiol, 74: 6663-6671.

Takai, K., & Horikoshi, K. (2000) Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol, 66: 5066-5072.

Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W., & Hedderich, R. (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat Rev Microbiol, 6: 579-591.

Valentine, D. L., & Reeburgh, W. S. (2000) New perspectives on anaerobic methane oxidation. Environ Microbiol, 2: 477-484.

Vanwonterghem, I., Evans, P. N., Parks, D. H., Jensen, P. D., Woodcroft, B. J., Hugenholtz, P., & Tyson, G. W. (2016) Methylotrophic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. Nat Microbiol, 1: 16170.

Wang, J., Cai, C., Li, Y., Hua, M., Wang, J., Yang, H., et al. (2019) Denitrifying anaerobic methane oxidation: A previously overlooked methane sink in intertidal zone. Environ Sci Technol, 53: 203-212.

Wilkins, D., Lu, X.-Y., Shen, Z., Chen, J., Lee, P. K. H., & Nojiri, H. (2015) Pyrosequencing of *mcrA* and archaeal 16S rRNA genes reveals diversity and substrate preferences of methanogen communities in anaerobic digesters. Appl Environ Microbiol, 81: 604-613.

Yanagawa, K., Matsumoto, R., & Suzuki, Y. (2012) Subseafloor biosphere mediating global methane cycle. Jour Japan Assoc Petrol Technol, 77: 374-383.

Yanagawa, K., Shiraishi, F., Tanigawa, Y., Maeda, T., Mustapha, N. A., Owari, S., et al. (2019) Endolithic microbial habitats hosted in carbonate nodules currently forming within sediment at a high methane flux site in the sea of Japan. Geosciences, 9:463