Distribution of Methanotrophic Bacteria in the Coastal Marine Sediments

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(Received November 9, 1998)

Methanotrophic bacteria attract attention from the viewpoints of biology, geochemistry and bioremediation. However, the ecology of marine methanotrophs is not well known in spite of their possible important role in the environment, partly because they are difficult to cultivate. Enrichment cultures of methanotrophic bacteria were obtained from coastal marine sediment under 1:1 methane and air conditions. The cultures utilized methane and oxygen and released carbon dioxide. Under TEM observation the cultures contained bacteria with the stacked intracytoplasmic membrane system, which is characteristic of group I methanotrophic bacteria. MPN method with tentative growth confirmation by TEM observation was applied to enumerate methanotrophic bacteria in the sediments from Otsuchi Bay, Tokyo Bay, and Aburatusbo Inlet. The count had positive correlation with the viable count of heterotrophic bacteria. Vertical distributions in sediment samples at two stations in Tokyo Bay showed a high count of methanotrophic bacteria and low methane concentration in shallow sediment, which suggested the methane profile was caused by the activity of methanotrophs. The highest count of methanotrophic bacteria was 2400 MPN in 1 ml of the marine sediment.

Key words: methane, methanotroph, methane oxidation, MPN

Methanotrophic bacteria are a group of bacteria defined by their ability to grow on methane as the sole carbon and energy source. Several studies have shown methanotrophs may play important ecological roles in marine environments in the view points of a greenhouse effect gas,1) macrofauna communities in deep-sea seeping areas2) and biodegradation of halogenated volatile organic substances.3) Although their ecological roles are possibly wide and important in environments, only a limited number of methanotrophs from the marine environment have been isolated.4-8) Furthermore, studies on their population are quite limited. In this report, sediment microorganisms enriched and cultured with methane were characterized and they were enumerated at three coastal areas in Japan using MPN method with combination of TEM observation.

Materials and Methods

Samples
Sediment samples were collected on December 16-18, 1987, June 24, 1988, and August 6, 1988, from Otsuchi Bay in Iwate Prefecture, Aburatsuibo Inlet in Kanagawa Prefecture and Tokyo Bay, respectively. Sampling stations in Tokyo Bay are shown in Fig. 1. Samples from Tokyo Bay were obtained with a box corer during the cruise of

Fig. 1. Sampling stations in Tokyo Bay.

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the R/V Tansei-maru (KT-88-14). Sub-cores (36 mmΦ) were then taken from the box core samples. The upper 9 cm layer of each sub-core was divided into three portions at 3 cm intervals. Samples from Aburatsubo Inlet and Otsuchi Bay were obtained with Ekman-Birge Sampler except some samples from Otsuchi Bay which were obtained by Plieger Core Sampler. Only upper 3 cm sections of these samples were used for analysis and inoculation.

**Methane Concentration in Sediment**

Methane concentrations in the interstitial water of the sediments were analyzed by head space gas chromatography.39 Interstitial water was separated from sediment by centrifuge (1500 × g, 20 min), 5 ml of which was introduced into evacuated sterile 35 ml serum vial equipped with a butyl rubber stopper and sealed with an aluminum seal. After shaking for five minutes at room temperature, the vials were equilibrated to atmospheric pressure with a needle. Methane in 1 ml gas phase in the vial was measured with gas chromatograph (Shimadzu GC-12A with Molecular Sieve 5A column 2 m long, 3 mm I.D., 100°C column temp.) fitted with FID. Nitrogen gas was used as carrier.

**Culture Media**

B728 medium which is a modification of NMS medium30 was prepared as follows: (i) basal salts: NaCl, 25 g; MgSO₄, 2 g; KCl, 0.5 g; CaCl₂, 2H₂O, 1.0 g; KNO₃, 0.5 g; FeSO₄·7H₂O, 20 mg; CuSO₄·5H₂O, 0.1 mg; ZnSO₄·7H₂O, 0.1 mg; MnCl₂·4H₂O, 0.1 mg; CoCl₂·6H₂O, 0.1 mg; Na₂MoO₄·2H₂O, 0.1 mg; distilled water, 900 ml (ii) buffer: Na₂HPO₄·12H₂O, 0.3 g and KH₂PO₄, 0.2 g dissolved in 100 ml of distilled water and adjusted to pH 7.6. Solutions (i) and (ii) were mixed after separate autoclave. Fifteen g/l of agar (Difco) was added to this medium for preparation of slants and plates.

For the viable count of heterotrophic bacteria, modified ZoBell 2216E medium31 was used, which contained the following components: bactopeptone (Difco), 0.5 g; yeast extract (Difco), 0.1 g; Fe-EDTA, 0.1 g; agar (Difco), 15 g; sea water 1000 ml. The pH was adjusted to 7.6.

**Enrichment Culture**

For the enrichment culture of methanotrophic bacteria from coastal sediments, 1 ml of each sediment sample was inoculated into a 1000 ml volume suction bottle in which 100 ml of B728 medium was prepared in advance. The inoculated bottles were then evacuated and methane gas was introduced through sterile cotton filter into the bottles up to a ratio of methane and air volume 1:1. The bottles were incubated at 35°C, shaking reciprocally at 120 rpm in the dark. A 1% portion of every culture was monthly inoculated into a 1000 ml volume suction bottle in which 100 ml of ZoBell 2216E medium in a 18 mmΦ-18 cm long test tube with a cotton plug. Five dilutions including this initial one were prepared in a decimal series dilutions in the same medium and test tubes. Trillicate series of dilutions were incubated without shaking at 20°C in the dark in the polycarbonate desiccator in which methane was introduced (methane:air = 1:1, atmospheric pressure). After a month of incubation, tubes with turbidity or pellicle formation on the medium surfaces were tested for the existence of methanotrophic cells by the same method as previous description except that cells were tested for the existence of methanotrophic cells by the same method as previous description except that cells were collected primarily from pellicle and pelletized by centrifuge. For viable count of heterotrophic bacteria, sediment samples were serially diluted with sterile seawater, and enumerated with the agar pour method using modified ZoBell 2216E medium after incubating for 14 days at 20°C.

**Transmission Electron Microscopy (TEM)**

Cells grown for 14 days under 50% methane atmosphere at 30°C under dark conditions with reciprocal shaking at 120 rpm were harvested by centrifuge (1000 × g, 5 min), and prefixed with 1% glutaraldehyde (0.1 m cacodylate pH 6.85, 0.25 m NaCl) for two hours at room temperature. After washing five times with 0.1 m cacodylate buffer (pH 6.85, 0.25 m NaCl), cells were fixed with 1% OsO₄ for two hours at room temperature, then washed five times with distilled water. Fixed cells were embedded in 1.2% agarose gel, which was cut and dehydrated in ethanol series, substituted with propylene oxide, then soaked in epon 812 (TAAB)/propylene oxide for 12 hours. Epon 812 was polymerized at 40°C for four hours, then at 60°C for 24 hours. Ultramicrotome (LKB Ultratome V, Porter MT-11) with a diamond knife was used to make thin sections. The samples were observed with TEM (JEOL, JEM-100X), after electron staining by saturate uranium acetate and 0.4% lead citrate.

**Enumeration of Bacteria**

MPN method32 was used to enumerate methane utilizing bacteria in the sediment samples. Each 1 ml sediment sample was suspended with 9 ml of B728 medium in a 18 mmΦ-18 cm long test tube with a cotton plug. Five dilutions including this initial one were prepared in a decimal series dilutions in the same medium and test tubes. Trillicate series of dilutions were incubated without shaking at 20°C in the dark in the polycarbonate desiccator in which methane was introduced (methane:air = 1:1, atmospheric pressure). After a month of incubation, tubes with turbidity or pellicle formation on the medium surfaces were judged positive. Some of the highest grown dilution tubes were tested for the existence of methanotrophic cells by the same method as previous description except that cells were collected primarily from pellicle and pelletized by centrifuge. For viable count of heterotrophic bacteria, sediment samples were serially diluted with sterile seawater, and enumerated with the agar pour method using modified ZoBell 2216E medium after incubating for 14 days at 20°C.
Results

Coastal sediment samples incubated under methane and air atmosphere (1:1) in B728 medium at 35°C with reciprocal shaking showed visible growth after one to two weeks. The growth sometimes showed pigmented flocking. Some cultures showed no growth after more than three weeks of incubation. Stable cultures were obtained from the surface sediments of Tokyo Bay at stations T-3 and T-5, and Aburatsubo Inlet at St.3. The cultures were named as T3a, T5a, and St3a, respectively, and subjected to further observation.

T5a showed aggregations pigmented with slightly brownish color in shaking culture as well as static culture. Other two cultures showed dispersed growth with slightly pink pigmented. Growths of the three cultures measured as protein increase after incubation of one week at 35°C under 50% methane atmosphere were 30-50 times more than under 100% air atmosphere. The cultures consumed methane and oxygen, and released carbon dioxide during growth. The consumed volume ratios of oxygen/methane by T3a, T5a, and St3a cultures were 1.6, 1.6, and 1.1, respectively. Harvested cells also consumed methane and oxygen and released carbon dioxide within several hours (Fig. 2). The consumption ratio of oxygen/methane by T3a, T5a and St3a were 1.0, 1.1, and 1.9 in the first 2 hours and 2.0, 3.7, 3.1 in the next five hours, respectively.

In order to confirm whether methanotrophic bacteria were present or not, thin sections of the cells in the cultures were observed under TEM. A as a result, the majority of the cells possessed the intracytoplasmic membrane system (Fig. 3). Cells with the membrane system were short rod and sized 0.8 – 1.1 μm × 1.1 – 2.5 μm. From all the cultures, cells possessing stacked intracytoplasmic membrane system, a characteristic of group I methanotrophic bacteria,14,15 were detected by TEM.

As the results above showed that enrichment culture of methanotrophic bacteria was successful, we tried to enumerate them using the MPN method. The observed range of methanotrophic bacteria in the sediments was 4.0×10²-2.4×10³ MPN/ml sediment in Tokyo Bay with this method (Fig. 4). Pellicles observed at the air-water interface of the culture tubes were suspected to be methanotrophic growth. Some of the pellicles in highest dilutions of positive cultures were tested for the existence of methanotrophs by TEM observation. All the pellicles tested contained methanotrophic cells similar to those observed in the enrichment culture. At every station, the numbers of methanotrophic bacteria were higher in the surface than lower depths of the sediments (Fig. 4). The addition of cycloheximide (20 μg/ml) in order to eliminate feeding by eucaryote6 after a month of incubation did not show any effect on the culture after further incubation (data not shown).

At station T-2 and station T-3, methane concentrations were high in deeper sediments. Methane in the interstitial water was not detected at station T-5E and T-7. Heterotrophic bacteria were counted from 10⁴ to 10⁷ cfu/ml sediment (Fig. 4). A positive correlation between the number of methanotrophic and the number of heterotrophic bacteria was observed in Tokyo Bay and Otsuchi Bay samples (Fig. 5). Heterotrophs in Aburatsubo Inlet samples were observed to be almost constant in cfu, while the MPN of methanotrophic bacteria varied widely (Fig. 5).

Discussion

Bacteria grew visibly when 1 ml of coastal sediment sample was incubated under methane and air atmosphere in B728 medium. This medium is a modification of NMS medium which Whittenbury et al.10 used to isolate methanotrophic bacteria from many terrestrial samples including soil, pond water, etc. They also used AMS medium which differs from NMS in substituting nitrate with ammonium as a nitrogen source. However, we used nitrate as a nitrogen source in B728 medium, because all known methanotrophic bacteria are capable of utilizing nitrate as a nitrogen source.

The growth was sometimes accompanied by brown, milky white, or pink pigmentation. Cultures of T3a, T5a, and St3a were chosen because of their stable growth. In order to ascertain whether methanotrophic bacteria were present in the cultures, biomass increase and methane con-
The bar in each figure shows 1 μm. The bundles of membranes in cell(s) in each figure are "intracytoplasmic membrane systems", typical of group I methanotrophic bacteria.

Harvested cells consumed methane and oxygen and released carbon dioxide (Fig. 2). The ratio of consumed oxygen/consumed methane for the harvested cells was lower in the initial two hours (1.0, 1.1, and 1.9 for T3a, T5a, and St3a, respectively) than in the following five hours (2.0, 3.7, and 3.1 for T3a, T5a, and St3a, respectively) (Fig. 2a–c). The increase of ratios in the latter phase may be attributed to higher heterotrophic activity. The ratios for T3a and T5a in the initial phase were lower than the value 1.8 reported for the pure culture,19 which suggested that in the initial phase harvested cells absorbed methane more than oxygen.

The methanotrophic bacteria are classified into two groups based on the type of the structure of intracytoplasmic membrane system.14 This classification is consistent with physiological, biochemical characters,15 and 16S
rRNA sequence. Only group I methanotrophs were observed under TEM in T3a, T5a, and S13a cultures (Fig. 3). It is interesting that all the methanotrophic bacteria of marine origin ever reported including symbionts have a type I membrane system.6,7,8,18)

As methanotrophic bacteria in marine sediment could grow under the culture conditions, methanotrophic bacteria in sediment samples were enumerated by the MPN method. Although there have been several reports of new methods to elucidate the distribution of methanotrophs by indirect fluorescent antibody-membrane filter technique19) and oligonucleotide probes20) and these methods may overcome the limitation that only culturable fraction are targeted, the former were reported to be too specific and the latter does not seem to be applicable for methanotrophs in marine sediment because their cultured strains and genetic information are limited. The MPN method seems the most suitable at the present time for routine counting of marine methanotrophs in sediment.

Methanotrophic bacteria were enumerated to be 4.0 × 10^2–2.4 × 10^5 MPN/ml in the sediment of Tokyo Bay (Fig. 4). The vertical distribution of methanotrophs and methane concentration in the Tokyo Bay sediment showed a negative correlation (Fig. 4, Station T-2, T-3). It can be explained that methanotrophic bacteria were responsible for the distribution of methane, on the assumption that diffusion and bioturbation were negligible.

A few attempts to enumerate methanotrophs by the MPN method have been reported. De Bont21) counted methane oxidizing bacteria of terrestrial waters and muds, and reported them to be 10^5 MPN/g. Heyer et al.22) enumerated the bacteria in various samples including sediments and waters of river, lakes, and sea in USSR and DDR. They reported, 1.7 × 10^5 MPN/g for Black Sea sediment, 3 × 10^5 cells/g for salt lake, and from 10^4 to 10^5 for sediments of eutrophic lakes. From their data and the data of the number of methanotrophs in this research, methanotrophs seem to be more abundant in the freshwater environment than in the marine environment. In the aquatic habitat, a large fraction of the methane produced is considered to be consumed by methane oxidation before it leaves to the air.23) The estimation of the population of methanotrophic bacteria is important in the prediction of potential methane oxidizing activity in marine environments.

Conventional viable cell count with plate cultures seemed almost impossible; we found it difficult to grow marine methanotrophs on solid media. This may be explained by their lack of potential to grow on solid media or need association with other bacteria. The latter reason seemed to be supported by observed positive correlation between the cfu of heterotrophs and the MPN of methanotrophs (Fig. 5). The MPN of methanotrophs in sediment may produce false results when the sediment contains relatively high organic compounds and a low number of methanotrophs. In this case heterotrophic growth on the sample organic compounds might mask methanotrophic growth. Although we did not meet the case in Tokyo Bay samples where growths were observed in high dilutions, confirmation of the existence of methanotrophic cells in pellicles by TEM were helpful especially for the low dilution tubes where heterotrophic growth on the inherent organic compounds was suspected.

Acknowledgments

We would like to thank Dr. K. Endow for technical instructions of TEM. This research was supported in part by the grant-in-aid from the Ministry of Education, Science, Sports and Culture, No. 62500184.

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19) W. M. Reed and P. R. Rasgon: Distribution of Methylococcales in Tokyo Bay samples where growths were observed in high dilutions, confirmation of the existence of methanotrophic cells in pellicles by TEM were helpful especially for the low dilution tubes where heterotrophic growth on the inherent organic compounds was suspected.

