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Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa

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ABSTRACT

Methane oxidation coupled to denitrification is mediated by '*Candidatus* Methylomirabilis oxyfera', which belongs to the candidate phylum NC10. The distribution of putative denitrifying methane-oxidizing bacteria related to "*M. oxyfera*" was investigated in a freshwater lake, Lake Biwa, Japan. In the surface layer of the sediment from a profundal site, a phylotype closely related to "*M. oxyfera*" was most frequently detected among NC10 bacteria in PCR analysis of the 16S rRNA gene. In the sediment, sequences related to "*M. oxyfera*" were also detected in a *pmoA* gene library. The presence of NC10 bacteria was also confirmed by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). Denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR indicated that the abundance of the "*M. oxyfera*"-related phylotype was higher in the upper layers of the profundal sediment. The horizontal distribution of the putative methanotrophs in lake sediment was also analyzed by DGGE, which revealed that their occurrence was restricted to deep water areas. These results agreed with those in a previous study of another freshwater lake, and suggested that the upper layer of the profundal sediments is the main habitat for denitrifying methanotrophs.

Introduction

Methane oxidation coupled to denitrification was first demonstrated in an enrichment culture [14]. The organism responsible for this reaction has been identified to date as '*Candidatus* Methylomirabilis oxyfera', which belongs to the candidate phylum NC10. Although it has not yet been isolated in pure culture, the genome sequence of "*M. oxyfera*" is now available [2], and it has been shown that this bacterium has the same enzyme system for methane oxidation as that used by well-known aerobic methane-oxidizing bacteria. Under anoxic conditions, "*M. oxyfera*" generates oxygen from nitrite to drive this system [2].

With its unique ability to generate molecular oxygen under anoxic conditions, "*M. oxyfera*" can couple denitrification to methane consumption. Anaerobic methane oxidation independent of sulfate has been overlooked, and thus it is not taken into consideration in the global methane budget. Presently, the distribution of denitrifying methane-oxidizing bacteria in natural environments is hardly known. Major phylogenetic clusters in candidate phylum NC10, group *a* and group *b*, have been recognized [3], and involvement of group *a* in methane oxidation has been suggested from studies of enrichment cultures [2,5,6,10]. In a previous study performed in an oligotrophic freshwater lake, Lake Constance, nitrate-dependent methane oxidation and an overwhelming dominance of a specific phylotype among NC10 bacteria were observed in the profundal sediment [1]. In that study, sequences of the particulate methane monooxygenase gene (*pmoA*) related to "*M. oxyfera*" were also detected. On the basis of these results, and compared with those of the littoral sediment, it had been deduced that the dominant phylotype, belonging to group *a*, was responsible for the observed anaerobic methane oxidation and that it possessed the detected *pmoA* gene [1].

In the present study, the distribution of putative denitrifying methanotrophs was investigated in a mesotrophic freshwater lake, Lake Biwa, Japan, where one of the closest relatives of "*M. oxyfera*" was detected in a previous study [8]. DGGE, quantitative PCR, and CARD-FISH were used to study the horizontal and vertical distributions of NC10 bacteria in lake sediment, in order to help determine the habitat preference of denitrifying methanotrophs.

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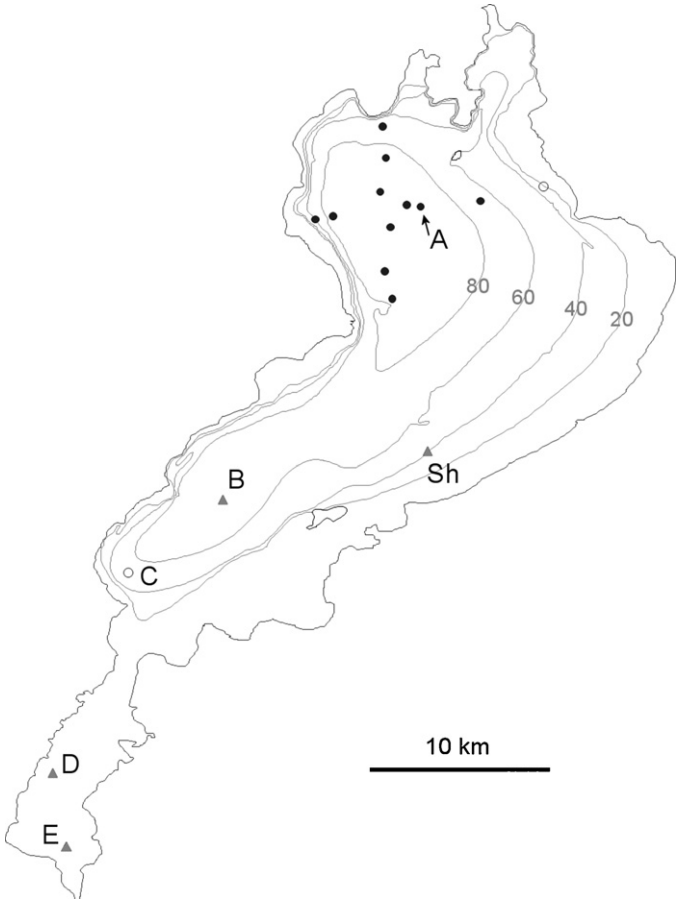

Fig. 1. Map showing the sampling sites and distribution of NC10 bacteria in Lake Biwa, based on the survey performed in 2009 (except for Sh, in 2004). Contour lines represent water depth in meters. Symbols correspond to detected NC10 bacteria; closed circle, group *a*; triangle, group *b*; open circle, not detected.

Materials and methods

Sample collection

Samples were obtained from Lake Biwa, a mesotrophic freshwater lake in Japan. In September 2004, sediment samples were obtained from two sites, site A and site Sh [9,15]. Sediment samples were also obtained in October 2010 from site A and site C (Fig. 1). For the investigation of the horizontal distribution, samples were obtained from 16 sites, including sites A and C, as well as B, D and E, in the period from November to December 2009 (Fig. 1, and supplementary Table S1). All sediment samples were obtained using a core sampler equipped with an acrylic tube (inside diameter 110 mm), and they were subcored (diameter 41 mm) immediately after collection. The subcore samples were kept at 4 °C in the dark until further processing in the laboratory.

Chemical analyses

Interstitial water samples were obtained by centrifugation of the sediment samples sectioned into five layers (the top 2 cm and at 3-cm intervals below that). The concentrations of chloride, nitrate, and sulfate were measured with an ion chromatograph (DX-120; Dionex, Sunnyvale, CA) equipped with a column for anion analyses (IonPac AS4ASC; Dionex). The concentrations of ammonium, nitrite, and ferrous iron were determined colorimetrically by the indophenol method, naphthyl ethylene diamine method, and ferrozine method, respectively.

To extract methane, portions of sediment (1.2–1.8 mL) were thoroughly mixed with 0.4 g NaCl and 1 mL distilled water in gas-tight vials. The resulting slurries were heated at 60 °C for 30 min to enhance release of methane and suppress microbial activities. The concentration of methane released into the head space was measured by using a gas chromatograph (model 2014; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. The stable carbon isotope ratio of methane was determined with a gas-chromatograph-combustion isotope ratio mass spectrometer (GC/C/IRMS, Finnigan MAT-252).

To examine oxygen penetration into the sediment, an oxygen microprofile was investigated using an intact sediment sample from site A obtained in 2010, which was stored for two days at 4 °C. During the measurement, the subcore sample was kept in a water bath at 7 °C, which was the *in situ* temperature of the sediment surface. To compensate for any decrease in oxygen during storage, overlying lake water (*ca.* 5 cm depth) was briefly aerated by bubbling air prior to the measurement. The concentration of oxygen was measured with a Clark-type oxygen microsensor (OX-25; Unisense, Århus, Denmark) connected to a picoammeter (PA 2000, Unisense).

Clone library analysis

The diversity of NC10 bacteria in the profundal site was analyzed by constructing clone libraries of 16S rRNA and *pmoA* genes (Table 2). From the surface layer of the sediment obtained from site A, genomic DNA was extracted using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA). From the extracted DNA, fragments of 16S rRNA genes were amplified using primers designed for the specific amplification of 16S rRNA genes from NC10 bacteria [3]. To maximize the recovery of a diversity of NC10 bacteria, two specific pairs, 202F and 1043R, were used in combination with universal bacterial primers 1492R and 27F, respectively (Table 2). Fragments of the 16S rRNA gene were also amplified with the universal primer pair 27F/1492R. All PCR amplifications were initiated with 2 min denaturation at 94 °C. Each thermal cycle consisted of 30 s denaturation at 94 °C, 30 s annealing, and elongation at 72 °C. The annealing temperature, extension time, and total cycle number for each primer pair are shown in Table 2. Additional extension was carried out for 10 min at 72 °C. The amplified fragments were ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and the resulting vectors were transformed into competent TOP10 cells (Invitrogen).

From the resulting three libraries of 16S rRNA genes, clones of NC10 were selected for further analysis. Based on the alignment of the region shared by all clones (822 sites), pairwise genetic distances were calculated using the Tajima-Nei model. Subsequently, the clones were grouped into operational taxonomic units (OTUs) in such a way that the distances between the clones of the same OTU did not exceed 0.01 in any combination.

A clone library was also constructed from PCR-amplified fragments of the genes for particulate methane monooxygenase (*pmoA*). For specific amplification of the *pmoA* gene related to "*M. oxyfera*", a new primer, 682_NC10 (5′-AAATCCGGCGAAGAACGA-3′) was designed at the position of primer A682 [4]. This primer, perfectly matching the gene sequence of "*M. oxyfera*", was used in combination with A189 [4]. The PCR amplification and subsequent cloning steps were carried out as described above.

PCR-denaturing gradient gel electrophoresis

The vertical and horizontal distributions of the NC10 bacteria were investigated by denaturing gradient gel electrophoresis (DGGE). Depth-related changes within the sediment were investigated for the samples from site A and site Sh obtained in 2004. With