Chapter 4

Determination of the structure of a methane-dependent denitrifying bacterial community in a sewage treatment system by DNA stable-isotope probing

Summary

Methane is used as an alternative carbon source in the denitrification of wastewater lacking organic carbon sources because it is nontoxic and may be efficiently produced by anaerobic biological processes. Methane-dependent denitrification (MDD) in the presence of oxygen requires the co-occurrence of methanotrophy and denitrification. We identified key bacterial populations involved in a MDD ecosystem by using DNA-based stable-isotope probing (DNA-SIP). Sewage sludge was incubated with ^{13}C-labeled methane in either a nitrate-containing medium or a nitrate-free medium. Then, bacterial and methanotrophic populations were analyzed by cloning analysis and T-RFLP analysis targeting 16S rRNA gene and cloning analysis targeting pmoA genes. DNA-SIP analysis of the 16S rRNA gene revealed an association of Gammaproteobacterial methanotrophs (in the family Methylococcaceae) and Alphaproteobacterial methylotrophs (in the family Hyphomicrobiaceae). Furthermore, supplementation with nitrate had significant effects on methane consumption and the activity of methanotrophic populations (i.e. the stimulation of uncultivated relatives of distinct groups of the Methylococcaceae). In particular, uncultured type-X methanotrophs of Gammaproteobacteria were dominant when nitrate was added, i.e. in the MDD incubations. On the other hand, most methanotrophs (type I, type II, and type X methanotrophs) were found to have been labeled with ^{13}C under nitrate-free conditions.

4.1 Introduction

Denitrification requires an oxidizing nitrogen compound as electron acceptor and organic matter, hydrogen, or sulfur as electron donor (Zumft, 1997). For wastewater that has a low C/N ratio or lacks readily biodegradable carbon sources, various organic compounds as external carbon sources, such as acetate, ethanol, glucose, or methanol, may be added to achieve a satisfactory degree of denitrification (Akunna *et al.*, 1993). However, the use of such compounds incurs significant costs in an industrial-scale plant. Recently, methane has been proposed as an alternative, inexpensive, and effective carbon source because it is non-toxic, is produced as a biogas by anaerobic treatment, and is available in numerous existing treatment plants (Amaral *et al.*, 1995; Davies, 1973; Eisentraeger *et al.*, 2001; Thalasso *et al.*, 1997; Werner and Kayser, 1991).

Methane-dependent denitrification (MDD) in the presence of oxygen has been demonstrated in many studies, but the mechanism of this process and the key microbial populations responsible are not yet known (Amaral *et al.*, 1995; Costa *et al.*, 2000; Knowles, 2005; Thalasso *et al.*, 1997; Waki *et al.*, 2002; Werner and Kayser, 1991). So far, although functional genes for dissimilatory nitrite and nitric oxide reductases have been found in some methanotrophic bacteria, there has no evidence that they can carry out denitrification (Ye and Thomas, 2000). Thalasso *et al.* (1997) demonstrated that nitrate depletion in MDD could not be attributed only to nitrogen assimilation, but may also be in part due to denitrification. Thus, it has been suggested that MDD in the presence of oxygen occurs due to the coexistence of methanotrophic bacteria producing organic intermediates in the metabolism of methane and denitrifiers using the organic intermediates as electron donors. Some research groups have suggested that intermediates produced by methanotrophic bacteria could be acetate (Costa *et al.*, 2000), citrate (Rhee and Fuhs, 1978), formaldehyde, methanol (Mechsner *et al.*, 1985), polysaccharides and proteins (Nesterov *et al.*, 1988).

Stable-isotope probing (SIP) techniques have been developed to identify active

bacterial populations in complex natural environments. These techniques are based on the incorporation of stable isotopes into the DNA of cells consuming a labeled substrate of interest (Radajewski *et al*., 2000). To date, the feasibility of SIP has been demonstrated for a wide range of substrates and environments (Dumont and Murrell, 2005; Neufeld *et al*., 2007b). SIP techniques using one-carbon compounds such as methane and methanol have been successfully applied to identifying active methanotrophic or methylotrophic populations in natural environments (Lin *et al*., 2004; Lu *et al*., 2005; Lueders *et al*., 2004a; Neufeld *et al*., 2007a; Radajewski *et al*., 2000 and 2002). Recently, SIP has been used to identify the active denitrifiers in wastewater treatment systems (Ginige *et al*., 2004 and 2005; Osaka *et al*., 2006).

Methanotrophic bacteria include species in the *Alphaproteobacteria* (type II methanotrophic bacteria) and in the *Gammaproteobacteria* (types I and X methanotrophic bacteria). The oxidation of methane to methanol is catalyzed by either a soluble form or a particulate form of methane monooxygenase (sMMO and pMMO, respectively) (Hanson and Hanson, 1996). The *pmoA* gene encoding the α-subunit of the pMMO can be used as a functional phylogenetic marker for the identification of methanotrophic bacteria and is present in almost all known methanotrophic bacteria, except in some members of the genus *Methylocella* (Holmes *et al*., 1995). The *pmoA* gene phylogeny is generally consistent with the 16S rRNA-based phylogeny of methanotrophic bacteria (Costello and Lidstrom, 1999; Murrell *et al*., 1998).

MDD in the presence of oxygen may be hypothesized to be carried out by the interaction between two different types of microorganisms: methanotrophs, which play a role in producing some electron donors in the metabolism of methane, and denitrifiers, which use organic intermediates as electron donors for denitrification. In this study, the microbial populations involved in MDD were identified by DNA-SIP using $^{13}CH_4$.

4.2 Materials and Methods

4.2.1 Pure Cultures

Hyphomicrobium denitrificans ATCC 51888 was grown on ATCC medium 784 AMS supplemented with 100 mM ^{12}C-methanol or ^{13}C-methanol (99% ^{13}C; Sigma, St. Louis, MO, USA) in the dark at 30°C.

4.2.2 Methane-dependent denitrification (MDD) assay

A sludge sample was taken from an urban wastewater treatment plant, the Ariake Water Reclamation Center in Tokyo, Japan, which uses an anaerobic-anoxic-oxic system. The basal medium contained (mg·l^{-1}): KNO_3, 570; KH_2PO_4, 300; $MgSO_4 \cdot 7H_2O$, 60; $CaCl_2 \cdot 2H_2O$, 40; $FeCl_2 \cdot 4H_2O$, 1; $MnCl_2 \cdot 4H_2O$, 1; $CoCl_2 \cdot 6H_2O$, 0.2; H_3BO_3, 0.12; $ZnCl_2$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.02; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MO_4 \cdot 2H_2O$, 0.02; and Na_2SeO_4, 0.02, resulting in a pH of 6.8. The sludge was pre-incubated in a nitrate-containing medium under anoxic conditions in the dark at 20°C for 3 days to eliminate residual organic carbon sources, and the total absence of soluble carbon sources in the supernatant was confirmed by using a TOC analyzer (model TOC-5000A; Shimadzu Corp., Kyoto, Japan). The supernatant was removed from the sludge by centrifugation at 6,000 rpm for 10 min, and the sludge was resuspended in the basal medium. The sludge incubations were conducted using 20 ml of this sludge (MLSS, 3,200 mg·l^{-1}; MLVSS, 2,500 mg·l^{-1}) and 35 ml of the basal medium in a 155 ml glass vial that was crimp-sealed with a butyl rubber stopper. Methane (10 ml, 0.4 mmol) was injected into the headspace of each vial, and each vial was incubated while being shaken at 100 rpm in the dark at 30°C. After >90% of the initial CH_4 had been consumed, the vials were opened, flushed with air (500 ml) to remove evolved gas (e.g. CO_2) and to replenish air in the headspaces, and were resealed. Then, methane (10 ml) was reinjected into the headspace. The incubation was continued under the same conditions until approximately 4.5 mmol of CH_4 had been consumed. The assays for methane-dependent

denitrification and the control test (**Table 4.1**) were performed in triplicate incubation runs.

Table 4.1 Experimental set up and incubation conditions

	Carbon source	Headspace	Nitrate	Assay
Run 1	CH_4	CH_4, Air	+	Methane-dependent denitrification in the presence of oxygen
Run 2	CH_4	CH_4, Air	+	Sterilized inoculum
Run 3	CH_4	CH_4, Air	+	No inoculum
Run 4	CH_4	CH_4, Air		Methane consumption in the absence of nitrate
Run 5		Air	+	Nitrate consumption under microaerophilic conditions
Run 6	CH_4	CH_4, Argon	+	Methane-dependent denitrification under anoxic conditions
Run 7		Argon	+	Endogenous denitrification under anoxic conditions
Run 8	CH_3OH	Argon	+	Denitrification with methanol

4.2.3 Incubation for stable-isotope probing (SIP) analysis

Microcosms for SIP analysis were incubated under the same conditions as described above. Two types of sludge incubation were conducted using 20 ml of the sludge (i.e. 50 mg-MLVSS) placed in a 155 ml crimp-sealed glass vial: Run 1-type involved incubation with a nitrate-containing medium; and Run 4-type involved incubation with a nitrate-free medium. The $^{12}CH_4$ or $^{13}CH_4$ (10 ml, 0.4 mmol 99% ^{13}C; Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was injected into the headspace of each vial, and each vial was incubated while being shaken at 100 rpm in the dark at 30°C. The incubation was continued in the same way until approximately 4.5 mmol of CH_4 had been consumed. Samplings for analysis were performed at several points during the experiment: Run 1-type at 0.42 mmol, 1.6 mmol, and 4.1 mmol of methane consumption; and Run 4-type at 0.39 mmol, 1.6 mmol, and 3.9 mmol of methane consumption. At each sampling, a microcosm was sacrificed and the sludge was stored at -80°C prior to DNA extraction.

4.2.4 Analytical Methods

Methane concentrations in the headspaces of each vial were measured using a

GC380 gas chromatograph equipped with a flame ionization detector (GL Science, Inc., Tokyo, Japan). Water samples were filtered through a Whatman GF/C glass microfiber filter (Whatman International, Ltd., Maidstone, UK) and were stored at 4ºC until analysis. Nitrate and nitrite concentrations were measured with an ICS-3000 ion chromatograph (Dionex, Sunnyvale, CA, USA). Ammonium concentrations were measured by colorimetry using the indophenol method (Weatherburn, 1967). Total nitrogen (T-N) and dissolved total nitrogen (DT-N) were measured at the beginning and end of incubation as nitrate-nitrogen by colorimetry using the salicylic acid method (Cataldo *et al*., 1975) after alkaline peroxodisulfate digestion (Ebina *et al.*, 1983). Particulate nitrogen (P-N) was calculated as the difference between T-N and DT-N. Dissolved organic nitrogen (DO-N) was determined by subtracting ammonium-nitrogen, nitrate-nitrogen, and nitrite-nitrogen from DT-N. The contribution of denitrification to DT-N removal was calculated using the following equation.

Denitrification contribution [%] = $[(T\text{-}N_{beginning} - T\text{-}N_{end}) / (DT\text{-}N_{beginning} - DT\text{-}N_{end})] \times 100$

Moreover, the contribution of assimilation to the DT-N removal was calculated using the following equation.

Assimilation contribution [%] = 100 – (Denitrification contribution)

4.2.5 DNA extraction, CsCl density gradient centrifugation, fractionation and PicoGreen assay

Total DNA was extracted from 0.15 g (wet weight) of a sludge pellet using Isoplant (Nippon Gene, Inc., Toyama, Japan) according to the manufacturer's instructions. The DNA was purified using a phenol/chloroform/isoamyl alcohol (25/24/1) solution, and was precipitated by adding ethanol and sodium acetate. DNA (ca. 5 μg) was spun in CsCl gradients with an average density of 1.740 $g \cdot ml^{-1}$. The density was determined with an AR200 digital refractometer (Reichert, Inc., Depew, NY, USA). Centrifugation was conducted at 45,000 rpm and 20°C for >36 h (Lueders *et al*. 2004b). Centrifuged

gradients were fractionated from bottom to top into 12 fractions (ca. 400 µl) by displacement with water by using a syringe pump (Harvard Apparatus, Inc., Holliston, MA, USA) at a flow rate of 800 $\mu l \cdot min^{-1}$. The density of each fraction was determined with an AR200 digital refractometer (Leica Microsystems, Inc., Buffalo, NY, USA). DNA was precipitated from each fraction by adding two volumes of polyethylene glycol at 20°C for 2 h and centrifuging at 15,000 rpm at 4°C for 15 min. DNA pellets were washed once with 70% ethanol and were dissolved in 40 µl TE buffer. Subsequently, the total DNA concentration of each fraction was determined using the PicoGreen dsDNA Assay kit (Molecular Probes, Inc., Eugene, OR, USA).

4.2.6 PCR characterization

The following primer sets were used for PCR amplification: (i) forward primer 8f (Amann *et al*., 1995) and reverse primer 926r (Muyzer *et al*., 1995) for the amplification of 16S rRNA gene fragments, (ii) forward primer A189 and reverse primer A682 (Holmes *et al*., 1995) for the amplification of *pmoA* gene fragments. The PCR mixture contained 0.5 µM concentrations of each primer, 200 µM concentrations of dNTP, 1.5 mM concentrations of $MgCl_2$ for the 16S rRNA gene and *pmoA* gene, 2.5 U of rTaq DNA polymerase (Toyobo, Osaka, Japan), and 5 µl of 10× PCR buffer for rTaq. The PCR amplifications of the 16S rRNA gene and *pmoA* gene were performed in a total volume of 50 µl in 0.2 ml reaction tubes by using a model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following programs: (i) 16S rRNA gene, 3 min at 94°C, 30 cycles (40 sec at 94°C, 40 sec at 56°C, 40 sec at 72°C), and 3 min at 72 °C; (ii) *pmoA* gene, a touchdown PCR program with annealing temperatures decreasing from 62°C to 52°C (decrease by 1°C after 2 cycles), 3 min at 94°C (40 sec at 94°C, 40 sec at 62–52°C, 40 sec at 72°C), 15 cycles (40 sec at 94°C, 40 sec at 52°C, 40 sec at 72°C), and 3 min at 72°C. The presence of PCR products was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide.

4.2.7 Cloning, sequencing, and phylogenetic analysis

PCR products were purified by eluting the bands from 2% agarose gels using a Wizard SV gel and a PCR clean-up system (Promega, Madison, WI, USA). The PCR amplicons were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions. Then, colonies were randomly picked up with a needle and transferred to Insert Check Ready Solution (Toyobo). Clones were sequenced by an ABI PRISM 3100-*Avant* DNA sequencing system (Applied Biosystems) using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The 16S rRNA gene sequences with more than 97% identity were considered to belong to the same operational taxonomic unit (OTU). The *pmoA* gene sequences exhibiting more than 98% identity were considered to belong to the same OTU (Yan *et al*., 2006). A database search was conducted using BLAST from the DDBJ (DNA Data Bank of Japan). Sequences determined in this study and those retrieved from the database were aligned using Clustal W (Thompson *et al*., 1994). Phylogenetic trees were constructed using a neighbor-joining algorithm (Saitou and Nei, 1987).

4.2.8 T-RFLP analyses

T-RFLP analysis of 16S rRNA genes was carried out using the forward primer 8f labeled at the 5′ end with the dye 6-carboxy-fluorescein and the reverse primer 926r. After purification of PCR products with a Wizard SV gel and a PCR clean-up system (Promega), 4 μl of the PCR-products were digested with 10 U of the restriction enzyme *Msp*I (TaKaRa) in the manufacturer's recommended reaction buffers for 4 h at 37°C. The enzyme was subsequently inactivated by incubation at 65°C for 20 min. Aliquots of the digested amplicons were desalted by ethanol precipitation. Desalted digests were suspended in 15 μl of Hi-Di formamide (Applied Biosystems) containing GeneScan-1000 size standard (Applied Biosystems), denatured (5 min at 94°C), cooled

on ice, and resolved by an ABI PRISM 3100-*Avant* Genetic Analyzer automated sequence analyzer (Applied Biosystems) using the GeneScan software (Applied Biosystems).

4.2.9 Nucleotide sequence accession numbers

16S rRNA gene sequences determined in this study were deposited under accession numbers AB280265–AB280414. *PmoA* sequences determined in this study were deposited under accession numbers AB280415–AB280427.

4.3 Results

4.3.1 Consumption of methane and nitrate

The consumption of methane was greatly affected by supplementation with nitrate (**Fig. 1-a**). In Run 1, a sludge with nitrate supplementation consumed >90% of the initial concentration of methane after 54 h of incubation. In contrast, in Run 4, a sludge without nitrate supplementation took 113 h of incubation to consume >90% of the initial CH_4. The addition of nitrate resulted in differences in the incubation time it took for approximately 4.5 mmol of methane to be consumed: in Run 1, it took 134 h, whereas in Run 4, it took 415 h. In addition, consumption of methane was never observed under anoxic conditions during experimental periods. This might be due to the extremely slow growth of a microbial consortium exhibiting anoxic methane oxidation (AOM) coupled to denitrification (Raghoebarsing *et al.*, 2006) or to the absence of such a consortium in the sludge. Nitrate concentrations obviously decreased with consumption of methane in Run 1 (**Fig. 1-b**). In addition, decrease of nitrate was not observed in the absence of external carbon sources (Run 5). In Runs 6 and 7, the decrease of nitrate may have been due to denitrification with organic substrates produced by self-digestion of the sludge or endogenous denitrification. In the assay with methanol as the carbon source, the sludge exhibited the strongest denitrification activity (Run 8).

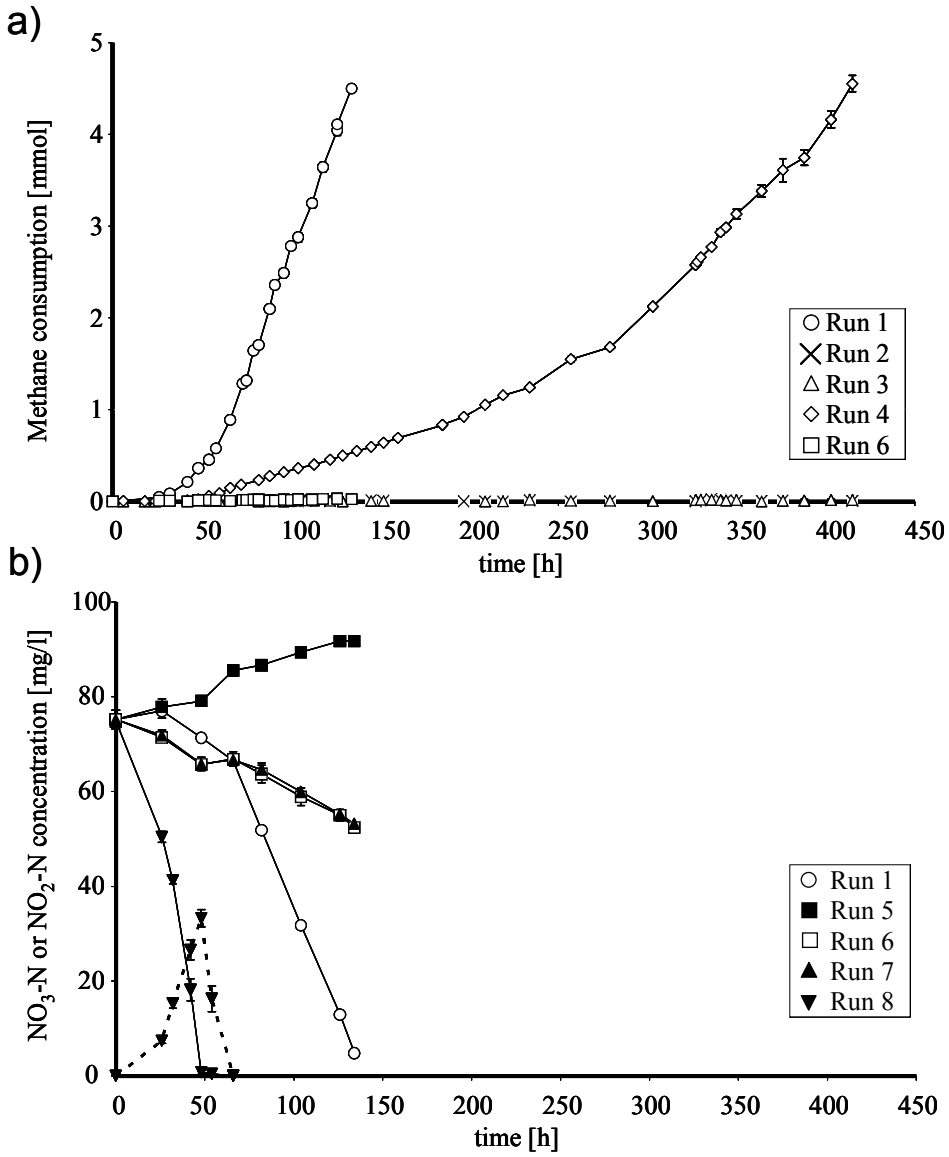

Fig. 4.1. Time course of consumption of methane (a) and NO_3-N and NO_2-N (b). In a panel (b), NO_3-N is shown by the solid line and NO_2-N is shown by the broken line; NO_2-N was detected only in Run 8. Some of the error bars, which indicate standard deviations (n=3), are smaller than the symbols.

4.3.2 Nitrogen balance

Nitrogen balance at the beginning and at the end of incubation is shown in **Fig. 4.2**. When the vials were incubated with methane as the external carbon source (Run 1), total nitrogen (T-N) concentrations in the medium decreased more than in incubated vials that were free of external carbon (Run 5). This suggested that the sludge in Run 1 exhibited methane-dependent denitrification (MDD) activity. However, the T-N removal rates by MDD (22.1 ± 2.5%; n = 3) were less than that by denitrification with methanol as a carbon source (52.8 ± 1.5%; n = 3). These differences might be attributed to the different fates of depleted nitrate in each of the incubations. Denitrification with methane contributed 40.1 ± 3.1% (n = 3) to the dissolved total nitrogen (DT-N) removal from the medium, whereas 59.9 ± 3.1% (n = 3) of the removed DT-N accumulated in the sludge (Run 1). This suggests that most of the nitrate in the medium was assimilated for bacterial growth in MDD. On the other hand, denitrification with methanol contributed 87.5 ± 5.4% (n = 3) to the dissolved nitrogen (DT-N) removal from the medium, whereas 12.5 ± 5.4% (n = 3) of the removed DT-N was accumulated as biomass in the sludge (Run 8).

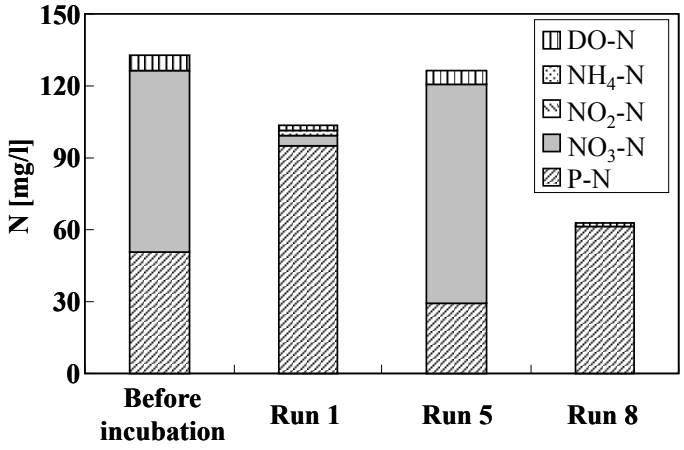

Fig. 4.2. Nitrogen balance at beginning and end of incubation. Particulate nitrogen (P-N) was calculated as the difference between total nitrogen and dissolved total nitrogen (DT-N). Dissolved organic nitrogen (DO-N) was calculated from subtraction of ammonium-nitrogen, nitrate-nitrogen, and nitrite-nitrogen from DT-N.

4.3.3 CsCl density gradient centrifugation for separation of ^{12}C-DNA and ^{13}C-DNA

Characterization for separation of ^{12}C-DNA and ^{13}C-DNA was carried out by fluorometric determination of total DNA of each fraction ranging from 1.69 to 1.78 g·ml^{-1} CsCl buoyant density. The fractionation using an isopycnic centrifugation is the most important step in SIP analysis. Therefore, we demonstrated successful separation of ^{12}C-DNA and ^{13}C-DNA extracted from pure cultures of *Hyphomicrobium denitrificans* (**Fig. 4.3-a**). Furthermore, we also showed the profiles of the incubation controls with $^{12}CH_4$ (4.1 mmol consumption) (broken lines in **Figs. 4.3-b** and **4.3-c**). We analyzed samples at several points during the experiment: Run 1 at 0.42 mmol, 1.6 mmol, and 4.1 mmol; and Run 4 at 0.39 mmol, 1.6 mmol, and 3.9 mmol. Although the position of DNA extracted from sludge consuming the initial CH_4 was almost the same as that of the incubation controls with $^{12}CH_4$, DNA peaks were steadily shifted to the "heavy" density fraction with the consumption of $^{13}CH_4$ (**Figs. 4.3-b** and **4.3-c**).

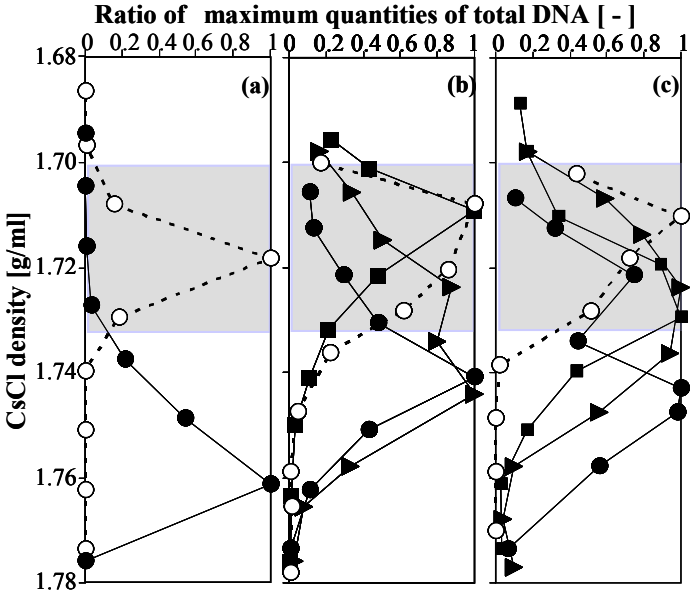

Fig. 4.3. Quantitative profiles of CsCl density gradient fractions by PicoGreen assays. (a) Quantitative distribution of total DNA extracted from fully ^{13}C-labeled (●) and unlabeled *H. denitrificans* (○). (b) Quantitative distribution of total DNA extracted from $^{13}CH_4$- or $^{12}CH_4$-incubated sludge samples at different consumption amounts of methane (0.42 mmol of $^{13}CH_4$, ■; 1.6 mmol of $^{13}CH_4$, ▲; 4.1 mmol of $^{13}CH_4$, ●; 4.1 mmol of $^{12}CH_4$, ○) in Run 1. (c) Quantitative distribution of total DNA extracted from $^{13}CH_4$-incubated sludge samples at different consumption amounts of methane (0.39 mmol of $^{13}CH_4$, ■; 1.6 mmol of $^{13}CH_4$, ▲; 3.9 mmol of $^{13}CH_4$, ●; 4.1 mmol of $^{12}CH_4$, ○) in Run 4. The density-range characteristics for "light" DNA are shaded in gray.

4.3.4 T-RFLP fingerprinting of bacterial populations in gradient fractions

T-RFLP fingerprinting of bacterial populations was performed from gradient fractions at three different points in time of $^{13}CH_4$ consumption. For early points in time of $^{13}CH_4$ consumption in Run 1 (0.42 mmol of $^{13}CH_4$), T-RFLP profiles for three "light" fractions and one "heavy" fraction are shown in **Fig. 4.4-a**, because only one T-RFLP profile was obtained from the "heavy" fractions (1.741 g·ml^{-1}). For other points in time, T-RFLP profiles for two "light" and two "heavy" fractions are shown in **Figs. 4.4-a and 4.4-b**. At an early point in time in Run 1, some characteristic T-RFs (i.e. 135, 141, 436, 437, 439, 455, 487 and 490 bp) were detected from the "heavy" fraction (1.741 g·ml^{-1}), indicating key bacterial populations involved in MDD in sewage sludge. T-RFLP profiles also showed that the diversity of T-RFs in this "heavy" fraction was clearly reduced in comparison to those of the "light" fractions. After 1.6 mmol of $^{13}CH_4$ consumption in Run 1, the buoyant density of the 135, 141, 437, 439 and 455 bp T-RFs further increased to 1.758 g·ml^{-1} with the increase of $^{13}CH_4$ consumption. After 4.1 mmol of $^{13}CH_4$ consumption in Run 1, some additional characteristic T-RFs (e.g. 529 and 541 bp) other than the above-mentioned T-RFs, were contained in the "heavy" fractions (1.741 and 1.751 g·ml^{-1}). In T-RFLP analysis of sludge samples consuming $^{13}CH_4$ under nitrate-free conditions (Run 4), two major T-RFs (436 bp and 487 bp) and some minor T-RFs (e.g. 135, 141 and 152 bp) were detected in the "heavy" fraction (1.740 g·ml^{-1}) at an early time point (0.39 mmol of $^{13}CH_4$ consumption). T-RFLP profiles of the "heavy" fractions changed dramatically due to the further ^{13}C labeling in Run 4, which resulted in many T-RFs (e.g. 114, 430 and 455 bp) in the "heavy" fractions. At the point in time of approximately 4 mmol of $^{13}CH_4$ consumption, T-RFLP profiles of the "heavy" fraction in Run 1 (1.751 g·ml^{-1}) and Run 4 (1.748 g·ml^{-1}) were quite different. Additionally, it was evident that the above-mentioned characteristic T-RFs shifted to "heavy" fractions in comparison with fingerprints generated as $^{12}CH_4$ incubation controls (**Figs. 4.4-c** and **4.4-d**).

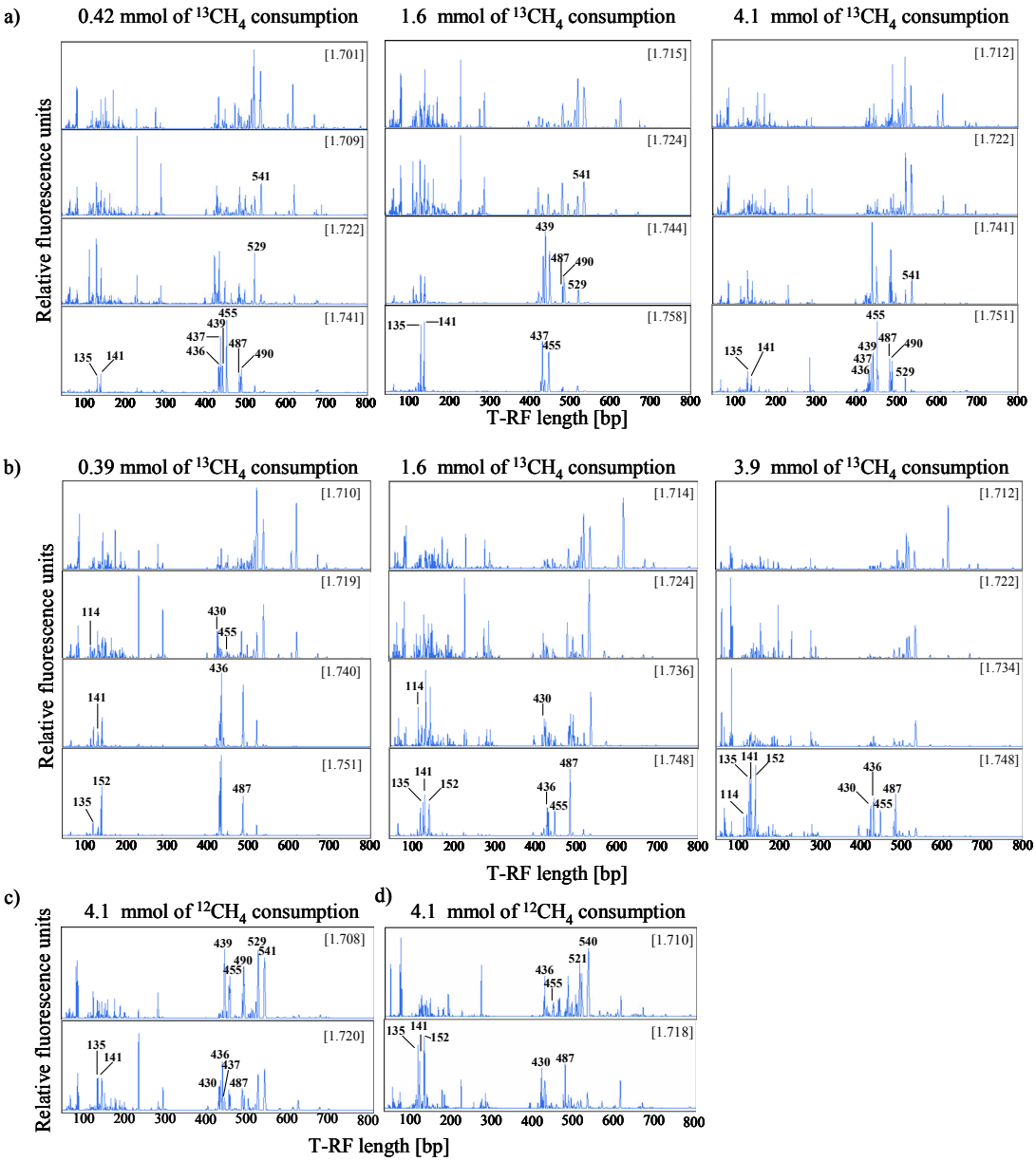

Fig. 4.4. T-RFLP profiles of density-fractionated bacterial communities. T-RFLP profiles were generated from selected "light" and "heavy" DNA gradient fractions at three different points in time in each ^{13}C-incubation run: (a) is Run 1, (b) is Run 4. T-RFLP profiles of ^{12}C-incubation controls in each run are also shown: (c) is Run 1; (d) is Run 4. Amplicons with 8f-FAM/926r primers and digested with *MspI*. CsCl buoyant densities ($g \cdot ml^{-1}$) of fractions are given in brackets. Sizes of important T-RFs (bp) are given.

4.3.5 Phylogenetic analysis of bacterial populations and assignment to T-RFs

After approximately 4 mmol of $^{13}CH_4$ consumption, 16S rRNA gene clone libraries (denoted R1.13 and R4.13, respectively) were generated from the "heavy" fraction (Run 1, 1.751 g·ml^{-1}; Run 4, 1.758 g·ml^{-1}) in order to identify the likely key players in each incubation and to assign phylogenetic groups to distinct T-RFs. Additionally, the 16S rRNA gene clone library (denoted R1) was also constructed from DNA of Run 1 (4.1 mmol $^{13}CH_4$ consumption) before isopycnic centrifugation. The phylogenetic affiliations of sequenced clones and their affiliations with distinct T-RFs are summarized in **Table 4.3**.

Table 4.3. Phylogenetic affiliations and numbers of 16S rRNA gene clones.[a]

	R1[b]	R1.13[c]		R4.13[c]	
Phylogenetic group	No. of clones	No. of clones	T-RF length (bp)[d]	No. of clones	T-RF length (bp)[d]
Alphaproteobacteria	9	5	150, 152, **403**	13	**79**, **113**, **132**, **152***, 403, 441
Hyphomicrobiaceae	4	24	**436**, **437**, **439**	2	436*, 439
Methylocystaceae (type II MOB)	1	1	151	5	**150**, **152***
Betaproteobacteria	2			11	**140, 141***, **430***, 488,
Comamonadaceae	3	4	**139**	2	139*, 490*
Methylophilaceae	1	2	490, 493	3	**114**
Rhodocyclaceae	3	4	**529**	1	370
Gammaproteobacteria	4	6	**123,** 224**,** 238, **487***	10	87, 193, **240**, 436*, 490*
Methylococcaceae (type I & X MOB	1	28	**135, 141**, 444, **455**, **487***	19	**135**, 141*, 144, **444**, 455, 456, **487**
Deltaproteobacteria, *Myxococcales*	4	5	77, **78**, 491,	6	**78**, 151, 152*, 506
Acidobacteria	1			5	**171**, 201, **285**
Actinobacteria	2				
Bacteroidetes	12			9	**91**, 93, **181, 541**, 542
Chloroflexi	6			4	147, 164, 512, 518
Cyanobacteria	3	2	32	1	495
Gemmatimonadetes	1	3	130, **129**	2	77, 131
Nitrospirae	1				
Planctomycetes				3	207, 268, 291*
Verrucomicrobia	1				
Candidate division OP11				1	166
Candidate division Termite group 1				2	**291**
Unidentified bacteria	3				

[a]Characteristic T-RFs for different clone groups are given.
[b]Clone library "R1" was constructed from DNA of Run 1 without CsCl density gradient centrifugation.
[c]Clone libraries "R1.13" and "R4.13" were constructed from ^{13}C-DNA separated by CsCl gradient centrifugation.
[d]Terminal restriction fragment length of each clone are shown in base pairs. T-RFs detected for more than one clone within one phylogenetic group are indicated in boldface. T-RFs detected in more than one phylogenetic group are marked with an asterisk (*).

In contrast to the clone library R1 (52 OTUs; *n* = 62), the clone library R1.13 (33 OTUs; *n* = 84) had a completely different bacterial composition and diversity. The clone library R1.13 showed a clear predominance of sequences (65% of all clones) related to the *Gammaproteobacterial* methanotrophs (types I and X methanotrophs) and to *Alphaproteobacterial* methylotrophic denitrifiers. Methanotroph-related and Methylotroph-related sequences are shown in **Fig. 4.5**. Thirty-three percent of all clones were related to the *Methylococcaceae* of the *Gammaproteobacteria* (e.g. T-RF of 135, 141, 455, and 487 bp). *Methylococcaceae* are obligate methanotrophic bacteria that have the ability to utilize methane as the sole carbon and energy source. Of these clones, 20 clones were affiliated with the novel uncultured type X methanotrophs related to the *Methylocaldum*. Only one clone was closely related to the 16S rRNA sequence of *Methylosinus* (T-RF of 151 bp), which is known as one of the *Alphaproteobacterial* methanotrophs (type II methanotrophs). A further 28% of all clones clustered in the *Hyphomicrobiaceae* of the *Alphaproteobacteria* (T-RF of 436, 437, and 439 bp), which are capable to utilizing one-carbon compounds (e.g. CH_3OH) except for methane. Two of the clones were related to the *Methylophilaceae* of the *Betaproteobacteria* (T-RF of 490 and 493 bp), known as the methylotrophs. Also, some sequences of non-methanotrophs and non-methylotrophs (e.g. *Comamonadaceae*, *Rhodocyclaceae*) were detected within this library (34%).

In comparison with the clone library R1.13, the clone library R4.13 (65 OTUs; *n* = 99) showed more diverse bacterial populations. Most of the clone sequences (73%) were related to sequences other than those of methanotrophs and methylotrophs. However, predominant clone sequences (19%) were related to the *Methylococcaceae* of the *Gammaproteobacteria* (e.g. T-RF of 135, 141, 455 and 487 bp) as well as the clone library R1.13. Furthermore, some sequences detected in the clone library R4.13 were related to other methanotrophs and methylotrophs, i.e. *Methylocystaceae* (5% of all clones; T-RF of 150 and 152 bp), *Hyphomicrobiaceae* (2% of all clones; T-RF of 436

and 439 bp) and *Methylophilaceae* (3% of all clones; T-RF of 114 bp).

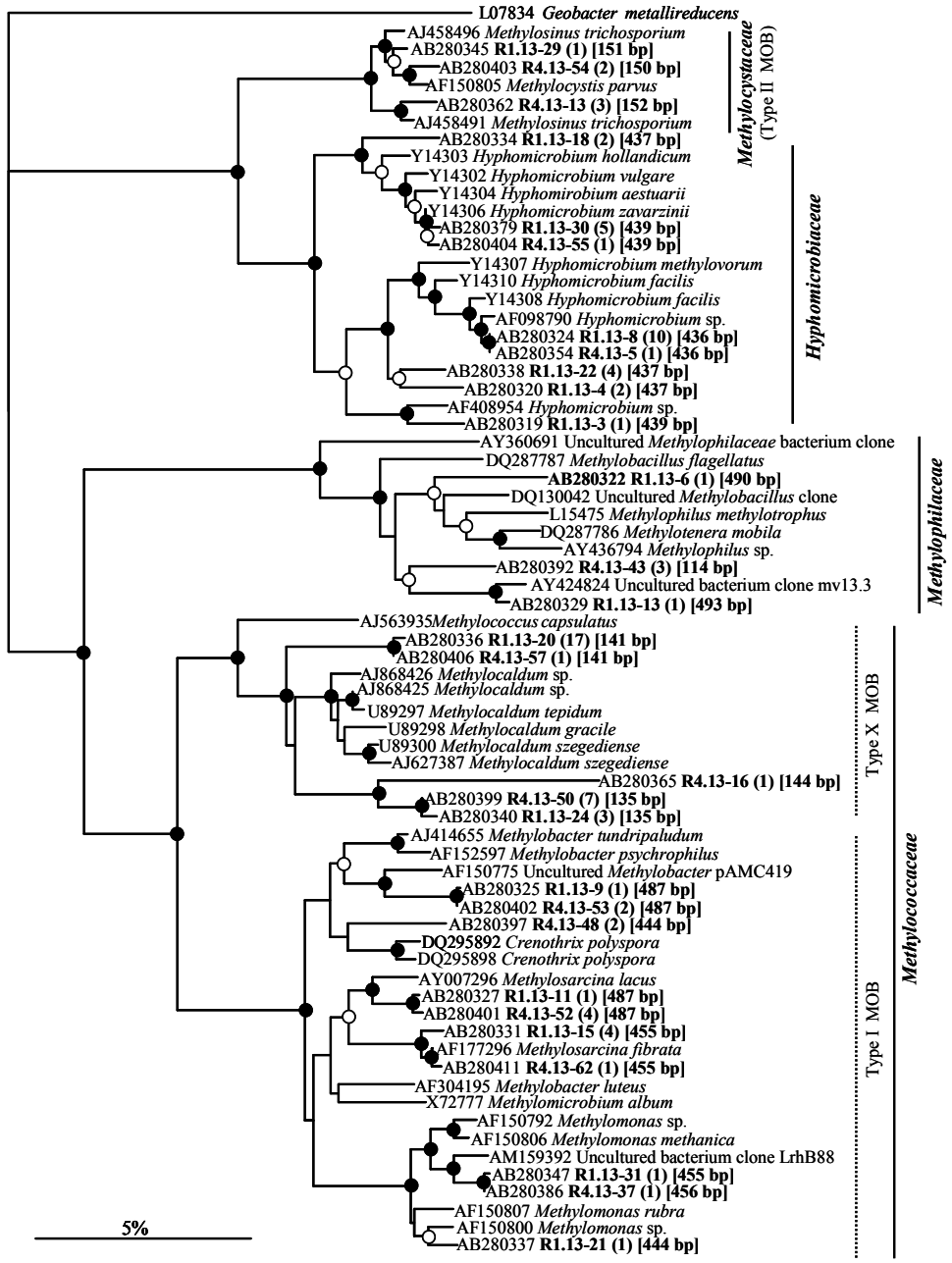

Fig. 4.5. Phylogenetic affiliation of clones derived from methanotrophs and methylotrophs by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from the ^{13}C-DNA fraction in Run 1 and ^{13}C-DNA fraction in Run 4 are labeled "R1.13-", and "R4.13-", respectively. The number of clones assigned to each sequenced OTU with greater than 97% identity is shown in parentheses. *Geobacter metallireducens* (accession no. L07834) is used as the outgroup. Closed circles (i.e., bootstrap values, >75% derived from 1000 replicates) and open circles (i.e., bootstrap values, 50% to 75% derived from 1000 replicates) are indicated at branch points. The scale bar represents 5% sequence divergence.

4.3.6 Identification of active methanotrophs based on *pmoA* gene clone library

Our results show that supplementation with nitrate affected the rate of methane consumption by the sludge (**Fig. 4.1-a**). To characterize how the methanotrophic populations in the sludge were affected by the supplementation with nitrate, *pmoA* gene clone libraries were constructed from the same DNA templates as the 16S rRNA gene clone libraries: DNA template before isopycnic centrifugation of Run 1 (denoted "R1.PmoA", 44 clones), the "heavy" gradient fraction from Run 1 (denoted "R1.13.PmoA", 46 clones), and the "heavy" gradient fraction from Run 4 (denoted "R4.13.PmoA", 45 clones). The cloned sequence types with more than 98% identity were considered to belong to the same OTU (Yan *et al.*, 2006). Of these clones, 3 OTUs were obtained from the "R1.PmoA" library, 1 OTU was obtained from the "R1.13.PmoA" library, and 9 OTUs were obtained from the "R4.13.PmoA" library. The amino acid sequences of each OTU were divided into six clusters, and the phylogenetic analysis of three *pmoA* clone libraries revealed that the methanotrophic populations were strongly affected by the supplementation with nitrate (**Fig. 4.6**).

Four OTUs from the "R4.13.PmoA" library belonged to cluster I, which were closely related to the *pmoA* of type II methanotrophs (*Methylocystis* and *Methylosinus*). The OTU (R4.13.PmoA-3) had 98.2% similarity with the *pmoA* of *Methylocystis* sp. (AJ489800), two OTUs (R4.13.PmoA-7 and R4.13.PmoA-9) had 98–100% similarity with the *pmoA* of *Methylocystis parvus* (AJ459042), and the OTU (R4.13.PmoA-6) had 98.9% similarity with the *pmoA* of an uncultured type II methanotrophic bacterium (AY355393). In addition, the *pmoA* of type I methanotrophs might belong to cluster II. In cluster II, two OTUs (R1.PmoA-1 and R1.PmoA-3) had 96.4–99.4% similarity with the *pmoA* of *Methylomonas* sp. (AF150801). The *pmoA* of type X methanotrophs might belong to clusters III to VI. In cluster III, the OTUs (R4.13.PmoA-8) had 96% similarity with the *pmoA* of an uncultured methanotrophic bacterium (AB222915). In cluster IV, the OTUs (R4.13.PmoA-4) had 91% similarity with the *pmoA* of an uncultured

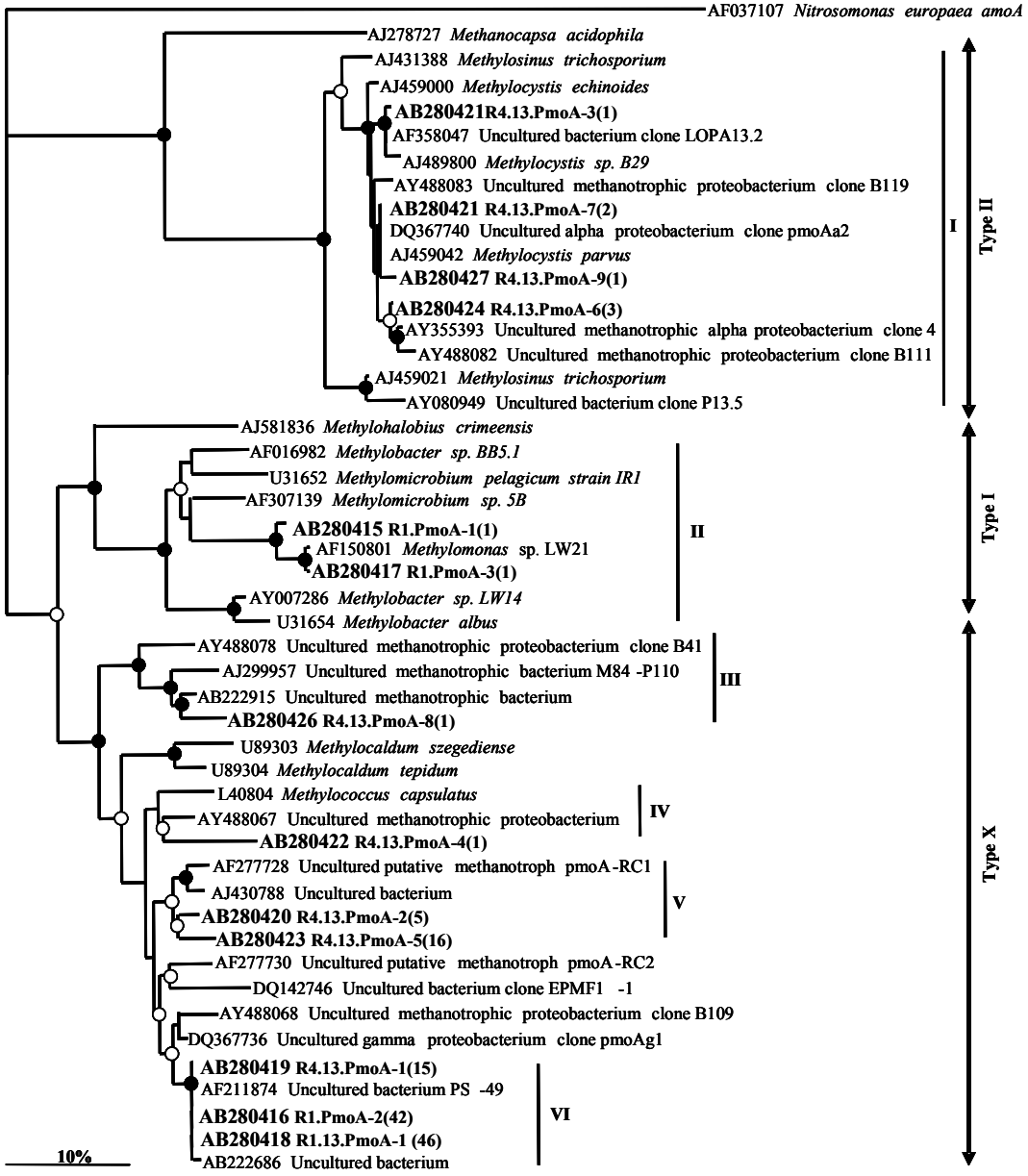

Fig. 4.6. Neighbor-joining analysis of partial *pmoA* gene products (177 amino acids) from each clone library. The partial *pmoA* sequences obtained from non-SIP treated DNA sample and ^{13}C-DNA fraction in Run 1 and ^{13}C-DNA fraction in Run 4 are labeled "R1.PmoA-", "R1.13.PmoA-", and "R4.13.PmoA-", respectively. The number of clones assigned to each sequenced OTU with greater than 98% identity is shown in parentheses. The *amoA* from *Nitrosomonas europaea* (accession no. AF037107) is used as the outgroup. Closed circles (i.e., bootstrap values, >75% derived from 1000 replicates) and open circles (i.e., bootstrap values, 50% to 75% derived from 1000 replicates) are indicated at branch points. The scale bar = 10% amino acid substitution.

methanotrophic bacterium (AY488067) and 88.7% similarity with the *pmoA* of *Methylococcus capsulatus* (L40804). Cluster V consisted of two major OTUs in the "R4.13.PmoA" library. These OTUs (R4.13.PmoA-2 and R4.13.PmoA-5) had 93.3–96.3% similarity with the *pmoA* of an uncultured methanotrophic bacterium (AJ430788). Cluster VI consisted of one OTU in the "R1.PmoA" library and one OTU in the "R1.13.PmoA" library and one OTU in the "R4.13.PmoA" library. The clones of Cluster VI (R1.PmoA-2, R1.13.PmoA-1 and R4.13.PmoA-1) were most abundant in all clone libraries, and they were closely related to the *pmoA* of an uncultured bacterium (AF211874).

4.4 Discussion

In this study, DNA stable-isotope probing (DNA-SIP) was used to identify the active microorganisms in wastewater that were involved in methane-dependent denitrification (MDD) in the presence of oxygen. The clone library R1.13, which was generated from a "heavy" fraction after approximately 4 mmol of $^{13}CH_4$ consumption, contained a high number of clones related to the *Methylococcaceae* and *Hyphomicrobiaceae*. Additionally, clones other than those of these bacteria were also present in a "heavy" fraction (e.g. *Betaproteobacteria*). This was probably due to the prolonged supply of $^{13}CH_4$, i.e. heavy ^{13}C labeling led to enrichment and selection of certain bacterial populations. Thus, time-course T-RFLP analysis combined with DNA-SIP was conducted in order to obtain information on key bacteria at early points in time. T-RFLP profiles of a "heavy" fraction at an early point in time showed the predominance of the T-RFs derived from methanotrophs (135, 141, 455 and 487 bp) and methylotrophs (i.e. 436, 437 and 439 bp), which supported the idea that the *Methylococcaceae* and *Hyphomicrobiaceae* are key bacterial populations in the MDD ecosystem in this study.

DNA-SIP results showed an association of methanotrophs (i.e. *Methylococcaceae*) and methylotrophs (e.g. *Hyphomicrobiaceae* and *Methylophilaceae*) in the MDD ecosystem. The high degree of ^{13}C labeling of methylotrophs was due to indirect labeling effects via one-carbon organic metabolites from methanotrophs. Although it is difficult to identify key substrates for this trophic link from only the ecological data of DNA-SIP, we would like to suggest the following. In the metabolism of methanotrophs, possible one-carbon organic metabolites are methanol, formaldehyde, and formate (Hanson and Hanson, 1996). Methanol is formed from methane by methane monooxygenases when methanotrophs utilize NADH as a reducing agent. Then, methanol is further oxidized to formaldehyde, which plays a central role as an intermediate in assimilation for biosynthesis of cell materials and in dissimilation.

Formaldehyde is oxidized sequentially to formate and carbon dioxide, when most of the reducing power (i.e. NADH) required for the metabolism of methane is produced. From the point of view of energetics in methanotrophs, the oxidation of methanol is an important step in the production of formaldehyde to gain reducing power. Therefore, formate might be a key substrate for trophic links in MDD ecosystems, because it is implausible that these compounds were transferred from methanotrophs to methylotrophs. However, it is known that methanol and formaldehyde accumulate in methanotrophic cultures (Hanson and Hanson, 1996). Therefore, we cannot rule out the possibility that methanol and formaldehyde are key substrates involved in trophic interactions of methanotrophs and methylotrophs in MDD ecosystems. Nevertheless, the identities of key substrates for trophic links must be confirmed by experiments using pure cultures and co-cultures of methanotrophs and methylotrophs.

Nitrogen balance in Run 1 showed the occurrence of MDD in the presence of oxygen, i.e. 40% of the initial dissolved total nitrogen, mostly nitrate-nitrogen, was removed from the medium in some gas form. Known methanotrophs have not been observed to perform denitrification, although some of them have functional genes involved in denitrification (Ye and Thomas, 2000). This, in turn, suggests that denitrification in MDD ecosystems may be carried out by some of the ^{13}C-labeled bacteria other than the methanotrophs. The major phylotype within non-methanotrophs was related to the *Hyphomicrobiaceae* (T-RF of 436, 437 and 439 bp), which are restricted facultative methylotrophs and grow using one-carbon compounds such as methanol and formate as a carbon source and oxygen or nitrate/nitrite as the terminal electron acceptor. To date, some members of the *Hyphomicrobiaceae* have been identified as methylotrophic denitrifiers in sewage sludge (Holm *et al*., 1996; Osaka *et al*., 2006). Therefore, our results indicate that these bacteria may play a major role in denitrification in MDD ecosystems. Additionally, the uncultured *Betaproteobacteria* (e.g. *Methylophilaceae*, *Rhodocyclaceae*) were also detected in ^{13}C clone library

profiles and T-RFLP profiles in “heavy” fractions. In some previous studies, these bacteria were shown to be important denitrifying bacterial populations in wastewater treatment systems (Ginige *et al*., 2005; Osaka *et al*., 2006; Wagner and Loy, 2002). Therefore, these *Betaproteobacteria* are also candidate denitrifiers in MDD ecosystems. However, we cannot rule out that these ^{13}C-labeled non-methanotrophs may have assimilated intermediates aerobically, because denitrifiers are usually facultative anaerobes. For example, isolates of the *Methylophilaceae* are known to be unable to perform denitrification, i.e. methylotrophic aerobes (Jenkins *et al*., 1984; Urakami and Komagata, 1986), although the uncultured *Methylophilaceae* have been identified as important methylotrophic denitrifiers in sewage sludge in other recent SIP studies (Ginige *et al*., 2004; Osaka *et al*., 2006).

It has long been believed that denitrifying activity and enzyme synthesis are completely suppressed by oxygen because denitrifiers normally gain energy by oxygen-dependent respiration under aerobic conditions and conduct denitrifying metabolism only when in oxygen-depleted environments (Knowles, 1982; Zumft, 1998). Thus, it is conceivable that denitrification was suppressed under our experimental conditions, i.e. the initial oxygen concentrations (18% v/v) of 90% air saturation in headspaces. Although the oxygen in the vials was mostly consumed to oxidize methane (10% v/v) by the methanotrophs, microorganisms are required to perform denitrification in the presence of oxygen. Nevertheless, previous studies on MDD (Amaral *et al.*, 1995; Costa *et al.*, 2000; Knowles, 2005; Thalasso *et al.*, 1997; Waki *et al.*, 2002; Werner and Kayser, 1991) have shown evidence of methane-dependent denitrification in the presence of oxygen. We will now discuss possible explanations for denitrification in the presence of oxygen. First, the spatial arrangement of methanotrophs and denitrifiers may be due to a dissolved oxygen gradient in sludge flocs (Schramm *et al*., 1999; Rittmann and Langeland, 1985), which has also been reported in sludge in which there is co-occurring methanotrophy and denitrification (Waki *et al*., 2002). Another

possibility is that aerobic denitrification was conducted by certain species of bacteria capable of co-respiring oxygen and nitrogen oxides and producing N_2 (Meiberg *et al*., 1980; Patureau *et al*., 1994; Robertson and Kuenen, 1984). In contrast to common denitrifiers, aerobic denitrifiers appear to have an ecological advantage in niches with frequent aerobic/anoxic shifts (Frette *et al*., 1997). Additionally, it has been suggested that some aerobic denitrifiers prefer one-carbon sources such as methanol and formate (Takaya *et al*., 2003; Zhao *et al*., 1999). In fact, aerobic denitrification is observed in some methylotrophs, such as *Hyphomicrobium denitrificans* (Meiberg *et al*., 1980) and *Paracoccus denitrificans* (Robertson and Kuenen, 1984). Furthermore, Knowles (2005) showed that some methanotrophs produce gaseous nitrogen oxides (e.g. NO and N_2O) during growth on nitrate when oxygen is depleted through methane oxidation. Therefore, MDD ecosystems might exhibit these phenomena.

Our results show that supplementation with nitrate had strong effects on methane consumption (**Fig. 4.1-a**) and methanotroph community structure (**Fig. 4.5** and **Fig. 4.6**). The *pmoA* phylogeny is generally consistent with the 16S rRNA phylogeny of methanotrophs because it is said that there is no evidence of horizontal gene transfer of *pmoA* among methanotrophic bacteria (Costello and Lidstrom, 1999; Murrell *et al*., 1998). Thus, six clusters derived from *pmoA* clones obtained in this study were classified in three different groups (i.e. types I, II, and X) according to the phylogenetic indications of *pmoA* genes and 16S rRNA genes. The phylogenetic analyses showed that types I and X methanotrophs were stimulated by supplementation with nitrate, whereas type II methanotrophs were minor components of the population in the presence of nitrate (Run 1). On the other hand, a variety of methanotrophs, including type II methanotrophs, took up methane in the absence of nitrate (Run 4). Mohanty *et al.* (2006) demonstrated that nitrogenous fertilizers stimulated methane consumption and the growth of type I methanotrophs, whereas type II methanotrophs were generally inhibited. Other previous studies describe type I methanotrophs as tending to dominate

in eutrophic lakes, whereas type II methanotrophs tend to dominate in oligotrophic lakes (Hanson and Wattenberg, 1991; Saralov, *et al*., 1984). Growth of the type II methanotrophs was favored under nitrogen-limiting conditions due to the ability to fix nitrogen, although type X methanotrophs (e.g. *Methylococcus capsulatus*) are also able to fix nitrogen (Hanson and Hanson, 1996). Therefore, it was suggested that type I methanotrophs tend to dominate under nutrient-rich conditions that allow rapid growth, whereas type II methanotrophs tend to be abundant under nutrient-poor conditions that limit growth. It should be noted, however, that the types of methanotrophs in each habitat are not only determined by nutrient conditions, but also by various other factors, such as the concentrations of methane and oxygen (Amaral *et al*., 1995; Henckel *et al*., 2000) and the temperature (Sundh *et al*., 2005). However, there were discrepancies in the results for the 16S rRNA genes and the *pmoA* genes; i.e. there were high frequencies of putative *pmoA* sequences derived from type X methanotrophs. This might be indicative of small sample size of the clone, PCR bias or cloning bias (Bodrossy *et al*., 2003; Cebron *et al*., 2007; Horz *et al*., 2001). Methanotrophic populations should also be investigated by using PCR-independent methods (e.g. FISH analysis and PLFA analysis).

In addition, we detected bacterial groups with unique functions (*Myxococcales* of the *Deltaproteobacteria*). This order of bacteria contains groups described as "micropredators", which exhibit gliding motility and are able to degrade other organisms (e.g. bacteria, yeast) by means of a variety of hydrolytic exoenzymes, such as proteases, lipases, and cell wall lytic enzymes (Shimkets *et al*., 2005). In recent studies, the members of the *Myxococcales* have been found to play a significant role in the turnover of biomass carbon in soil (Lueders *et al*., 2006; Reichenbach, 1999). Therefore, it has been suggested that these bacteria incorporate ^{13}C by degrading active or dead ^{13}C-labeled cells (e.g. methanotrophs) in sludge exhibiting MDD activity, although it is not possible to determine whether there is gliding motility merely from

16S rRNA sequence similarity. These indirect labelings of microorganisms other than methanotrophs and methylotrophs could be due to heavy ^{13}C labeling of sludge. Nevertheless, DNA-SIP was superior to analysis without SIP treatment in Run 1, and thus, it was difficult to identify the key players in the MDD ecosystem from the clone library R1 of DNA samples before isopycnic centrifugation. On the other hand, the clone library R4.13 consisted of diverse taxonomic bacterial groups. In spite of similar levels of methane consumption, there were significant differences in the diversity levels between the clone libraries R1.13 and R4.13. This might not be due so much to the supplementation of nitrate as to differences in incubation time: Run 1, 134 h; Run 4, 415 h. Therefore, most bacteria may have been labeled due to the heavy labeling process and the long incubations.

4.5 Conclusions

Our results show the occurrence of methane-dependent denitrification (MDD) in the presence of oxygen. In MDD ecosystems, nitrate consumption is attributed not only to nitrogen assimilation for growth, but also to denitrification. However, it was also found that 60% of the initial DT-N accumulates as biomass in sludge, which is attributed to bacterial growth. This suggests that the assimilation of nitrate for the growth of active bacteria (methane-dependent assimilation: MDA) is a primary pathway of nitrate-depletion in this experiment. Thus, MDA may be thought of not as removing nitrogen from wastewater, but rather as moving nitrogen from the main treatment to the sludge treatment. Furthermore, DNA-SIP reveals that MDD requires an association of *Gammaproteobactrial* methanotrophs (i.e. the family *Methylococcaceae*) and methylotrophic denitrifiers (i.e. the genus *Hyphomicrobium*). Advances in understanding the metabolomics of methanotrophic bacteria under various conditions and further research into the determinants of methanotroph community structure will significantly improve the characterization of trophic links in MDD ecosystems.

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