Chapter 2

Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing

Summary

In this study, stable-isotope probing (SIP) analysis was applied to identify the acetate- or methanol-assimilating bacteria under nitrate-reducing conditions in activated sludge. A sludge sample obtained from wastewater treatment systems was incubated in a denitrifying batch reactor fed with synthetic wastewater containing [^{13}C]-acetate or [^{13}C]–methanol as the main carbon source and nitrate as the electron acceptor. We evaluated how growth of bacterial populations was stimulated by acetate or methanol as the external carbon source in nitrogen removal systems. Most of the acetate- or methanol-assimilating bacteria identified by SIP analysis have been known as denitrifiers in wastewater treatment systems. In the case of using acetate as the carbon source, 16S rRNA gene sequences retrieved from [^{13}C]-DNA were closely related to the 16S rRNA genes of Comamonadaceae (e.g. Comamonas and Acidovorax) and Rhodocyclaceae (e.g. Thauera and Dechloromonas) of Betaproteobacteria, and Rhodobacteraceae (e.g. Paracoccus and Rhodobacter) of Alphaproteobacteria. In the case of using methanol as the carbon source, 16S rRNA gene sequences retrieved from [^{13}C]-DNA were affiliated with Methylophilaceae (e.g. Methylophilus, Methylobacillus, and Aminomonas) and Hyphomicrobiaceae. Rarefaction curves for clones retrieved from [^{13}C]-DNA showed that the diversity levels for methanol-assimilating bacteria were considerably lower than those for acetate–assimilating bacteria.

2.1 Introduction

Biological nitrate and/or nitrite removal from wastewater is achieved by denitrification, which involves the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas (Zumft, 1997). Denitrification requires an oxidizing nitrogen compound as electron acceptor and a carbon source. 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, and methanol, are added to achieve a satisfactory degree of denitrification. Methanol has been often selected because of its relatively low cost and the small sludge production compared with other organic compounds, although only some bacteria can utilize methanol as the carbon source (Nyberg *et al*., 1992).

Conventional analysis of microbial communities in wastewater treatment systems depends on culture-dependent methods. However, a limitation of the culture-dependent method is that it is possible that only a fraction on the bacteria is detected due to cultivation bias. Thus, in recent years, molecular biological analysis has been applied to exploring microbial ecology in wastewater treatment systems regarded as a “black box”. For example, the 16S rRNA gene-based approach is the most widely used technique for organism identification and community analysis because it provides phylogenetic information based on a large database of sequence information.

Recently, molecular techniques using radio isotopes or stable isotopes in biomarkers have been developed and applied to identifing active microbial populations. The combination of microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) is used to simultaneously determine the phylogenetic composition and *in situ* substrate uptake patterns of complex microbial populations in wastewater treatment systems (Andreasen and Nielsen, 1997; Ito *et al*., 2002; Lee *et al*., 1999). Radajewski *et al.* (2000) established the stable-isotope probing (SIP) technique for linking the identity of active bacteria with their function in complex natural environments. DNA

synthesized during microbial growth on a substrate labeled with stable isotopes (e.g. ^{13}C), stable isotope becomes sufficiently labeled to be separated from unlabeled DNA by CsCl density gradient centrifugation. To date, the SIP technique has been successfully applied to identifing the active methylotroph populations in an oak forest soil (Moris et al., 2002; Radajewski *et al*., 2000; Radajewski *et al*., 2002), active ammonia oxidizers in lake sediment (Whitby *et al*., 2001), active phenol-degrading microbial populations in an aerobic industrial bioreactor (Manefield *et al*., 2002), and active denitrifiers in wastewater treatment systems (Ginige *et al*., 2004).

In this study, SIP analysis was applied to clarifying how growth of bacterial populations was stimulated by acetate or methanol as the external carbon source under nitrate-reducing conditions for a better understanding of the relationship between the carbon uptake pattern and the phylogenetic positions within denitrifying populations.

2.2 Material and Methods

2.2.1 Incubation with ^{13}C-substrates under denitrification conditions

Activated sludge samples were collected from an anoxic-oxic activated sludge system removing carbon and nitrogen from municipal sewage. To identify the microbial populations of the original sludge, some of the collected activated sludge was stored at –80°C. A sludge pellet (wet weight; 8 g) was suspended in synthetic wastewater containing ^{13}C-substrates. In the acetate experiment , $^{13}CH_3{}^{13}COONa$ (99% ^{13}C; Cambridge Isotope Laboratories, Inc., Cambridge, Mass.) was added, and $^{13}CH_3OH$ (99% ^{13}C; Sigma, Saint Louis, Mo.) was added in the methanol experiment. The composition of synthetic wastewater was as follows: NaCl (6.60 mg/l), $MgSO_4 \cdot 7H_2O$ (8.20 mg/l), KH_2PO_4 (18.6 mg/l), KCl (13.4 mg/l), dextrin (30.5 mg/l), bactopeptone (65.5 mg/l), yeast extract (65.4 mg/l), meat extract (74.6 mg/l), $NaHCO_3$ (191 mg/l), $NaNO_3$ (607 mg/l, i.e., 100 mg of NO_3-N/l), $^{13}CH_3OH$ (550 mg/l, i.e., 216 mg of ^{13}C /l), and $^{13}CH_3{}^{13}COONa$ (691 mg/l, i.e., 216 mg of ^{13}C /l). The artificial wastewaters used for both the acetate and methanol experiments were purged with pure N_2 gas (> 99.9%) to eliminate the dissolved oxygen maintaining the temperature at 25 °C. During incubation periods (6 days), it was confirmed using a dissolved oxygen meter TOX-98E (Toko Chemical Laoratories Co., Ltd, Tokyo, Japan) that dissolved oxygen concentration was almost zero. Furthermore, $NaNO_3$ (100 mg of NO_3-N /l) and ^{13}C-substrates (100 mg of ^{13}C /l) were added every 24 h. Sludge samples were collected in the acetate and methanol experiments every 24 h and stored at –80 °C. Nitrate, nitrite and acetate concentrations were measured with a DX-100 ion chromatograph (Dionex, Sunnyvale, California, USA), and methanol concentration was measured with a GC380 gas chromatograph (GL Science Inc.Tokyo, Japan).

2.2.2 DNA extraction

Total DNA was extracted from 0.15 g (wet weight) of sludge pellet using Isoplant

(Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. DNA was purified using a phenol/chloroform/isoamyl alcohol (25/24/1) solution, and precipitated by adding ethanol and sodium acetate.

2.2.3 CsCl gradient density centrifugation

CsCl-ethidium bromide density gradient centrifugation (55,000 rpm, 20 h, 20 °C) was used for the separation and collection of [^{13}C]-DNA from [^{12}C]-DNA fraction as described by Radajewski *et al* (2002). Ethidium bromide was extracted from DNA fractions with an equal volume of isoamyl alcohol saturated with water. Purified DNA was dialyzed in a large volume of TE buffer to remove CsCl, precipitated by adding ethanol and sodium acetate overnight at –20°C, and dissolved in 30 µl of TE buffer.

2.2.4 PCR characterization

The oligonucleotide primer sets eub341f-univ907r (Muyzer *et al*., 1998) were used for PCR amplification of 16S rRNA gene fragments. The PCR mixture contained 0.5 µM concentrations of each primer, 200 µM concentrations of dNTP, 2 mM concentrations of $MgCl_2$, 2.5 U of rTaq DNA polymerase (Toyobo, Osaka, Japan), and 2 µl of 10× PCR buffer for rTaq. The PCR amplifications were performed in a total volume of 20 µl in 0.2 ml reaction tubes by using a model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following programs: 2 min at 94 °C, 25 cycles (40 sec at 94 °C, 40 sec at 54 °C, 40 sec at 72 °C), 2 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.

2.2.5 Cloning, sequencing, rarefaction analysis, 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 Corp., Madison, Wis.). The PCR

amplicons were cloned using a QIAGEN PCR cloning kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Then, single colonies were picked up and soak the needle into Insert Check Ready Solution (Toyobo) for a few seconds. Amplified inserts were run on 2% agarose gels. Samples including inserts of estimated sizes were used for the subsequent sequencing. A DNA insert was amplified and used as template DNA in a cycle sequencing reaction with a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. The 16Sr RNA gene fragments were sequenced with an ABI PRISM 3100-*Avant* DNA Sequencing system (Applied Biosystems). 16S rRNA gene sequences with more than 99% identity were considered to belong to the same operational taxonomic unit (OTU). A database search was conducted using BLAST from 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) and were displayed using tree view (Page, 1996). Rarefaction calculations were carried out using Analytic Rarefaction software version 1.3 (Stratigraphy Laboratory, University of Georgia).

2.2.6 Nucleotide sequence accession numbers

16S rRNA gene sequences determined in this study were deposited under accession numbers AB205646-AB206036.

2.3 Results

2.3.1 Stable-isotope probing of denitrifying consortia

Nitrate reduction was observed from the beginning of incubation in both the methanol and acetate experiments (**Fig. 2.1**). Acetate was consumed rapidly from the beginning of the incubation. In contrast, methanol was consumed slowly in the beginning of incubation and then rapidly after two days of incubation.

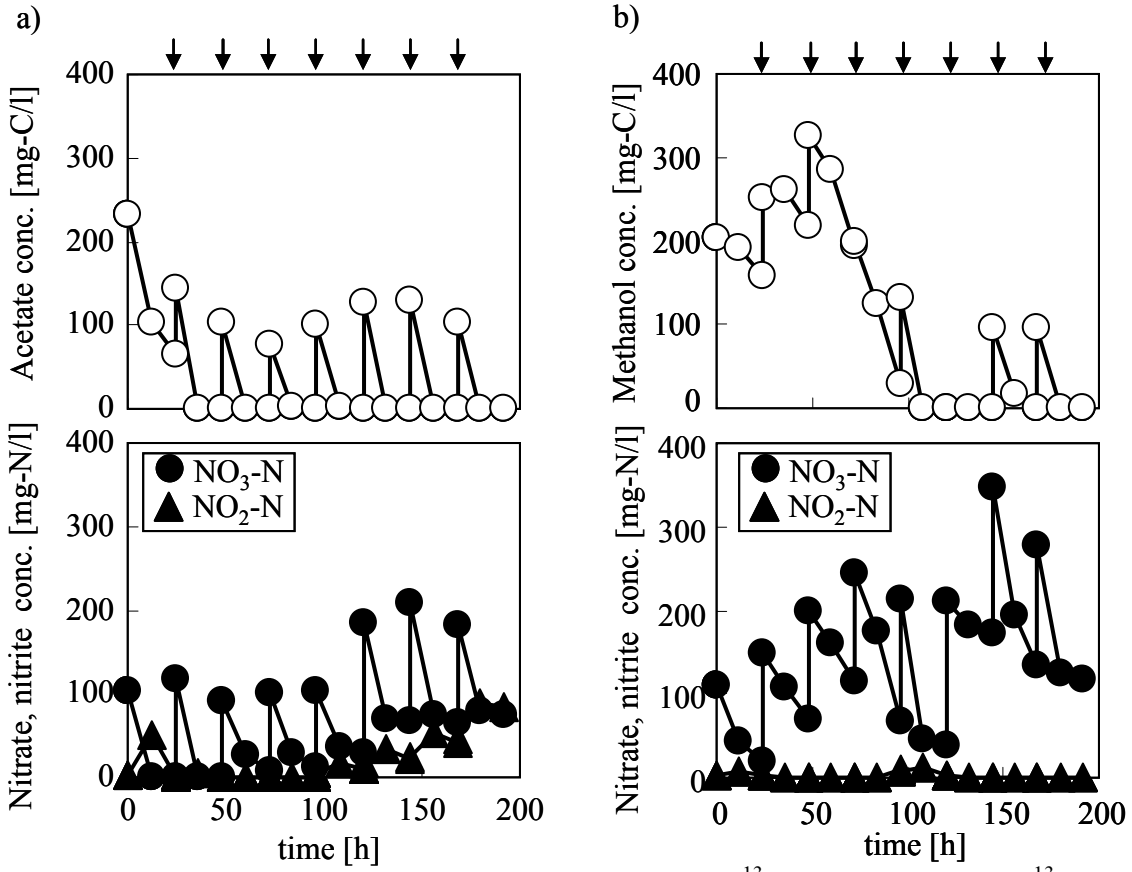

Fig. 2.1. Time courses of NO_3-N, NO_2-N, CH_3COONa-^{13}C (A), and CH_3OH-^{13}C (B) concentrations during incubation with ^{13}C-substrate under nitrate reducing conditions. Arrows indicated the seven additions of a ^{13}C-substrate and nitrate.

Total DNA was extracted from sludge samples after 1-6 days of incubation, and then ultracentrifuged. Double DNA bands were detected for the sludge sample incubated for three days with the ^{13}C-substrate, although a single DNA band was detected after two days of incubation with the ^{13}C-substrate. When total DNA extracted from the original sludge before substrate addition was ultracentrifuged as a control, a

single DNA band was detected. The position of the single DNA band, which was detected from the sludge sample after two days of incubation or before substrate addition, was almost the same as that of the upper DNA band. These results showed that the lower DNA band was generated due to an increase in buoyant density with the incorporation of ^{13}C into the DNA. Therefore, it was judged that the upper band corresponded to ^{12}C-DNA and the lower band to ^{13}C-DNA.

2.3.2 Evaluation of bacterial populations based on the 16S rRNA gene clone library

^{13}C-DNA and ^{12}C-DNA collected from sludge samples incubated for three days were chosen as the samples for the 16S rRNA gene clone library in both methanol and acetate experiments. Five 16S rRNA gene clone libraries were generated. The relative abundance of individual clones obtained from each sample was shown in histograms (**Fig. 2.2**). From acetate samples, 127 clones from ^{12}C-DNA and 201 clones from ^{13}C-DNA were obtained. In methanol samples, 130 clones from ^{12}C-DNA and 137 clones from ^{13}C-DNA were obtained. Additionally, the 16S rRNA gene clone library (131 clones) was also constructed from the original sludge before substrate addition. In contrast to clone libraries retrieved from ^{12}C-DNA fraction and the original sludge before incubation, clone libraries from ^{13}C-DNA fraction showed a completely different bacterial composition and diversity. In these experiments, the identification of active population using stable-isotope probing is a prerequisite for the incorporation of ^{13}C into the DNA. Consequently, the clone libraries from the ^{12}C-DNA fraction corresponded to organisms that did not grow with acetate or methanol under these conditions: those that were present in the original sludge and those that grew with carbon sources other than acetate and methanol.

Most of the clones were affiliated with the *Proteobacteria* in the clone libraries from ^{12}C-DNA fractions and the original sludge. The remaining clones were affiliated with *Acidobacteria*, *Actionobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*,

Nitrospairae, and *Planctomycetes*. In particular, many clones from ^{12}C-DNA were affiliated with *Actinobacteria*, including high G+C Gram-positive bacteria. In both the methanol and acetate samples, bacterial community structures were obviously different between ^{12}C-DNA and ^{13}C-DNA libraries. These findings confirmed the successful separation of ^{12}C-DNA and ^{13}C-DNA corresponding to an increase in buoyant density with the incorporation of ^{13}C into the DNA, and not to a difference in G+C content.

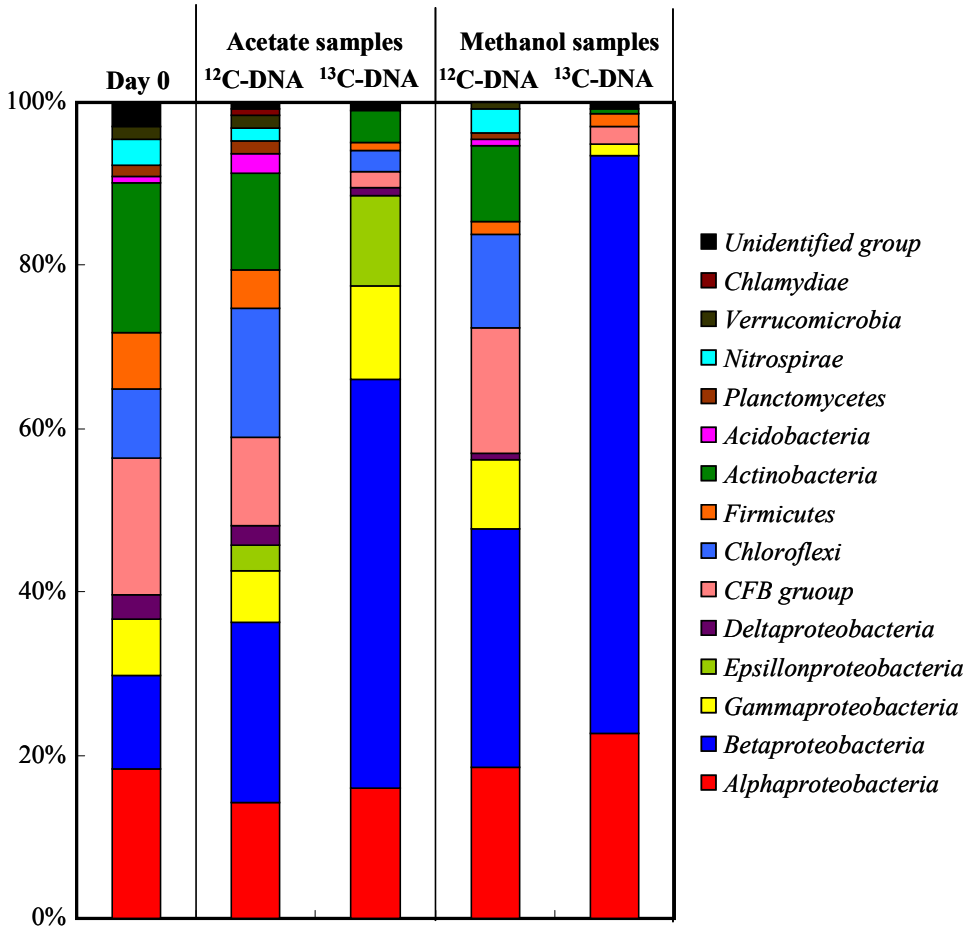

Fig. 2.2. Histograms of bacterial community structure based on bacterial 16S rRNA gene clones retrieved from DNA extracted from original sludge samples (before incubation) and each ^{12}C-DNA and ^{13}C-DNA (incubated 3 days) in acetate and methanol samples.

Table 2.1. Phylogenetic affiliations and numbers of 16S rRNA gene clones retrieved from the ^{12}C-DNA and ^{13}C-DNA obtained by CsCl density gradient centrifugation.

	Acetate samples		Methanol samples	
Phylogenetic group	**^{12}C-DNA**	**^{13}C-DNA**	**^{12}C-DNA**	**^{13}C-DNA**
Alphaproteobacteria				
Ancylobacter group	**1**			
Bradyrhizobiaceae	**1**		**1**	**1**
Hyphomicrobiumaceae	**1**		**3**	**29**
Type II Methanotroph			**1**	
Methylocystaceae			**1**	
Phyllobacteriaceae	**1**			
Rhizobiaceae	**1**	**2**		
Rhodobacteraceae	**8**	**29**	**13**	
Rhodospirillaceae	**3**		**1**	
Sphingomonadaceae	**2**		**1**	
unidentified bacteria		**1**	**3**	**1**
Betaproteobacteria				
Burkholderiaceae	**1**	**3**	**1**	**4**
Commamonadaceae	**11**	**50**	**17**	**2**
Methylophilaceae				**87**
Neisseriaceae	**2**	**7**		
Nitrosomonadaceae			**1**	
Oxalobacteraceae			**5**	
Rhdocyclaceae	**11**	**41**	**10**	**4**
unidentified bacteria	**3**		**4**	
Gammaproteobacteria				
Aeromonadaceae		**2**		
Altermonadaceae	**1**		**1**	
Enterobacteraceae	**1**	**4**		**2**
Methylococcaceae			**1**	
Pseudomonadaceae		**2**		
Thiothrix group			**3**	
Xanthomonadaceae	**1**	**3**	**2**	
unidentified bacteria	**5**	**12**	**4**	
Epsillonproteobacteria				
Campylobacteraceae	**4**	**22**		
unidentified bacteria			**1**	
Deltaproteobacteria				
Bdellovibrionaceae		**2**		
unidetified bacteria	**3**			
CFB gruoup	**14**	**4**	**20**	**3**
Chloroflexi	**20**	**5**	**15**	
Firmicutes	**6**	**2**	**2**	**2**
Actinobacteria	**15**	**8**	**13**	**1**
Acidobacteria	**3**		**1**	
Planctomycetes	**2**		**1**	
Nitrospirae	**2**		**4**	
Verrucomicrobia	**2**		**1**	
Chlamydia	**1**			
Unidentified group	**1**	**2**		**1**

About 90% of the clones retrieved from ^{13}C-DNA fraction were affiliated to *Proteobacteria*. This showed that *Proteobacteria* actively consumed acetate or methanol as the carbon source under nitrate-reducing conditions. The bacterial community structures in the ^{13}C-DNA fraction were obviously different between acetate and methanol samples. The phylogenetic affiliations of all analyzed clones are summarized in **Table 2.1**.

2.3.3 Phylogenetic analysis of acetate-assimilating bacteria in denitrifying sludge

A total of 201 clones from the ^{13}C-DNA fraction were assigned to 71 different OTUs (13C-A1 to 13C-A71). The cloned sequence types with more than 99% identity were considered to belong to the same OTU. The placements of OTUs within the ^{13}C-DNA clone library are shown in phylogenetic trees (**Fig. 2.3 - Fig. 2.6**). The ^{13}C-DNA clone library showed that acetate-assimilating bacteria were found in various phylogenetic groups. About 25% (50 clones) of all clones, including 11 different OTUs, were related to *Comamonadaceae* of *Betaproteobacteria*, such as the genera *Comamonas* and *Acidovorax*. The OTU 13C-A7 contained many clones (18 clones) closely related to *Comamonas denitrificans*. A further 20.4% (41 clones) of all clones, including 17 OTUs, were clustered within *Rhodocyclaceae* of *Betaproteobacteria*, such as the genera *Thauera*, *Dechloromonas*, *Zoogloea* and *Azonexus*. In particular, many clones related to the genus *Thauera* (e.g. *T. aromatica*, *T. selenatis*, and *T. terpenica*) were found (25 clones). Furthermore, 10 different OTUs (29 clones) were related to members of *Rhodobacteraceae* of *Alphaproteobacteria*, such as the genera *Paracoccus* and *Rhodobacter*, and OTU 13C-A71 (22 clones) was very similar to *Arcobacter cryaerophilus* of *Epsilonproteobacteria*. Nine different OTUs (23 clones) were clustered within *Gammaproteobacteria*, including the genera *Pseudomonas*, *Aeromonas*, *Thermomonas* and *Stenotrophomonas*. Furthermore, the remaining clones of this ^{13}C-DNA clone library were seemingly distributed randomly throughout the bacterial

domain, including *Actinobacteria*, *Bacteroidetes*, *Chlroflexi* and *Firmicutes*.

2.3.4 Phylogenetic analysis of methanol-assimilating bacteria in denitrifying sludge

One hundred thirty-seven clones from the ^{13}C-DNA were assigned to 22 different OTUs (OTU 13C-M1 to 13C-M22). The cloned sequence types with more than 99% identity were considered to belong to the same OTU. The phylogenetic positioning of OTUs obtained from the ^{13}C-DNA clone library are shown in phylogenetic trees (**Fig. 2.3** - **Fig. 2.6**). The ^{13}C-DNA clone library showed two predominant groups related to known methylotrophs. Sixty-three percent of all clones, including six different OTUs, were related to *Methylophilaceae* of *Betaproteobacteria*, such as *Methylophilus*, *Methylobacillus* and *Aminomonas*. Most of the *Methylophilaceae* are obligate methylotrophs, which have the capacity to grow in the presence of methanol or methylamine but not in that of methane. A further 21% of all clones, including four OTUs, were clustered within *Hyphomicrobiaceae* of *Alphaproteobacteria*. The remaining clones in this library were clustered in *Alphaproteobacteria* (e.g. *Blastobacter denitrificans*), *Betaproteobacteria* (e.g. *Ralstonia picketii*, *Thauera aromatica, Comamonas sp.*), *Gammaproteobacteria* (e.g. *Ewingella americana*) and *Actinobacteria* (e.g., *Carnobacterium piscicola* and *Nocardioides sp.*).

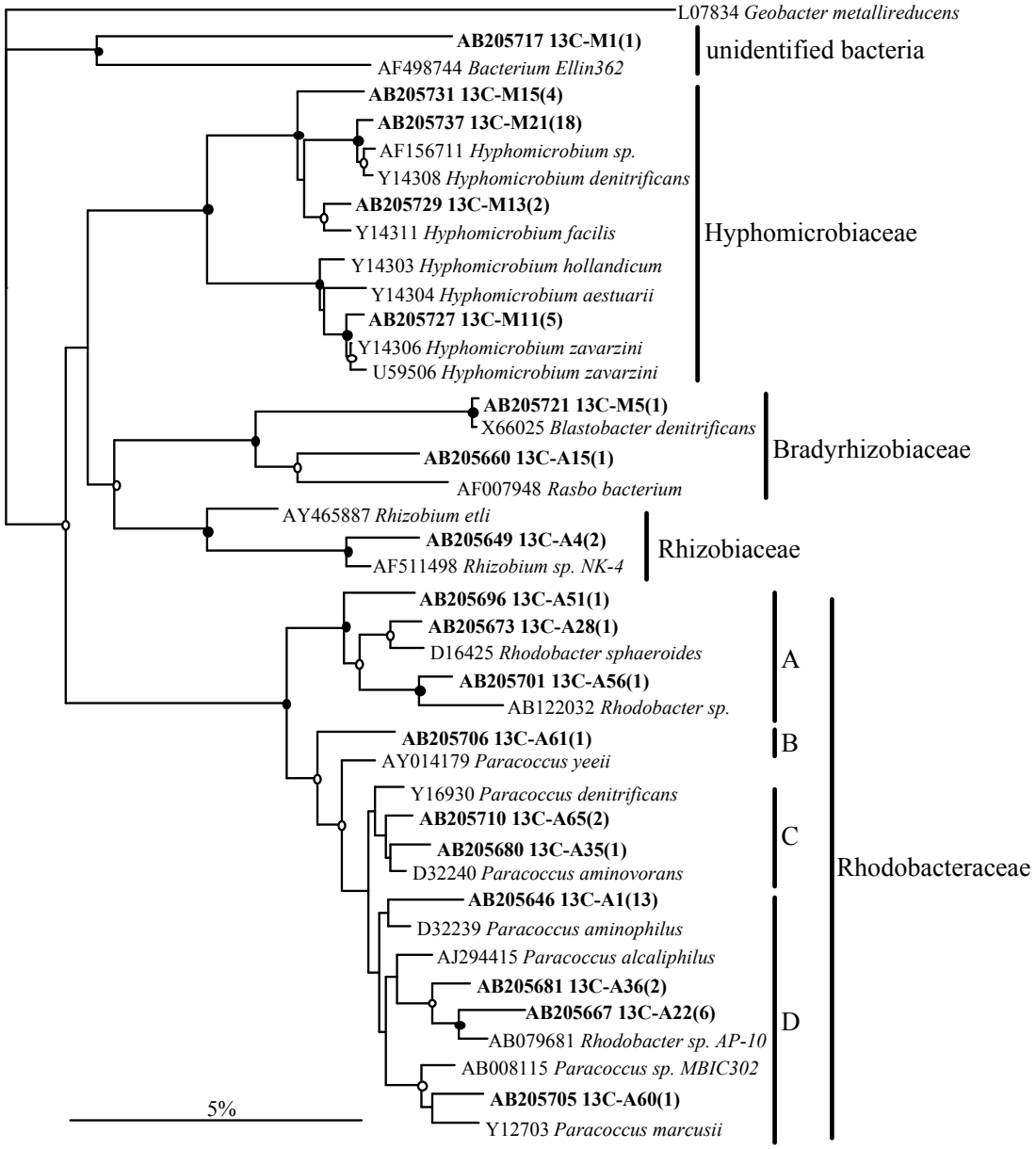

Fig. 2.3. Phylogenetic affiliation of the *Alphaproteobacterial* clones retrieved from each ^{13}C-DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanol samples are labeled as 13C-A and 13C-M, respectively. The number of clones assigned to each sequenced OTU with more than 99% identity is shown in parentheses. *Geobacter metallireducens* (accession no. L07834) are used as the outgroup. The bootstrap values >750 (closed circles) and in the range of 500 to 750 (open circles) are indicated at branch points. Scale bar = 5% nucleotide substitution.

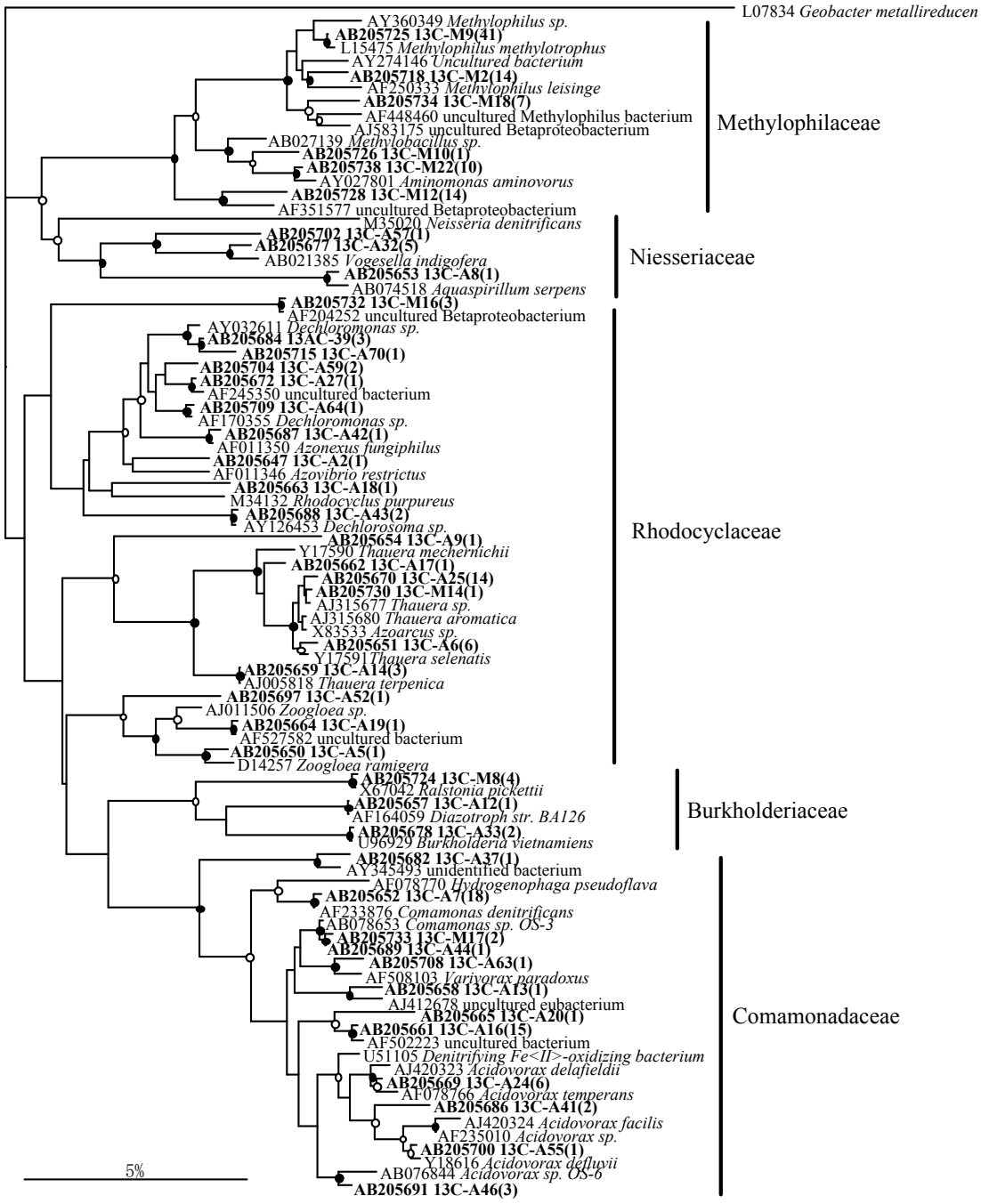

Fig. 2.4. Phylogenetic affiliation of the *Betaproteobacterial* clones retrieved from each ^{13}C-DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanol samples are labeled as 13C-A and 13C-M, respectively. The number of clones assigned to each sequenced OTU with more than 99% identity is shown in parentheses. *Geobacter metallireducens* (accession no. L07834) are used as the outgroup. The bootstrap values >750 (closed circles) and in the range of 500 to 750 (open circles) are indicated at branch points. Scale bar = 5% nucleotide substitution.

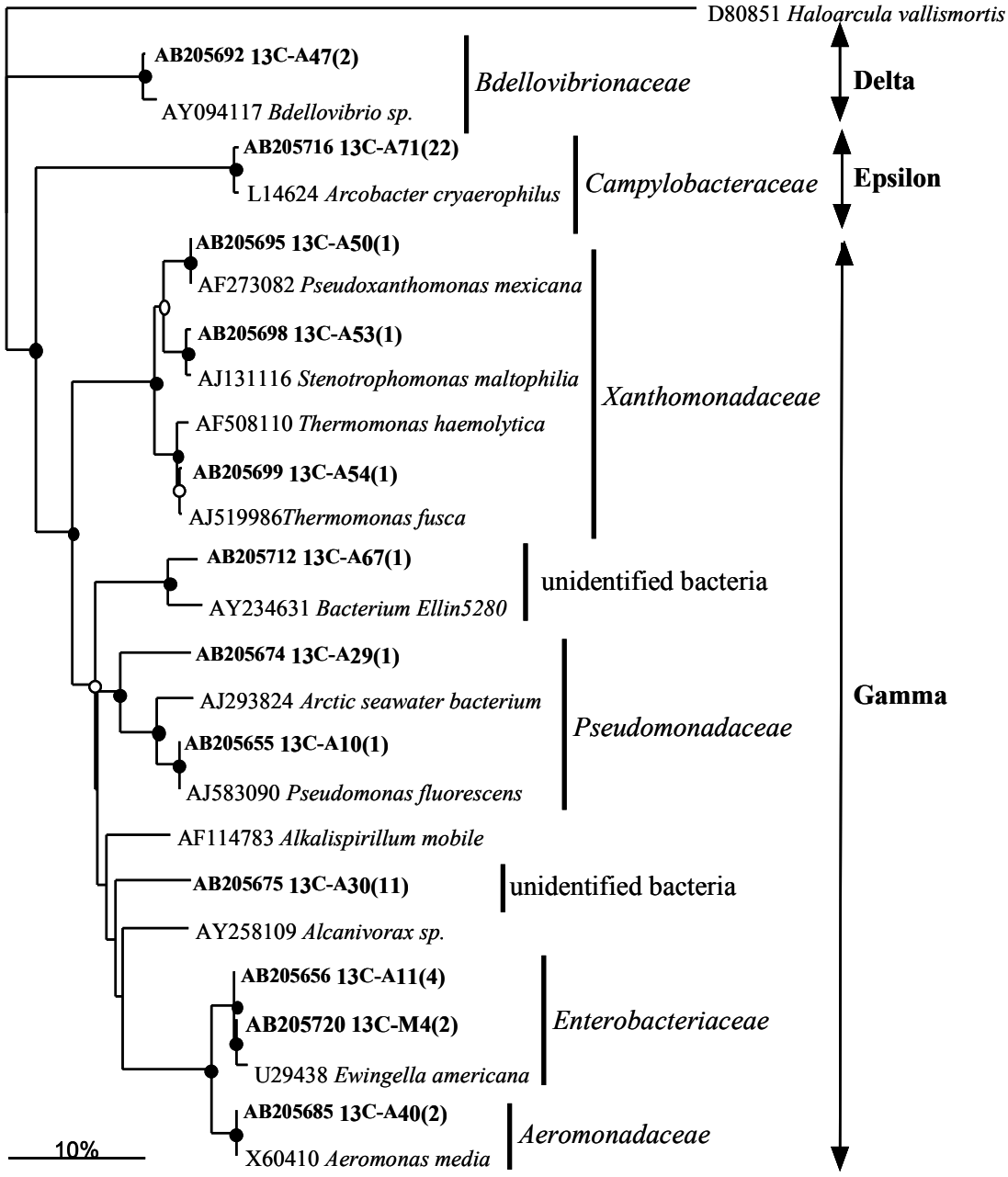

Fig. 2.5. Phylogenetic affiliation of the *Gamma*-, *Delta*-, *Epsilonproteobacterial* clones retrieved from each ^{13}C-DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanol samples are labeled as 13C-A and 13C-M, respectively. The number of clones assigned to sequenced OTU with more than 99% identity is shown in parentheses. *Haloarcula vallismortis* (accession no. D80851) are used as the outgroup. The bootstrap values >750 (closed circles) and in the range of 500 to 750 (open circles) are indicated at branch points. Scale bar = 10% nucleotide substitution.

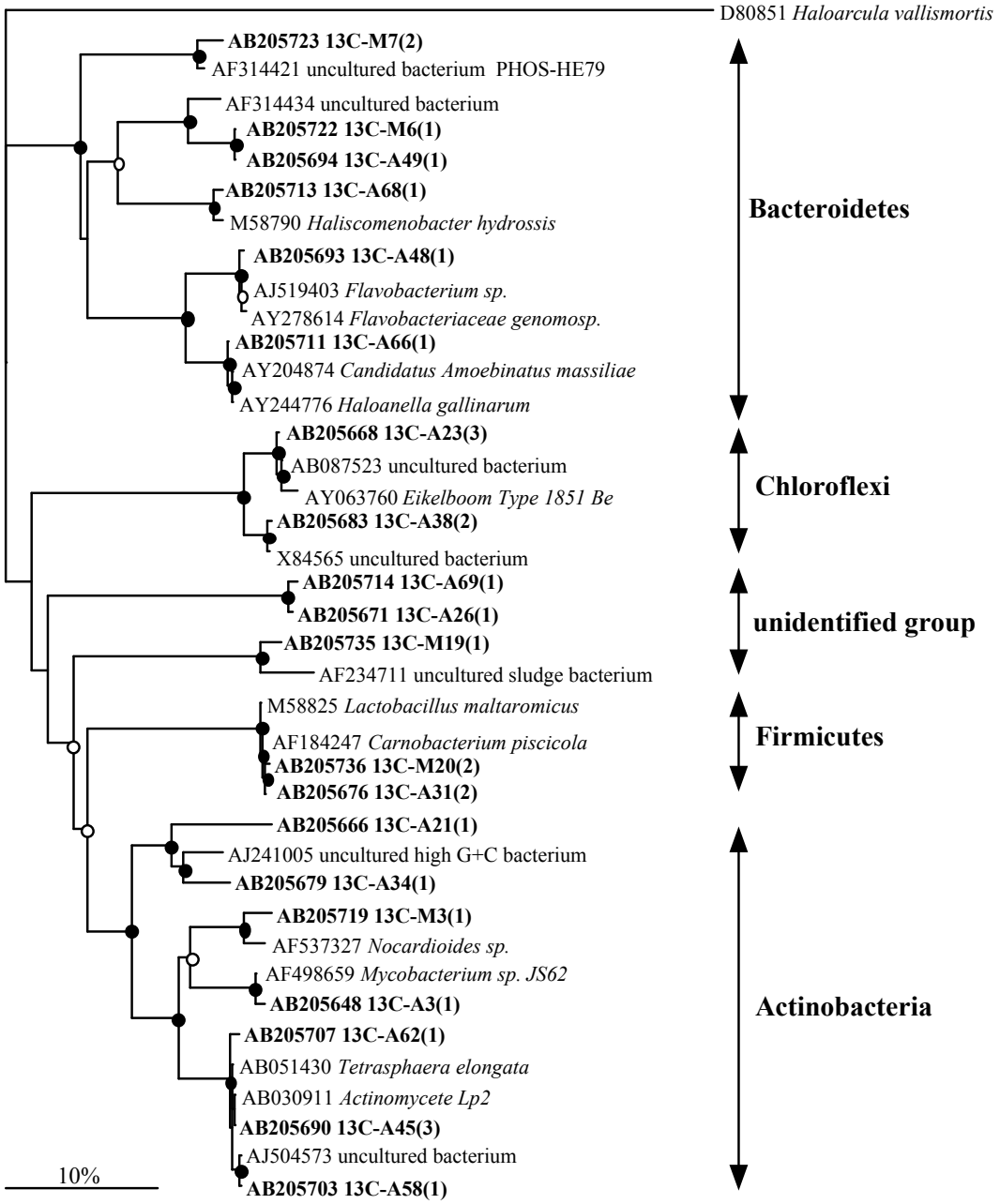

Fig. 2.6. Phylogenetic affiliation of the remaining bacterial clones except *Proteobacterial* clones retrieved from each ^{13}C-DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanolsamples are labeled 13C-A and 13C-M, respectively. The number of clones assigned to each sequenced OTU with more than 99% identity is shown in parentheses. *Haloarcula vallismortis* (accession no. D80851) are used as the outgroup. The bootstrap values >750 (closed circles) and in the range of 500 to 750 (open circles) are indicated at branch points. Scale bar = 10% nucleotide substitution.

2.3.5 Rarefaction analysis

The diversity of the 16S rRNA gene clones obtained from acetate and methanol samples and the original sludge sample was evaluated by rarefaction analysis (**Fig. 2.7**). The 16S rRNA gene clones retrieved from the ^{13}C-DNA indicated that the diversity level of methanol-assimilating bacteria was much lower than that of acetate-assimilating bacteria. There were no significant differences between the diversity levels of the 16S rRNA gene clones retrieved from the original sludge sample and the ^{12}C-DNA in both methanol and acetate samples. Therefore, these results implied that the three days of incubation in the synthetic wastewater had no significant effects on the bacterial diversity.

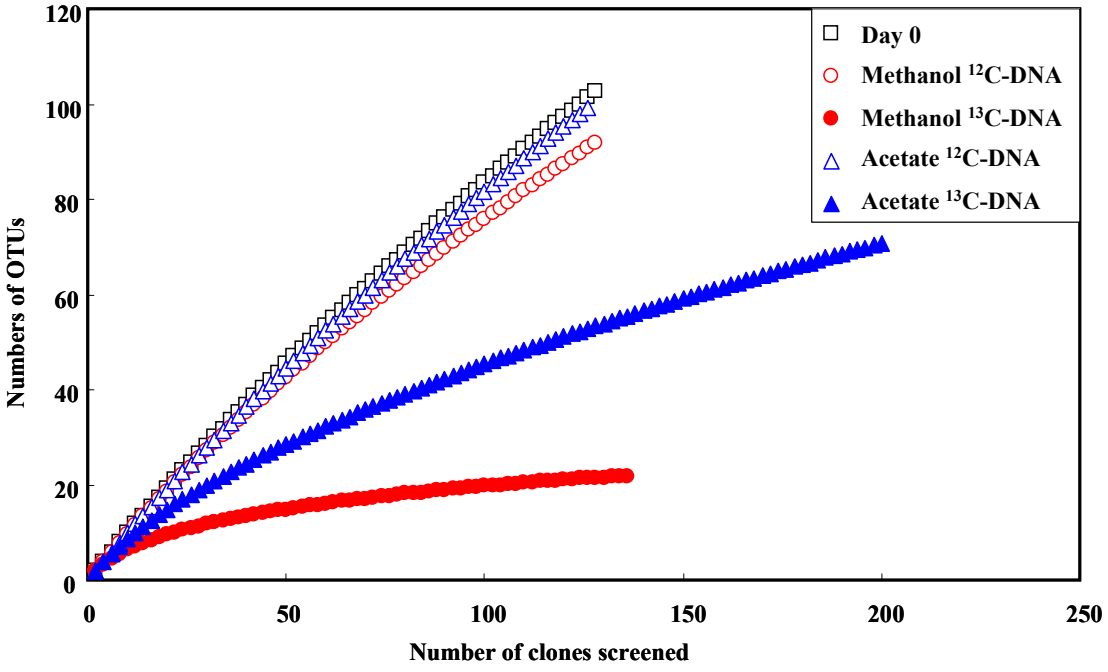

Fig. 2.7. Rarefaction curves indicating the diversity of denitrifying bacteria as OTU of 16S rRNA sequences retrieved from acetate and methanol samples and the original sludge sample.

2.4 Discussion

In this study, 16S rRNA gene targeted analysis combined with DNA based stable-isotope probing (DNA-SIP) revealed the identity and carbon uptake pattern of active denitrifying populations in activated sludge. Our results showed that the type of organic carbon source, such as acetate or methanol, had a strong impact on the active denitrifying populations. As for the community structure and ecological aspects of methanol-assimilating denitrifiers, some members of the genera *Hyphomicrobium*, *Paracoccus, Rhodobacter*, *Blastobacter* and *Hydrogenophaga* have been reported as methanol-assimilating denitrifiers in freshwater environments (Lemmer *et al*., 1997a; Lemmer *et al*., 1997b; Neef *et al*., 1996). However, active methanol-assimilating bacteria in a denitrifying consortium might have been missed because these members were identified by culture-dependent methods. Therefore, it is necessary to characterize active methanol-assimilating bacteria in a denitrifying consortium by molecular techniques linking microbial function with taxonomic identity (Labbe *et al*., 2003). Thus, we applied SIP to the identification of active methanol-assimilating bacteria under nitrate-reducing conditions and found the existence of two predominant methanol-assimilating populations in the ^{13}C-DNA clone library from methanol samples. One major phylotype was related to the members of the *Methylophilaceae* of *Betaproteobacteria*, such as the genera *Methyolphilus, Methylobacillus* and *Aminomonas*. Ginige *et al.* (2004) also reported on a methanol-fed denitrifying community in activated sludge using the SIP and MAR-FISH techniques. In their study, the dominant 16S rRNA gene phylotype in the ^{13}C-DNA clone library was closely related to those of the obligate methylotroph of *Methylophilaceae*, most of which assimilated ^{14}C-methanol in the MAR-FISH analysis. The other major phylotype was related to the genus *Hyphomicrobium*. The genus *Hyphomicrobium* spp. (hyphomicrobia) are restricted facultative methylotrophs and have been found in soils, aquatic habitats, and a sewage treatment plant (Poindexter, 1992). The diversity of

hyphomicrobia in a sewage treatment plant and its adjacent receiving lake has been examined by Holm *et al.* (1996). An important characteristic of certain hyphomicrobia is their ability to grow with methanol as the carbon source and nitrate as the terminal electron acceptor. Moreover, we also found some clones related to *Blastobacter denitrificans*, which was reported as a methanol-utilzing denitrifier (Trotsenko *et al*., 1989). Hence, it was confirmed that SIP could specifically identify a methanol-assimilating population under nitrate-reducing conditions in a complex microbial community, such as activated sludge.

No clones related to the members of the family *Rhodobacteraceae* (e.g. *Paracoccus* and *Rhodobacter*), which were reported as methanol-assimilating denitrifiers, were detected in the ^{13}C-DNA clone library generated from methanol samples. However, a number of clones related to the family *Rhodobacteraceae* were detected in the ^{13}C-DNA clone library from acetate samples and the ^{12}C-DNA clone library from both acetate and methanol samples. Seven sequences obtained from ^{12}C-DNA of acetate samples belonged to cluster A, and 13 sequences obtained from ^{12}C-DNA of methanol sample belonged to cluster A (9 clones) and cluster C (4 clones) (**Fig. 2.3**). These results implied that these bacterial groups preferred other organic compounds (e.g. acetate, dextrin, and peptone) to methanol as carbon sources. Indeed, these bacterial groups can utilize various organic compounds for denitrification (Kelly *et al*., 2001). On the other hand, Claus and Kutzner (1985) also reported that the members of *Paracoccus* grow poorly with methanol as the carbon source. Furthermore, they assumed that the growth of *Paracoccus* is supported by formate, which is produced by other bacteria (such as hyphomicrobia), or by organic compounds excreted by other bacteria. This assumption might lead to one limitation of SIP, that is, labeled intermediates and products might become incorporated into the DNA of other microbial communities when primary consumers metabolize the original substrates and excrete labeled metabolites. However, in this study, we considered this limitation unlikely

because no clone obtained from the ^{13}C-DNA isolated from methanol samples was related to the genus *Paracoccus*.

In contrast to methanol as an external carbon source, acetate was consumed quickly from the beginning of incubation. This implied that numerous heterotrophic bacteria in activated sludge can take up acetate under denitrifying conditions. MAR-FISH analysis conducted by Nielsen *et al*. (2002) revealed that wastewater treatment systems with nitrogen removal have relatively high numbers of acetate-assimilating bacteria (53-93%). The phylogeny of 16S rRNA gene sequences retrieved from the ^{13}C-DNA indicated that a diverse range of bacteria in the activated sludge assimilated acetate under nitrate-reducing conditions. From the viewpoint of the number of clones retrieved from the ^{13}C-DNA, the dominant acetate-assimilating bacteria were the *Acidovorax*-related groups (28 clones), *Paracoccus*-related groups (26 clones), *Thauera*-related groups (24 clones), *Arcobacter*-related groups (22 clones), *Comamonas*-related groups (19 clones) and *Dechloromonas*-related groups (8 clones). In previous studies, some denitrifying members within these genera except for the genus *Arcobacter* were isolated from activated sludge and were found to have the ability to degrade various xenobiotic compounds (e.g. aromatic compounds, hydrocarbons and chlorinated compounds) under nitrate-reducing conditions (Coates *et al*., 2001; Kelly *et al*., 2001; Manefield *et al*., 2002; Willems and Vos, 2001; Zhao and Ward, 1999). Wagner and Loy (2002) reported that the *Azoarcus-Thauera* group of Betaproteobacteria represents numerous denitrifiers in wastewater treatment systems. As for the genus *Arcobacter*, *A. cryaerophilus* was shown to be abundant in activated sludge (Snaidr *et al*., 1997). Some *Arcobacter spp.* were shown to be sulfide oxidizers (with the production of sulfur) and it has been suggested that they play a role in the sulfur cycle by reoxidizing sulfide formed by microbial sulfate or sulfur reduction (Teske *et al.,* 1996; Voordouw *et al*., 1996; Wirsen *et al*., 2002). Teske *et al*. (1996) reported that *Arcobacter* spp. use acetate as the electron donor and nitrate as the electron

acceptor. However, to date, their denitrification ability in the environment has not been well documented.

2.5 Conclusions

Stable-isotope probing (SIP) is applied for identifying the active denitrifying bacteria in sewage sludge. By molecular analysis targeting 16S rRNA gene with stable-isotope probing, we screen candidates for active acetate- or methanol-assimilating denitrifiers in the complex bacterial communities, such as activated sludge. As a result, a diverse range of bacteria in the activated sludge assimilates acetate under nitrate-reducing conditions. In particular, the *Comamonadaceae* and *Rhodocyclaceae* of *Betaproteobacteria*, and *Rhodobacteraceae* of *Alphaproteobacteria* are major acetate-assimilating populations. In contrast, active methanol-assimilating populations are two distinctive bacterial groups, i.e. the families *Hyphomicrobiaceae* and *Methylophilaceae*. In the future, the combination of SIP and other molecular techniques, such as MAR-FISH and real-time PCR, will develop quantitative understandings of active denitrifying populations in sewage sludge.

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