

## Identification of Acetate- or Methanol-Assimilating Bacteria under Nitrate-Reducing Conditions by Stable-Isotope Probing

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### Abstract

Stable-isotope probing (SIP) was used to identify 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 [<sup>13</sup>C]acetate or [<sup>13</sup>C]methanol as the main carbon source and nitrate as the electron acceptor. We analyzed 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 have been known as denitrifiers in wastewater treatment systems. When acetate was used as the carbon source, 16S rRNA gene sequences retrieved from <sup>13</sup>C-labeled DNA were closely related to the 16S rRNA genes of *Comamonadaceae* (e.g., *Comamonas* and *Acidovorax*) and *Rhodocyclaceae* (e.g., *Thauera* and *Dechloromonas*) of the Betaproteobacteria, and *Rhodobacteraceae* (e.g., *Paracoccus* and *Rhodobacter*) of the Alphaproteobacteria. When methanol was used as the carbon source, 16S rRNA gene sequences retrieved from <sup>13</sup>C-DNA were affiliated with *Methylophilaceae* (e.g., *Methylophilus*, *Methylobacillus*, and *Aminomonas*) and *Hyphomicrobiaceae*. Rarefaction curves for clones retrieved from <sup>13</sup>C-DNA showed that the diversity levels for methanol-assimilating bacteria were considerably lower than those for acetate-assimilating bacteria. Furthermore, we characterized nitrite reductase genes (*nirS* and *nirK*) as functional marker genes for denitrifier communities in acetate- or methanol-assimilating populations and detected the *nirS* or *nirK* sequence related to that of some known pure cultures, such as *Alcaligenes*, *Hyphomicrobium*, and

*Thauera*. However, most of the *nirS* or *nirK* sequences retrieved from <sup>13</sup>C-DNA were clustered in some unidentified groups. On the basis of 16S rRNA gene clone libraries retrieved from <sup>13</sup>C-DNA, these unidentified *nir* sequences might be identified by examining the *nir* gene in candidates for true denitrifiers (e.g., the families *Comamonadaceae*, *Hyphomicrobiaceae*, *Methylophilaceae*, and *Rhodobacteraceae*).

### 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 [49]. Denitrification requires an oxidizing nitrogen compound as the electron acceptor and a carbon source. For wastewater with a low carbon/nitrogen ratio or which lacks readily biodegradable carbon sources, various organic compounds acting as external carbon sources, such as acetate, ethanol, glucose, and methanol, are added to achieve a satisfactory degree of denitrification. Methanol has often been chosen because of its relatively low cost and the small amounts of sludge produced compared with other organic compounds, although only some bacteria can utilize methanol as a carbon source [27].

In recent years, molecular biological analysis has been applied to understanding microbial ecology in wastewater treatment systems. 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.

Denitrification ability is found in many bacterial species in a variety of taxonomic groups [49]. These

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bacteria have been found using primer sets specific for functional genes involved in denitrification: genes encoding periplasmic and membrane-bound nitrate reductases (*napA* and *narG*) [5, 8, 9], genes encoding cytochrome *cd<sub>1</sub>* and copper-containing nitrite reductases (*nirS* and *nirK*, respectively) [2, 3, 10, 18, 26, 31, 38, 47], nitric oxide reductase (*norB*) [4], and a gene encoding a nitrous oxide reductase (*nosZ*) [35, 36]. Polymerase chain reaction (PCR)-based methods using these primers revealed the diversity and community structure of denitrifiers in activated sludge [10, 38], marine sediment [2, 18, 35], and soil [26, 31, 36]. However, active denitrifiers in various environments cannot be identified by these methods.

Recently, molecular techniques using radio isotopes or stable isotopes in biomarkers have been developed and applied to identifying active microbial populations [1, 12, 15, 20–23, 28, 32, 33, 44]. 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 [1, 12, 15]. Radajewski *et al.* [32] 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 enriched with a heavy, stable isotope is sufficiently labeled to be separated from unlabeled DNA by CsCl density gradient centrifugation. To date, the SIP technique has been successfully applied to identifying the active methyloph populations in oak forest soil [32, 33], active ammonia oxidizers in lake sediment [44], active phenol-degrading microbial populations in an aerobic industrial bioreactor [21], and active methanol-assimilating denitrifiers in wastewater treatment systems [20].

In this study, SIP analysis was applied to clarify how growth of bacterial populations was stimulated by acetate or methanol as the external carbon source under nitrate-reducing conditions. Moreover, we characterized the community structure and diversity of functional genes (nitrite reductase genes, *nirS* and *nirK*) in acetate- or methanol-assimilating bacterial populations for a better understanding of denitrifying consortia.

## Methods

**Incubation with [<sup>13</sup>C] Substrates under Nitrate-Reducing Conditions.** Activated sludge samples were collected from an anoxic–oxic activated sludge system for 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 [<sup>13</sup>C] substrates. In

the acetate experiment, <sup>13</sup>CH<sub>3</sub><sup>13</sup>COONa (99% <sup>13</sup>C; Cambridge Isotope Laboratories, Inc., Cambridge, MA, USA) was added, and <sup>13</sup>CH<sub>3</sub>OH (99% <sup>13</sup>C; Sigma, St. Louis, MO, USA) was added in the methanol experiment. The composition of the synthetic wastewater was as follows: NaCl (6.60 mg/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (8.20 mg/L), KH<sub>2</sub>PO<sub>4</sub> (18.6 mg/L), KCl (13.4 mg/L), dextrin (30.5 mg/L), bactopectone (65.5 mg/L), yeast extract (65.4 mg/L), meat extract (74.6 mg/L), NaHCO<sub>3</sub> (191 mg/L), NaNO<sub>3</sub> (607 mg/L, i.e., 100 mg of NO<sub>3</sub>-N/L), <sup>13</sup>CH<sub>3</sub>OH (550 mg/L, i.e., 216 mg of <sup>13</sup>C/L), and <sup>13</sup>CH<sub>3</sub><sup>13</sup>COONa (691 mg/L, i.e., 216 mg of <sup>13</sup>C/L). The synthetic wastewaters used for both the acetate and methanol experiments were purged with pure N<sub>2</sub> gas (>99.9%) to eliminate the dissolved oxygen while maintaining the temperature at 25°C. During incubation periods (6 days), it was confirmed by using a dissolved oxygen meter TOX-98E (Toko Chemical Laboratories Co., Ltd., Tokyo, Japan) that dissolved oxygen concentration was almost zero. Furthermore, NaNO<sub>3</sub> (100 mg of NO<sub>3</sub>-N/L) and [<sup>13</sup>C] substrates (100 mg of <sup>13</sup>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, CA, USA), and methanol concentrations were measured with a GC380 gas chromatograph (GL Science Inc., Tokyo, Japan).

**DNA Extraction and Ultracentrifugation.** Total DNA was extracted from 0.15 g (wet weight) sludge pellets using Isoplant (Nippon Gene, 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.

CsCl–ethidium bromide density gradient centrifugation (270,000 × g, 20 h, 20°C) was used for the separation and collection of <sup>13</sup>C-DNA from <sup>12</sup>C-DNA fraction as described by Radajewski *et al.* [33]. 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 Tris–EDTA (TE) buffer to remove CsCl, precipitated by adding ethanol and sodium acetate overnight at –20°C, and dissolved in 30 μL of TE buffer.

**PCR Characterization.** The following primer sets were used for PCR amplification: (1) eub341f-univ907r [24] for the amplification of 16S rRNA gene fragments, (2) nirS1F-nirS6R [3] for the amplification of *nirS* fragments, and (3) nirK1F-nirK5R [3] for the amplification of *nirK* fragments. The PCR mixture contained 0.5 μM of each primer, 200 μM of dNTP, 2.0 mM MgCl<sub>2</sub> for the 16S rRNA gene and *nirK* and 1.5 mM MgCl<sub>2</sub> for *nirS*, 2.5 U of rTaq DNA polymerase

(Toyobo, Osaka, Japan) for the 16S rRNA gene and *nirS* and 1.25 U of rTaq DNA polymerase for the *nirK*, 2  $\mu$ L of 10 $\times$  PCR buffer for rTaq, and sterile water added to a final volume of 20  $\mu$ L. The PCR amplifications of the 16S rRNA gene, *nirS*, and *nirK* were conducted in a model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following protocols: (1) 16S rRNA gene: 2 min at 94°C, 25 cycles (40 s at 94°C, 40 s at 54°C, 40 s at 72°C), 2 min at 72°C; (2) *nirS*: 5 min at 94°C, 30 cycles (1 min at 94°C, 1 min at 54°C, 1 min at 72°C), 5 min at 72°C; (3) *nirK*: 5 min at 94°C, 30 cycles (30 s at 94°C, 40 s at 48°C, 40 s at 72°C), 5 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.

**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 cleanup system (Promega Corp., Madison, WI, USA). 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 by using a toothpick, and the toothpick was immersed in 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 16S rRNA gene and *nir* 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). The *nir* sequences exhibiting more than 97% identity were considered to belong to the same OTU. Rarefaction calculations were carried out using Analytic Rarefaction software [version 1.3, Stratigraphy Laboratory, University of Georgia (<http://www.uga.edu/strata/software/Software.html>)]. A database search was conducted using BLAST from the DNA Data Bank of Japan (DDBJ). Sequences determined in this study and those retrieved from the database were aligned using Clustal W [40]. Phylogenetic trees were constructed using a neighbor-joining algorithm [34] and were displayed using TreeView [29].

**Nucleotide Sequence Accession Numbers.** 16S rRNA gene sequences determined in this study were deposited under accession numbers AB205646–AB206036, *nirS* sequences were deposited under accession numbers

AB162225–AB162256, and *nirK* sequences were deposited under accession numbers AB162309–AB162341.

## Results

**SIP of Denitrifying Consortia.** Nitrate reduction was observed from the beginning of incubation in both the methanol and acetate experiments (data not shown). Acetate was consumed rapidly from the beginning of the incubation. In contrast, methanol was consumed slowly at the beginning of incubation and then rapidly after 2 days of incubation. Total DNA was extracted from sludge samples after 1 to 6 days of incubation and then ultracentrifuged. Double DNA bands were detected for the sludge sample incubated for 3 days with the [ $^{13}\text{C}$ ] substrate, although a single DNA band was detected after 2 days of incubation with the [ $^{13}\text{C}$ ] 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 2 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  $^{13}\text{C}$  into the DNA. Therefore, it was judged that the upper band corresponded to  $^{12}\text{C}$ -DNA and the lower band to  $^{13}\text{C}$ -DNA.

**Evaluation of Bacterial Populations Based on the 16S rRNA Gene Clone Library.**  $^{13}\text{C}$ -DNA and  $^{12}\text{C}$ -DNA collected from sludge samples incubated for 3 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 an individual clone obtained from each sample is shown as a histogram (Fig. 1). From acetate samples, 127 clones from  $^{12}\text{C}$ -DNA and 201 clones from  $^{13}\text{C}$ -DNA were obtained. In methanol samples, 130 clones from  $^{12}\text{C}$ -DNA and 137 clones from  $^{13}\text{C}$ -DNA were obtained. In addition, 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 the  $^{12}\text{C}$ -DNA fraction and the original sludge before incubation, clone libraries from the  $^{13}\text{C}$ -DNA fraction showed a completely different bacterial composition and diversity. In these experiments, the identification of active population using SIP is a prerequisite for the incorporation of  $^{13}\text{C}$  into the DNA. Consequently, the clone libraries from the  $^{12}\text{C}$ -DNA fraction corresponded to organisms that did not grow with acetate or methanol under these conditions, i.e., 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  $^{12}\text{C}$ -DNA fractions

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

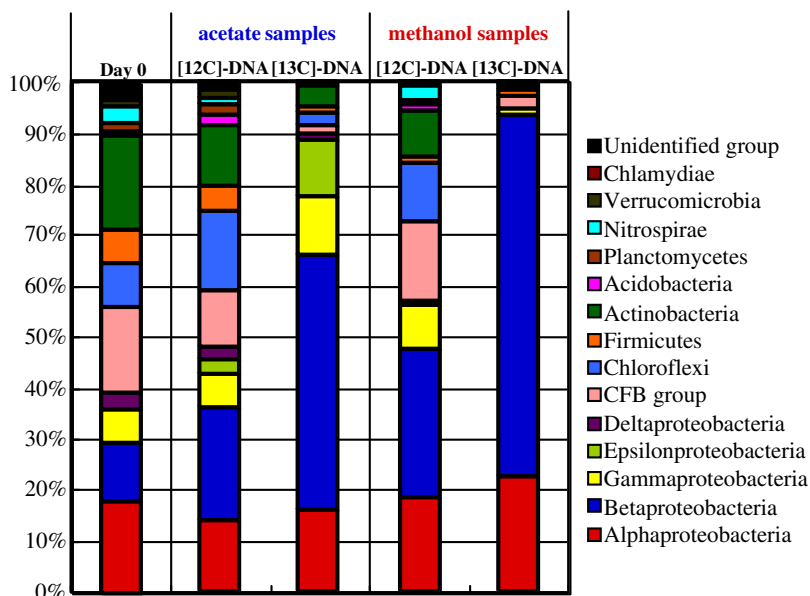
About 90% of the clones retrieved from the  $^{13}\text{C}$ -DNA fraction were affiliated with Proteobacteria. This showed that Proteobacteria actively consume acetate or methanol as a carbon source under nitrate-reducing conditions. The bacterial community structures in the  $^{13}\text{C}$ -DNA fraction were obviously different between acetate and methanol samples. The phylogenetic affiliations of all analyzed clones are summarized in Table 1.

**Phylogenetic Analysis of Acetate-Assimilating Bacteria in Denitrifying Consortium.** A total of 201 clones from the  $^{13}\text{C}$ -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  $^{13}\text{C}$ -DNA clone library are shown in phylogenetic trees (Figs. 2, 3, 4 and 5). The  $^{13}\text{C}$ -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 the 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 the Betaproteobacteria, such as the genera *Thauera*, *Dechloromonas*, *Zoogloea* and *Azonexus*. In particular, many clones related to the genus *Thauera* (e.g., *Thauera aromatica*, *Thauera selenatis*, and *Thauera terpenica*) were found (25 clones). Furthermore, 10 different OTUs (29 clones) were related to members of *Rhodobacteraceae* of the Alphaproteobacteria, such as the genera *Paracoccus* and *Rhodobacter*, and OTU 13C-A71 (22 clones) was very similar to *Arcobacter cryaerophilus* of the Epsilonproteobacteria. Nine different OTUs (23 clones) were clustered within the Gammaproteobacteria, including the genera *Pseudomonas*, *Aeromonas*, *Thermomonas*, and *Stenotrophomonas*. Furthermore, the remaining clones of this  $^{13}\text{C}$ -DNA clone library were seemingly distributed randomly throughout the bacterial domain, including the Actinobacteria, Bacteroidetes, Chloroflexi, and Firmicutes.

**Phylogenetic Analysis of Methanol-Assimilating Bacteria in Denitrifying Consortium.** One hundred thirty-seven clones from the  $^{13}\text{C}$ -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  $^{13}\text{C}$ -DNA clone library are shown in phylogenetic trees (Figs. 2 to 5).

## 16S rRNA gene clone library



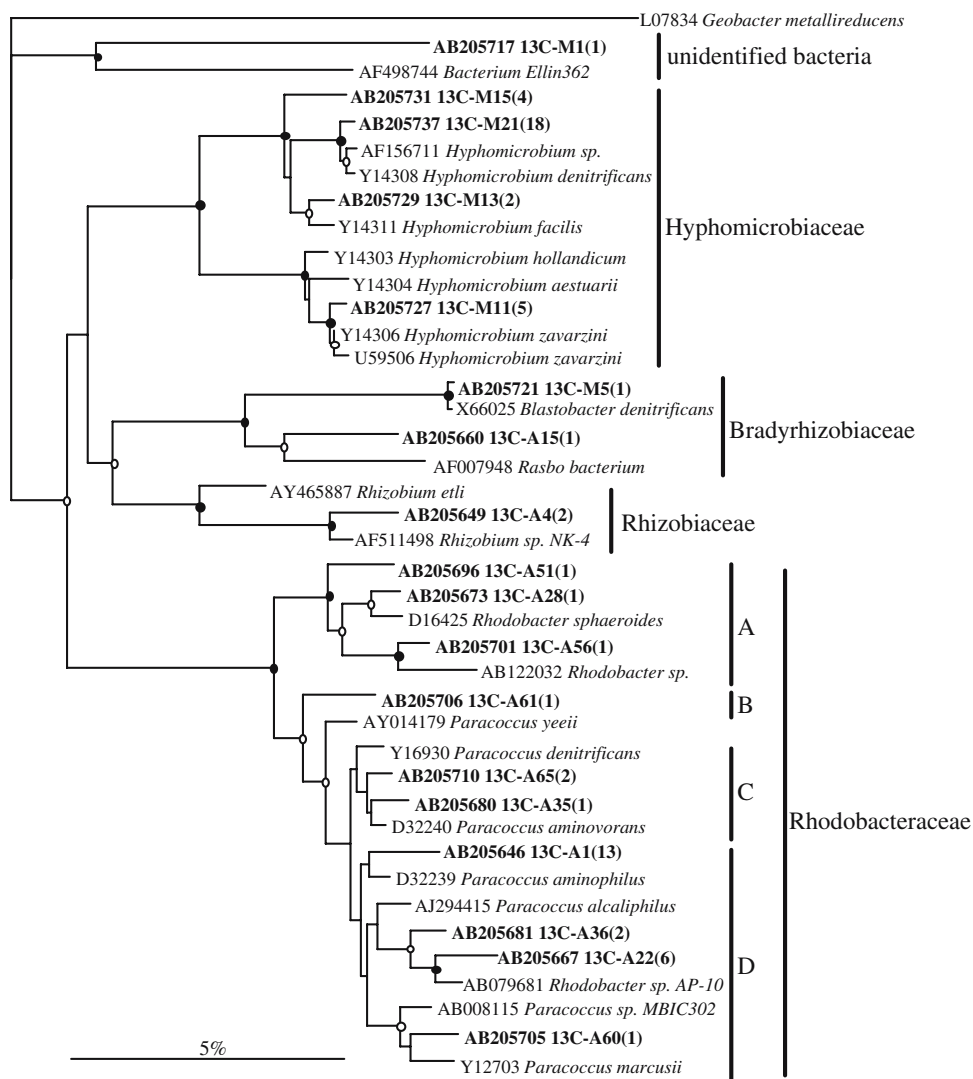
**Figure 1.** Histograms of bacterial community structure based on bacterial 16S rRNA gene clones retrieved from DNA extracted from original sludge samples (before incubation) and each  $^{12}\text{C}$ -DNA and  $^{13}\text{C}$ -DNA (incubated 3 days) in acetate and methanol samples.

**Table 1.** Phylogenetic affiliations and numbers of 16S rRNA gene clones retrieved from the  $^{12}\text{C}$ -DNA and  $^{13}\text{C}$ -DNA obtained by CsCl density gradient centrifugation

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

The  $^{13}\text{C}$ -DNA clone library showed two predominant groups related to known methyloproteobacteria. Of all the clones, 63%, including six different OTUs, were related to *Methylophilaceae* of the Betaproteobacteria, such as *Methylophilus*, *Methylobacillus*, and *Aminomonas*. Most of the *Methylophilaceae* are obligate methyloproteobacteria, which have the capacity to grow in the presence of methanol or methylamine but not in the presence of methane. A further

21% of all the clones, including four OTUs, were clustered within *Hyphomicrobiaceae* of the Alphaproteobacteria. The remaining clones in this library were clustered in the Alphaproteobacteria (e.g., *Blastobacter denitrificans*), Betaproteobacteria (e.g., *Ralstonia pickettii*, *Thaueria aromatica*, *Comamonas* sp.), Gammaproteobacteria (e.g., *Ewingella americana*), and Actinobacteria (e.g., *Carnobacterium piscicola* and *Nocardioides* sp.).



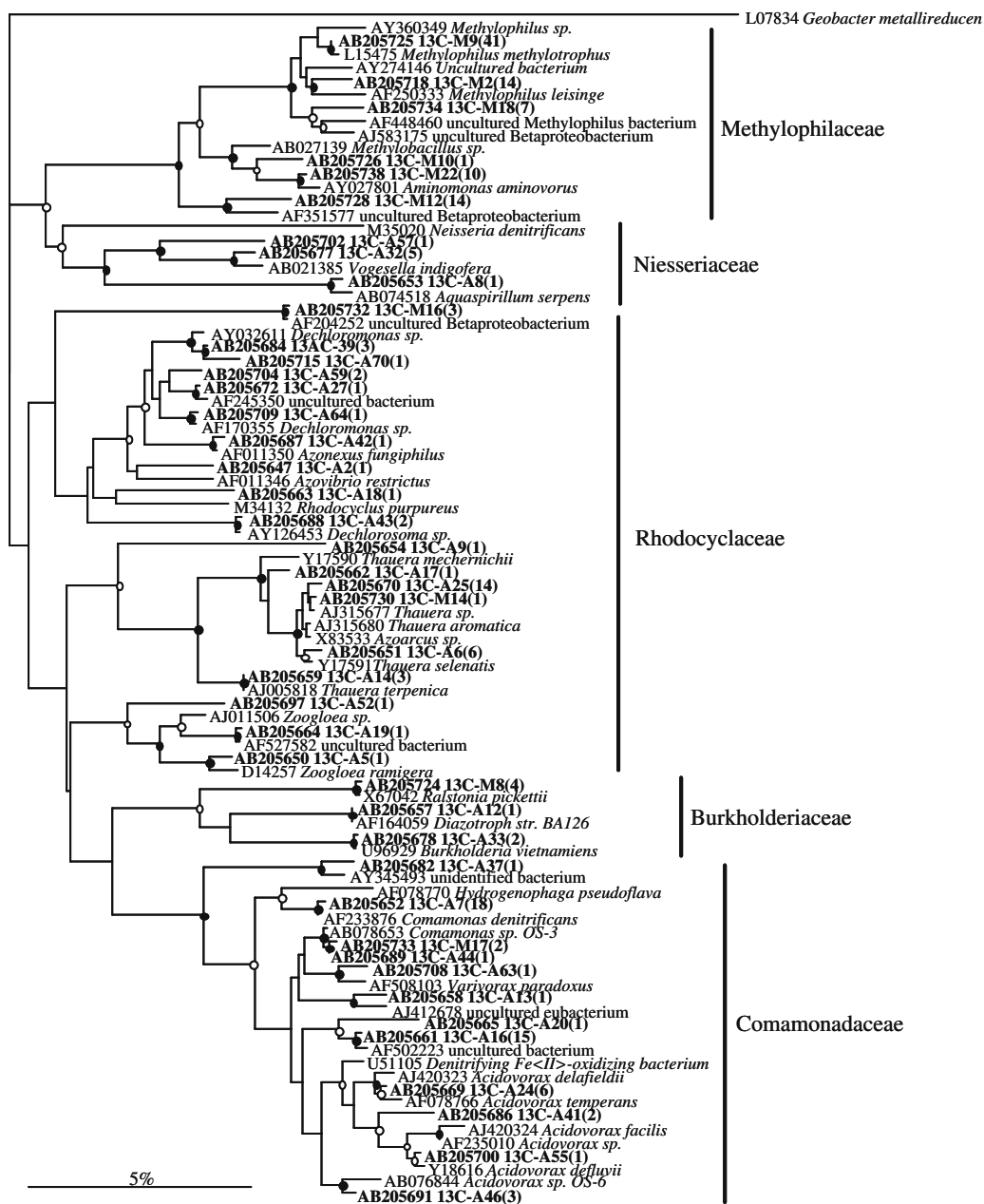
**Figure 2.** Phylogenetic affiliation of the alphaproteobacterial clones retrieved from each  $^{13}\text{C}$ -DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanol samples are labeled as 13C-A and 13C-M, respectively. The number of clones assigned to each sequenced OTU with more than 99% identity is shown in parentheses. *Geobacter metallireducens* (accession no. L07834) are used as the outgroup. 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.

**Nitrite Reductase Genes (*nirS* and *nirK*).** For the characterization of functional genes in acetate- and methanol-assimilating denitrifiers, the  $^{13}\text{C}$ -DNA fraction isolated in both experiments, in which activated sludge was incubated for 3 days, was used as a template for PCR amplification using the *nirS*- and *nirK*-specific primer pairs. Partial *nirS* and *nirK* sequences were determined and compared with those in the DDBJ database using BLAST. The phylogenetic analysis performed by the neighbor-joining method for all of the *nirS* and *nirK* clone sequences revealed that the denitrifying populations strongly depended on the type of organic carbon source (Figs. 6 and 7).

**(a) Nitrite Reductase Genes (*nirS* and *nirK*) in Acetate-Assimilating Populations.** One hundred seventeen *nirS* clones retrieved from the  $^{13}\text{C}$ -DNA were assigned to 27 OTUs (S-A1 to S-A27). About 88% of the *nirS* clones belonged to cluster II and were related to a *nirS* mRNA clone detected from estuarine sediment [26].

In particular, OTU S-A24 (56 clones) was the most dominant in the  $^{13}\text{C}$ -DNA retrieved from acetate samples. Cluster I was predominantly composed of *nirS* clones from acetate samples, which were related to the *nirS* genes of the genus *Thauera* (e.g., *T. aromatica* and *T. terpenica*). The *nirS* clones in cluster V were related to the *nirS* clone detected in cyanobacterial aggregates (AJ457201). Only *nirS* clones from the incubation with acetate medium were found in clusters III and VI. The *nirS* clones in cluster III were related to the *nirS* genes of *Azospirillum brasilense* (AJ224912) and the *nirS* clones in cluster VI were related to the *nirS* genes of *Ralstonia eutropha* (X91394).

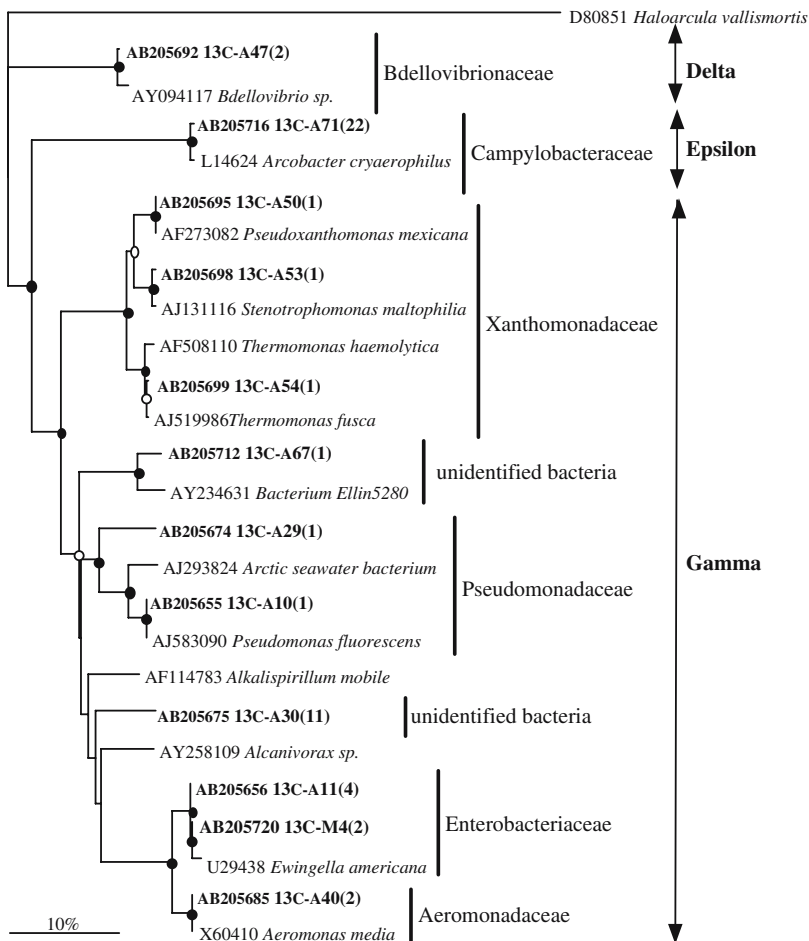
Eighty-seven *nirK* clones retrieved from the  $^{13}\text{C}$ -DNA were assigned to 27 OTUs (K-A1 to K-A29). Each of the *nirK* clones from the incubation with acetate medium belonged to all *nirK* clusters except for cluster V (I to IV and VI–XI). Most of the *nirK* clones showed less than 90% identity with *nirK* genes of isolated denitrifiers, such as *Mesorhizobium*, *Pseudomonas*, and *Rhizobium*. OTU



**Figure 3.** Phylogenetic affiliation of the betaproteobacterial clones retrieved from each  $^{13}\text{C}$ -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. 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.

K-A2 was highly related to the *nirK* genes of *Alcaligenes faecalis* (D13155). In the *nirK* clone library, the dominant OTUs were K-A3 (9 clones), K-A4 (14 clones), K-A17 (11 clones) and K-A19 (9 clones). OTUs K-A3 and K-A17 were related to the *nirK* clones detected in activated sludge and soil. OTU K-A4 was related to the *nirK* gene of *Alcaligenes* sp. (AB046603), and OTU K-A19 was related to the *nirK* gene of *Pseudomonas* sp. (M97294).

(b) *Nitrite Reductase Genes (*nirS* and *nirK*) in Methanol-Assimilating Populations.* Eighty-one *nirS* clones retrieved from the  $^{13}\text{C}$ -DNA were assigned to five OTUs (S-M1 to S-M5). About 96% of the total *nirS* clones belonged to cluster II and were related to a *nirS* mRNA clone detected from estuarine sediment [26]. In particular, OTU S-M4 (75 clones) was the most dominant in the  $^{13}\text{C}$ -DNA clone library generated from methanol samples. The *nirS* gene sequences of OTU S-M5 (1 clone)



**Figure 4.** Phylogenetic affiliation of the gamma-, delta-, and epsilonproteobacterial clones retrieved from each  $^{13}\text{C}$ -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. 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.

belonging to cluster I were related to the *nirS* genes of *T. aromatica* (AY078257). The remaining clones were related to the *nirS* clones detected in a cyanobacteria aggregate (AJ457201 and AJ457200).

Eighty *nirK* clones retrieved from the  $^{13}\text{C}$ -DNA were assigned to four OTUs (K-M1 to K-M4). The *nirK* clones from methanol samples were found in clusters III and V. OTU K-M4 (1 clone) was closely related to the *nirK* genes of *Hyphomicrobium zavarzinii* (AJ224902), and the remaining clones were related to the *nirK* genes of *Rhizobium hedysari* (U65658). In particular, K-M1 (71 clones) was the most dominant clone in the  $^{13}\text{C}$ -DNA clone library generated from methanol samples.

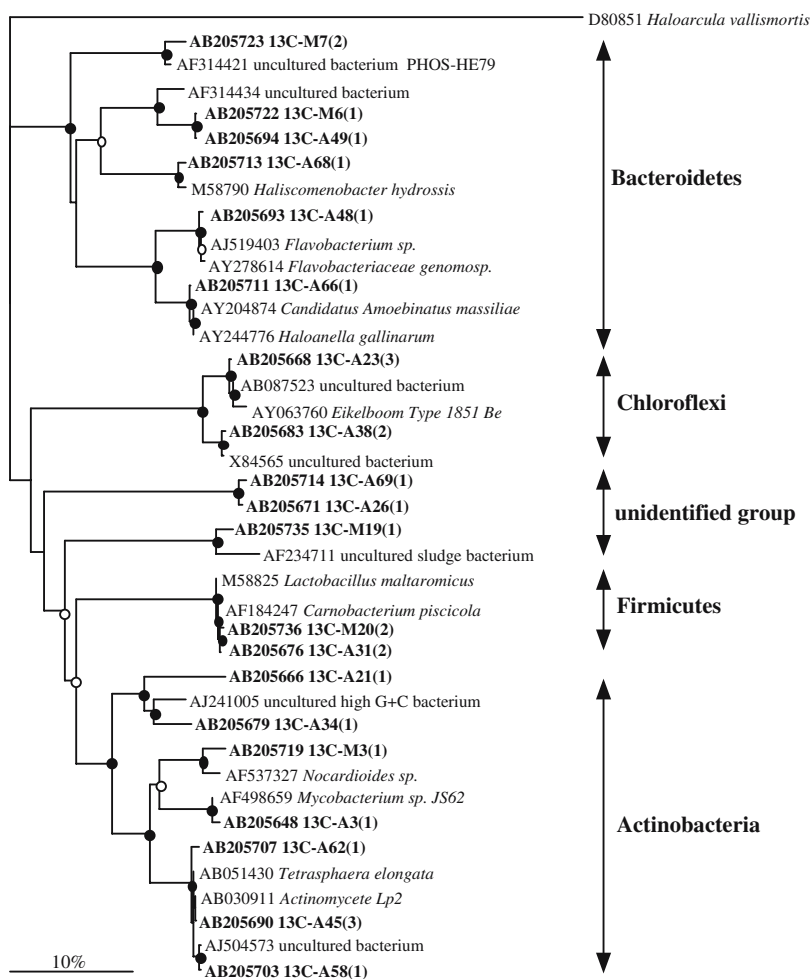
**Rarefaction Analysis.** The diversity of the 16S rRNA gene clones and *nir* clones isolated from acetate and methanol samples and the original sludge sample was evaluated by rarefaction analysis (Fig. 8). The 16S rRNA gene clones retrieved from the  $^{13}\text{C}$ -DNA indicated that the diversity level of methanol-assimilating bacteria was much lower than that of acetate-assimilating bacteria. Similarly, rarefaction analyses of *nirS* and *nirK*

clones showed that the diversity level of methanol-assimilating denitrifiers was much lower than that of acetate-assimilating denitrifiers. There were no significant differences between the diversity levels of the 16S rRNA gene clones retrieved from the original sludge sample and the  $^{12}\text{C}$ -DNA in both methanol and acetate samples. Therefore, these results implied that the 3 days of incubation in the synthetic wastewater had no significant effects on the bacterial diversity.

## Discussion

In this study, 16S rRNA gene- and functional gene (nitrite reductase gene)-targeted analysis combined with 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 effect 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-assim-



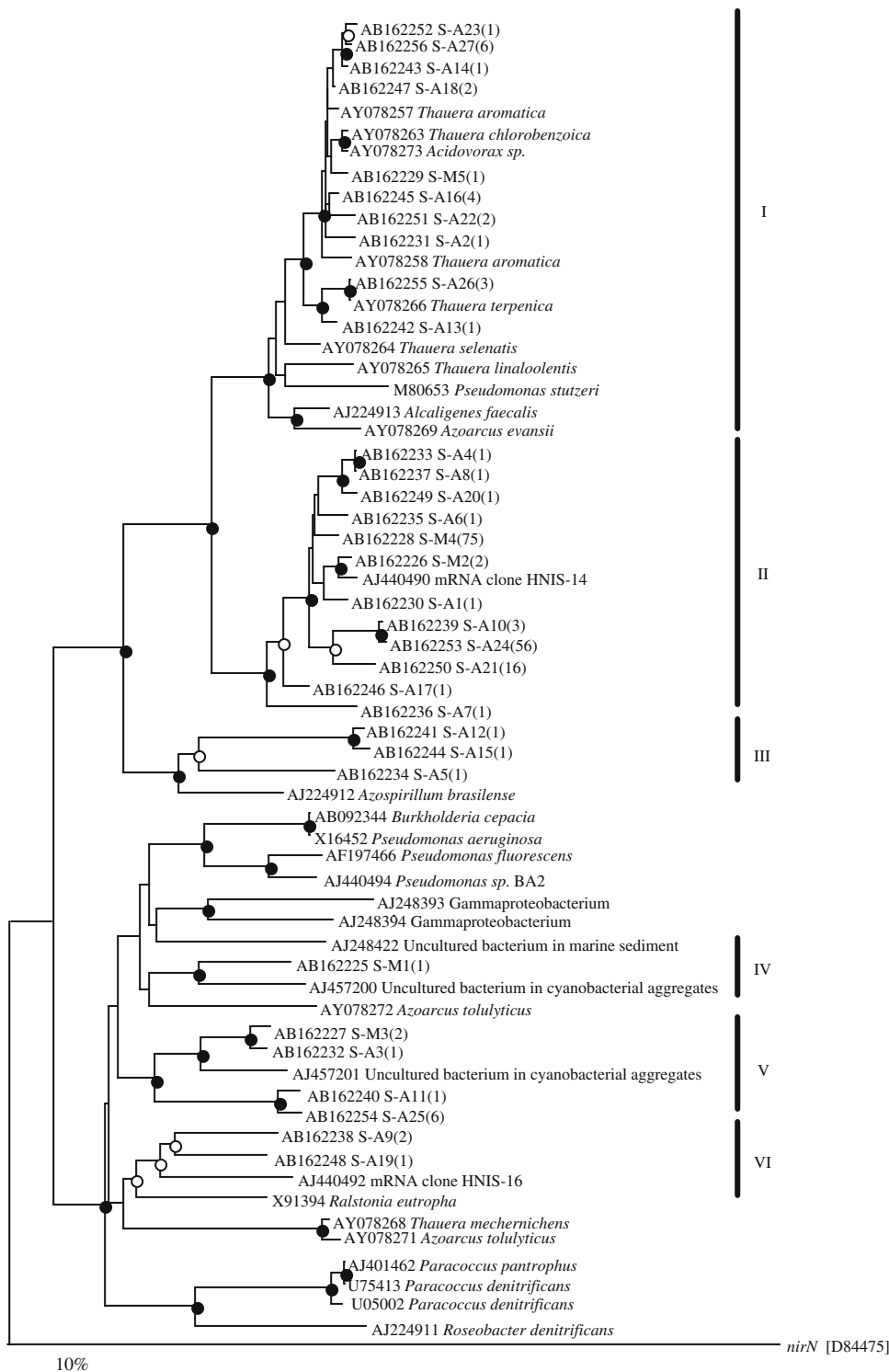


**Figure 5.** Phylogenetic affiliation of the remaining bacterial clones except proteobacterial clones retrieved from each  $^{13}\text{C}$ -DNA by neighbor-joining analysis. The partial 16S rRNA gene sequences obtained from acetate and methanol samples are labeled 13C-A and 13C-M, respectively. The 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. 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.

ilating denitrifiers in freshwater environments [16, 17, 25]. 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 [14]. Thus, we used SIP in the identification of active methanol-assimilating bacteria under nitrate-reducing conditions and found the existence of two predominant methanol-assimilating populations in the  $^{13}\text{C}$ -DNA clone library from methanol samples. One major phylotype was related to the members of the *Methylophilaceae* of the Betaproteobacteria, such as the genera *Methylophilus*, *Methylobacillus*, and *Aminomonas*. Maneesha *et al.* also reported on a methanol-fed denitrifying community in activated sludge using the SIP and MAR-FISH techniques [20]. In their study, the dominant 16S rRNA gene phylotype in the  $^{13}\text{C}$ -DNA clone library was closely related to those of the obligate methylophil of *Methylophilaceae*, most of which assimilated [ $^{14}\text{C}$ ]methanol in the MAR-FISH

analysis. The other major phylotype was related to the genus *Hyphomicrobium*. The genus *Hyphomicrobium* (hyphomicrobia) consists of restricted facultative methylophilic and has been found in soils, aquatic habitats, and a sewage treatment plant [30]. The diversity of hyphomicrobia in a sewage treatment plant and an adjacent receiving lake has been examined by Holm *et al.* [11]. 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 *B. denitrificans*, which was reported as a methanol-assimilating denitrifier [41]. 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.

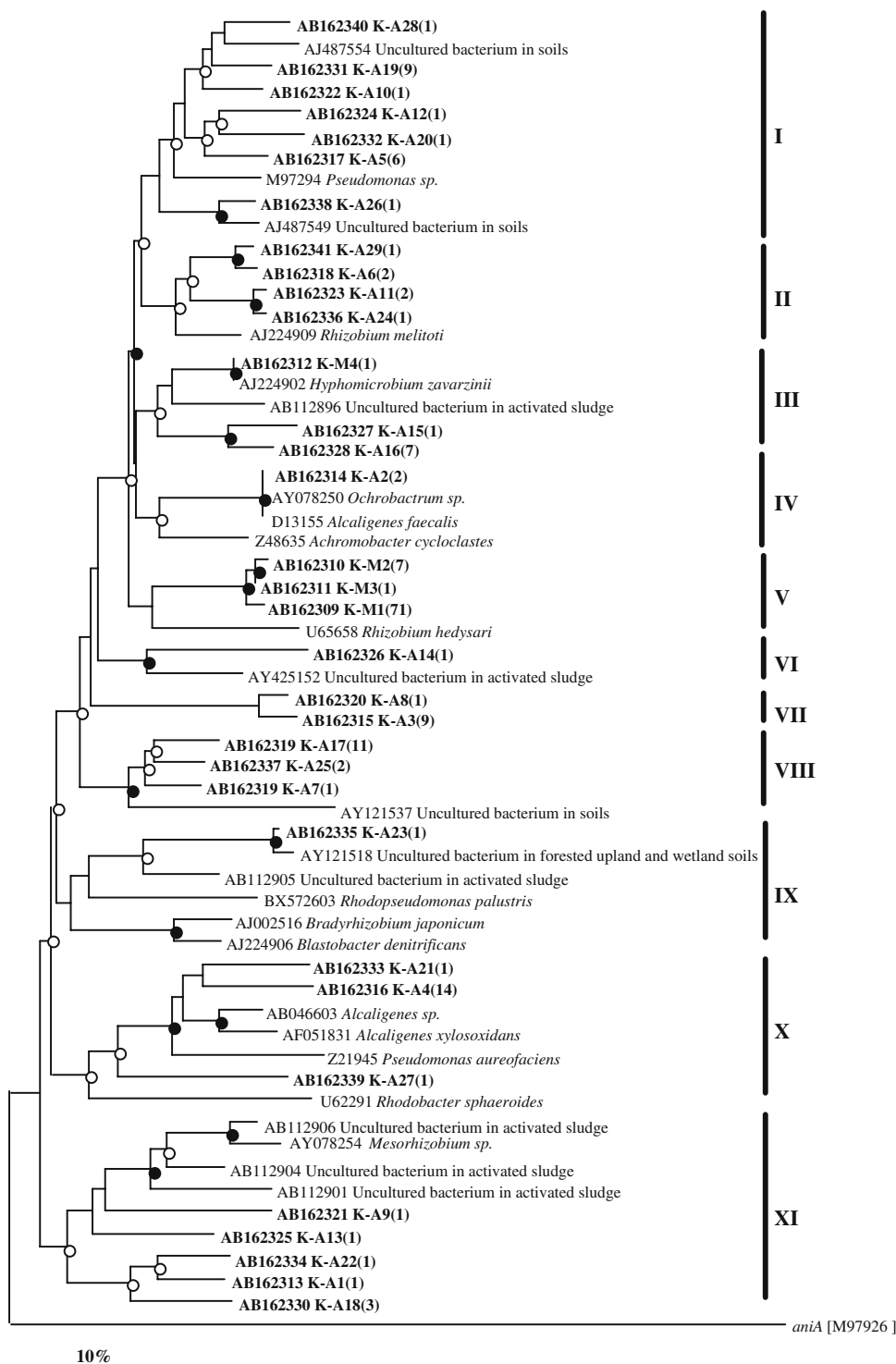
No clones related to the members of the family *Rhodobacteraceae* (e.g., *Paracoccus* and *Rhodobacter*), which were reported as methanol-assimilating denitrifiers, were detected in the  $^{13}\text{C}$ -DNA clone library generated from methanol samples. However, a number of clones related to the family *Rhodobacteraceae* were



**Figure 6.** Neighbor-joining analysis of partial *nirS* gene products (261 amino acids) from the  $^{13}\text{C}$ -DNA retrieved from acetate samples (S-A) and methanol samples (S-M). The number of clones assigned to each *nirS* sequence with more than 97% identity is shown in parentheses. *nirN* from *Pseudomonas aeruginosa* (accession no. D84475) was used as the outgroup. 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.

detected in the  $^{13}\text{C}$ -DNA clone library from acetate samples and in the  $^{12}\text{C}$ -DNA clone library from both acetate and methanol samples. Seven sequences obtained from the  $^{12}\text{C}$ -DNA of the acetate samples belonged to cluster A, and 13 sequences obtained from the  $^{12}\text{C}$ -DNA of the methanol samples belonged to cluster A (9 clones)

and cluster C (4 clones) (Fig. 2). 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 [13]. On the other hand, Claus and



**Figure 7.** Neighbor-joining analysis of partial *nirK* gene products (171 amino acids) from the  $^{13}\text{C}$ -DNA retrieved from acetate samples (K-A) and methanol samples (K-M). The number of clones assigned to each *nirK* sequence with more than 97% identity is shown in parentheses. *aniA* from *Neisseria gonorrhoeae* (accession no. M97926) was used as the outgroup. 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.

Kutzner [6] 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

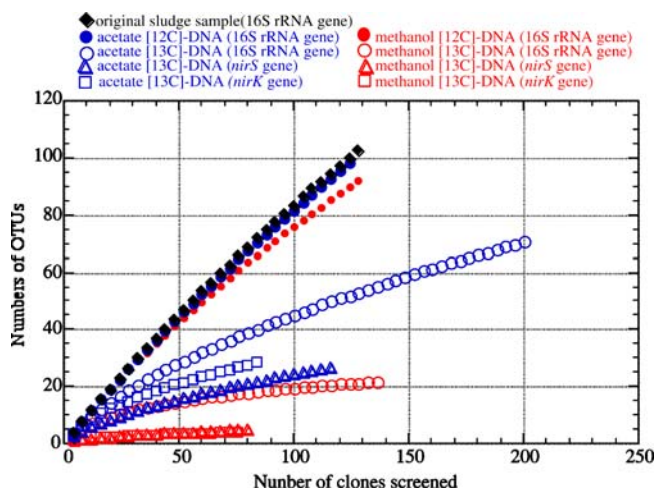
intermediates and products might become incorporated into the DNA of other microbial communities when primary consumers metabolize the original substrates and excrete labeled metabolites. However, in this study, we considered this limitation unlikely because no clone obtained from the  $^{13}\text{C}$ -DNA isolated from methanol samples was related to the genus *Paracoccus*.

In denitrifying functional gene analysis of methanol-assimilating bacterial populations, a *nirK* clone highly related to the *nirK* gene of *H. zavarzini* was detected only in the  $^{13}\text{C}$ -DNA retrieved from methanol samples. However, most of the *nirS* and the *nirK* clones were not closely related to the *nir* sequences of any known cultivated bacteria or *nir* clones found in various environments. Because the denitrification ability of methylotrophs was not previously characterized, there is a lack of *nirS* and/or *nirK* gene sequence data for most of the methylotrophs in the database. This might support the finding that many *nirS* and *nirK* clones found in this study, such as *nirS* clones S-M1 to S-M4 and *nirK* clones K-M1 to K-M3, were not related to any of the *nirS* and *nirK* gene sequences registered in the database. In future studies, we should obtain *nir* sequences from some pure cultures or isolates belonging to methanol-assimilating denitrifiers (e.g., *Methylophilaceae* and *Hyphomicrobiaceae*) identified by 16S rRNA gene clone libraries generated from the  $^{13}\text{C}$ -DNA clone library.

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 and Nielsen [22] 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  $^{13}\text{C}$ -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  $^{13}\text{C}$ -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 organisms in 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 [7, 13, 21, 45, 48]. Wagner and Loy reported that the *Azoarcus*–*Thauera* group of Betaproteobacteria represents numerous denitrifiers in wastewater treatment systems [43]. As for the genus *Arcobacter*, *A. cryaerophilus* was shown to be abundant in activated sludge [37]. 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 [39, 42, 46]. Teske *et al.* reported that *Arcobacter* spp. use acetate as the electron donor and nitrate as the electron

acceptor [39]. However, to date, their denitrification ability in the environment has not been well documented.

Most of the acetate- or methanol-assimilating bacteria identified by SIP have been known as denitrifiers in wastewater treatment systems. There were interesting findings for 16S rRNA gene and nitrite reductase gene clone libraries in  $^{13}\text{C}$ -DNA clone libraries. The *Thauera*-related groups and *Hyphomicrobium*-related groups were detected in both 16S rRNA gene- and nitrite reductase gene clone libraries. However, no *nirS* or *nirK* clone retrieved from  $^{13}\text{C}$ -DNA had *nir* sequences related to the *Paracoccus* isolates, which were found to be dominant by 16S rRNA gene analysis, whereas *nirK* clones retrieved from  $^{13}\text{C}$ -DNA were highly related to *A. faecalis* (99% identity), which was not detected by 16S rRNA gene analysis. Although it may not be appropriate to compare the results obtained by the 16S rRNA gene- and the nitrite reductase gene-based approach, we discuss possible explanations for these findings below. First, the 16S rRNA gene approach detects all organisms that are active under conditions that are nitrate reducing or anaerobic (e.g., fermentation), not only under denitrifying conditions. Second, there is insufficient analysis of a statistically significant number of clones required for understanding complex communities. In this study, some rarefaction curves did not reach a plateau; reaching a plateau would indicate that the diversity was sufficiently analyzed. Third, there may be bias due to PCR amplification and degenerate primers. Lueders and Friedrich [19] reported that the degeneracy of primers has a considerable impact on bacterial diversity. They showed a decrease in bacterial diversity with increase in annealing temperature within a bacterial community model. However, highly degenerate primers often have to be used for functional genes to cover a wide phy-



**Figure 8.** Rarefaction curves indicating the diversity of denitrifying bacteria as OTU of 16S rRNA sequences and functional gene (*nirS* and *nirK*) sequences retrieved from methanol and acetate samples and the original sludge sample.

logentic range because the sequence conservation of functional genes in comparison to 16S rRNA genes is constrained by the genetic code and its variability. Therefore, we should carefully characterize microbial community structures obtained by PCR-based analysis using highly degenerate primers, which might enhance the bias of PCR amplification. Other factors might be attributed to a lack of registered *nir* sequences of isolates with phylogenetic information from the database and to horizontal gene transfer. For instance, most of the *nirS* sequences registered in the database are retrieved from uncultured clones. Although only a part of the sequences are retrieved from pure cultures, most of these sequences are affiliated with limited bacterial groups (e.g., *Alcaligenes*, *Marinobacter*, *Pseudomonas*, *Paracoccus*, and *Thauera*). Thus, the primer sets used for detecting denitrifying bacteria, *nirS1F*–*nirS6R* and *nirK1F*–*nirK5R*, were designed on the basis of a limited number of sequences, mainly from laboratory strains. These primers were most commonly used, but this may need to be reassessed. Therefore, denitrifying gene sequences need to be obtained from many different isolates whose phylogenetic 16S rRNA positions have been identified. In particular, the denitrifying genes of candidates for true denitrifiers (e.g., the families *Comamonadaceae*, *Hyphomicrobiaceae*, *Methylophilaceae*, and *Rhodobacteraceae*) should be characterized because these bacteria are probably important populations in the nitrogen-removal system.

As for the horizontal gene transfer, Song and Ward reported an interesting correlation between 16S rRNA and nitrite reductase genes (*nirS*) and discussed the possibility of the horizontal gene transfer of *nirS* [38]. Most of the *nirS* genes from halobenzoate-degrading denitrifiers affiliated with the genera *Thauera*, *Acidovorax*, and *Azoarcus* of Betaproteobacteria are related to the *nirS* gene of *Pseudomonas stutzeri* of the Gammaproteobacteria, although phylogenetic relationships based on 16S rRNA genes from these genera are very different. The authors said that this might provide evidence for horizontal gene transfer of *nirS* among the genera *Pseudomonas*, *Thauera*, *Acidovorax*, and *Azoarcus* [38]. However, it was difficult to infer the horizontal gene transfer from our data sets at the community level.

This study showed how bacterial communities could be stimulated by external carbon sources (e.g., acetate and methanol) in denitrifying tanks. Moreover, SIP can be used for screening candidates for true denitrifiers from complex bacterial communities, such as activated sludge. Furthermore, the combination of SIP and other molecular techniques, such as MAR-FISH and real-time PCR, should significantly enhance our understanding of active denitrifying bacteria in activated sludge. Thus, in the future, the understanding of acetate- or methanol-assimilating bacteria under nitrate reducing conditions

will be useful for the efficient and stable operation and suitable maintenance of nitrogen-removal systems.

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