Chapter 3

Characterization of nitrite reductase (*nirK* and *nirS*) genes in acetate- or methanol-assimilating bacteria under nitrate reducing conditions

Summary

Using DNA-SIP in combination with analysis of denitrifiers-specific functional gene markers, we characterized the active denitrifying population in activated sludge. ^{13}C-methanol or ^{13}C–sodium acetate was used as the main organic carbon source under denitrification conditions. Phylogenetic analysis of nitrite reductase genes (nirS and nirK) indicated that the denitrifying bacterial population strongly depended on the type of organic carbon source. In methanol-assimilating denitrifying bacteria, many nirK and nirS sequences were newly detected, and a nirK sequence closely related to that of nirK genes of the genus Hyphomicrobium was also detected. In acetate-assimilating denitrifying bacteria, nirK sequences related to those of nirK genes of the genus Alcaligenes and nirS sequences closely related to those of nirS genes of the genus Thauera were detected, and many nirK and nirS sequences were newly detected. Rarefaction curves of nirK and nirS clones derived from ^{13}C-DNA showed that the diversity levels for methanol-assimilating denitirifying bacteria were extremely lower than those for acetate-assimilating denitrifying bacteria for both the nirS and nirK genes. Although previous studies showed that the diversity of the nirK gene was lower than that of the nirS gene in various environments, DNA-SIP analysis indicated that the diversity of the nirK gene in active denitrifying bacteria was almost equal to that of the nirS gene in active denitrifying bacteria in the activated sludge of a municipal sewage treatment system. Additionally, DNA-SIP analysis suggested that most of the nirS genes of the active denitrifying population in activated sludge might be derived from horizontal gene transfer.

3.1 Introduction

Denitrification ability is found among a wide variety of taxonomic groups (Zumft, 1997). Thus, denitrifying bacteria cannot be identified specifically on the basis of the 16S rRNA gene sequence. Recently, denitrifying bacteria belonging to different phylogenetic groups have been detected simultaneously using primer sets specific for the functional genes involved in denitrification; genes encoding periplasmic and membrane-bound nitrate reductase (*napA* and *narG*) (Cheneby *et al*., 2003; Flanagan *et al*., 1999; Gregory *et al*., 2000), genes encoding cytochrome cd_1 and copper-containing nitrite reductase (*nirS* and *nirK*, respectively) (Braker *et al*., 1998; Braker *et al*., 2001; Hallin and Lindgren, 1999; Liu *et al*., 2003; Nogales *et al*., 2002; Prieme *et al*., 2002; Song and Ward, 2003), and a gene encoding a nitrous oxide reductase (*nosZ*) (Scala and Kerkhof, 1998; Scala and Kerkhof, 1999). Then, PCR-based approaches using these primers revealed the ecophysiology of denitrifying bacteria in activated sludge (Hallin and Lindgren, 1999), marine sediment (Braker *et al*., 2001; Liu *et al*., 2003; Scala and Kerkhof, 1998) and soil (Nogales *et al*., 2002; Prieme *et al*., 2002; Scala and Kerkhof, 1999). However, truly active denitrifying bacteria in various environments cannot be identified by these approaches.

DNA-based stable-isotope probing (DNA-SIP) is a useful tool for the isolation of the genome of microorganisms that are acitively involved in ecologically important processes (Radajewski *et al*., 2000). Thus, DNA-SIP studies can be accessed not only SSU rRNA genes to identify the taxonomy of microorganisms involved in the process of interest but also functional genes that encode key enzyme in specific metabolic pathways when a specific metabolic function has been previously established for cultivated microorganisms (Borodina *et al*., 2005; Lin *et al*., 2004; Neufeld *et al*., 2007; Radajewski *et al*., 2002). In this study, we report here on the identification of nitrite reductase genes sequences which probably represent the active denitrifying populations that utilized acetate or methanol under denitrifying conditions in sewage sludge.

3.2 Materials and Methods

3.2.1 PCR amplification of nitrite reductase-encoding genes (*nirK* and *nirS*)

The fragments of *nirK* and *nirS* genes were obtained from the DNA extracted from the original sludge sample and the ^{13}C-DNA obtained from sludge samples incubated with ^{13}C-acetate or ^{13}C-methnol as described in chapter 2. The following primer sets were used for PCR amplification: (i) nirK1F-nirK5R (Braker *et al*., 1998) for the amplification of *nirK* fragments, and (iii) nirS1F-nirS6R (Braker *et al*., 1998) for the amplification of *nirS* fragments. The PCR mixture contained 0.5 μM of each primer, 200 μM of dNTP, 2.0 mM $MgCl_2$ for *nirK* and 1.5mM $MgCl_2$ for *nirS*, 1.25 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) for *nirK* and 2.5 U of rTaq DNA polymerase for the *nirS*, 2 μl of 10xPCR buffer for rTaq, and sterile water added to a final volume of 20 μl. The PCR amplifications of *nirK* and *nirS* were conducted in a model 9700 thermal cycler (Applied Biosystems, Foster City, Calif.) using the following programs: (i) *nirK*; 5 min at 94 °C, 30 cycles (30 sec at 94 °C, 40 sec at 48 °C, 40 sec at 72 °C), 5 min at 72 °C, (ii) *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. The presence of PCR products was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide.

3.2.2 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 *nir* gene fragments were sequenced with an ABI PRISM 3100-*Avant* DNA Sequencing system (Applied Biosystems). The *nir* gene sequences with more than 97% 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).

3.2.3 Nucleotide sequence accession numbers

The DDBJ accession numbers for the *nir* clones are as follows: *nirS*, AB162225 - AB162308; *nirK*, AB162309 - AB162341.

3.3 Results

3.3.1 Amplification of nitrite reductase-encoding genes (*nirK* and *nirS*)

For the characterization of functional genes in acetate- and methanol-assimilating denitrifiers, the ^{13}C-DNA fraction isolated from sludge samples incubated with ^{13}C-acetate or ^{13}C-methanol, in which activated sludge was incubated for three days, was used as a template for PCR amplification using the *nirK*- and *nirS*-specific primer pairs. Although nonspecific amplification products were detected for both reactors for *nirK* and *nirS*, PCR products of the expected sizes, which were about 515 bp for *nirK* and 890 bp for *nirS*, were clearly detected in sludge samples from acetate samples or methanol samples. Therefore, PCR products of the expected sizes were eluted from agarose gels and cloned, and subsequent sequence analysis was performed for these samples. The phylogenetic analysis of the *nirK* and *nirS* clone sequences detected from the ^{13}C-DNA revealed that the denitrifying populations strongly depended on the type of organic carbon source (**Fig. 3.1** - **Fig. 3.2**).

Additionally, DNA was extracted from activated sludge in municipal sewage treatment systems, from where sludge samples were originally collected, and then used as a template for PCR amplification using the *nirK*- and *nirS*-specific primer pairs. As a result, *nirK* genes could not be amplified from the original sludge samples although the *nirS* gene was amplified.

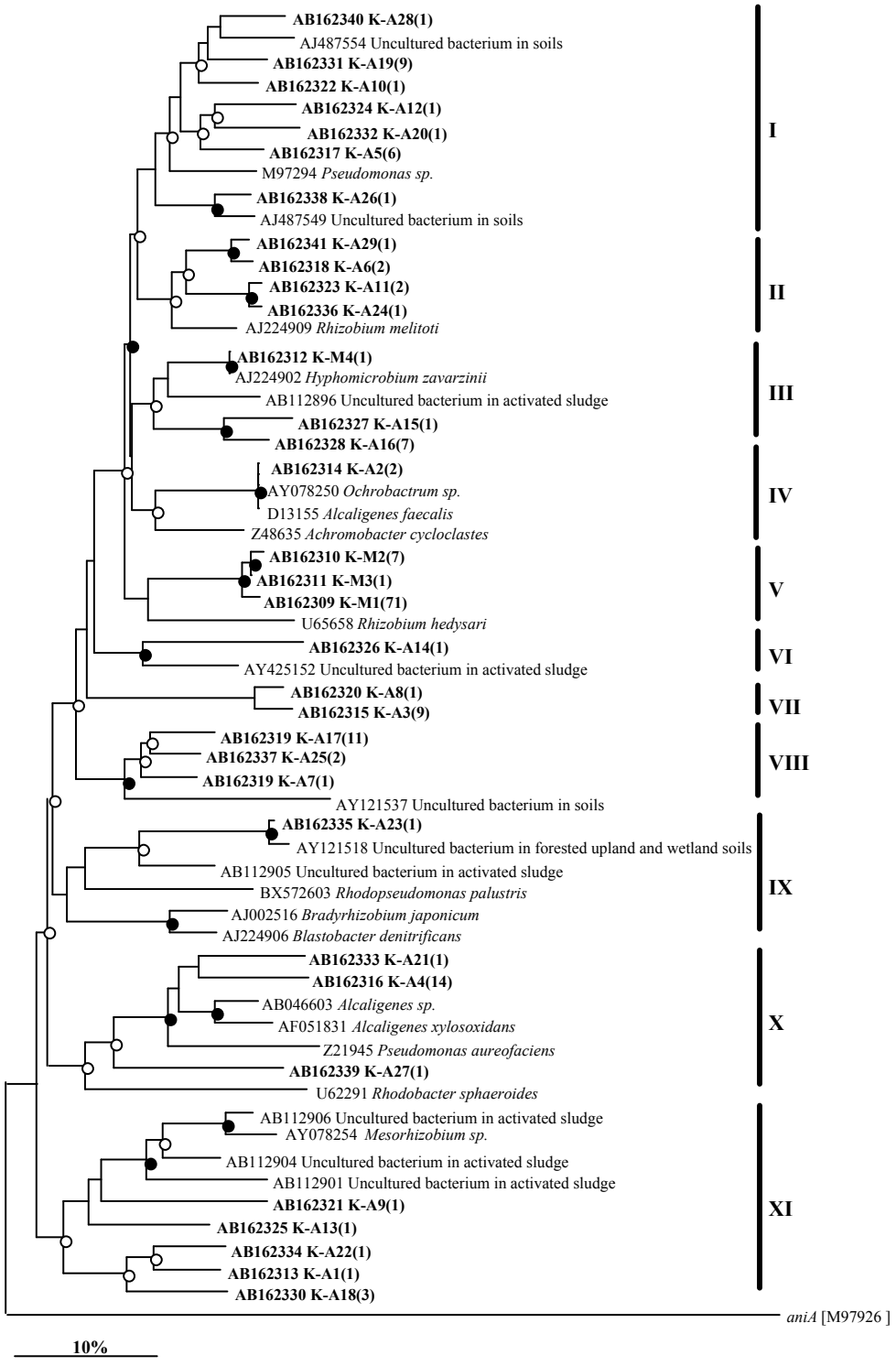

Fig. 3.1. Neighbor-joining analysis of partial *nirK* gene products (171 amino acids) from the [^{13}C]-DNA retrieved from acetate samples (symbol: K-A) and methanol samples (symbol: K-M). The number of clones assigned to each *nirK* sequences with more than 97% identity is shown in parentheses. *aniA* from *Neisseria gonorrhoeae* (accession no. M97926) was 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.

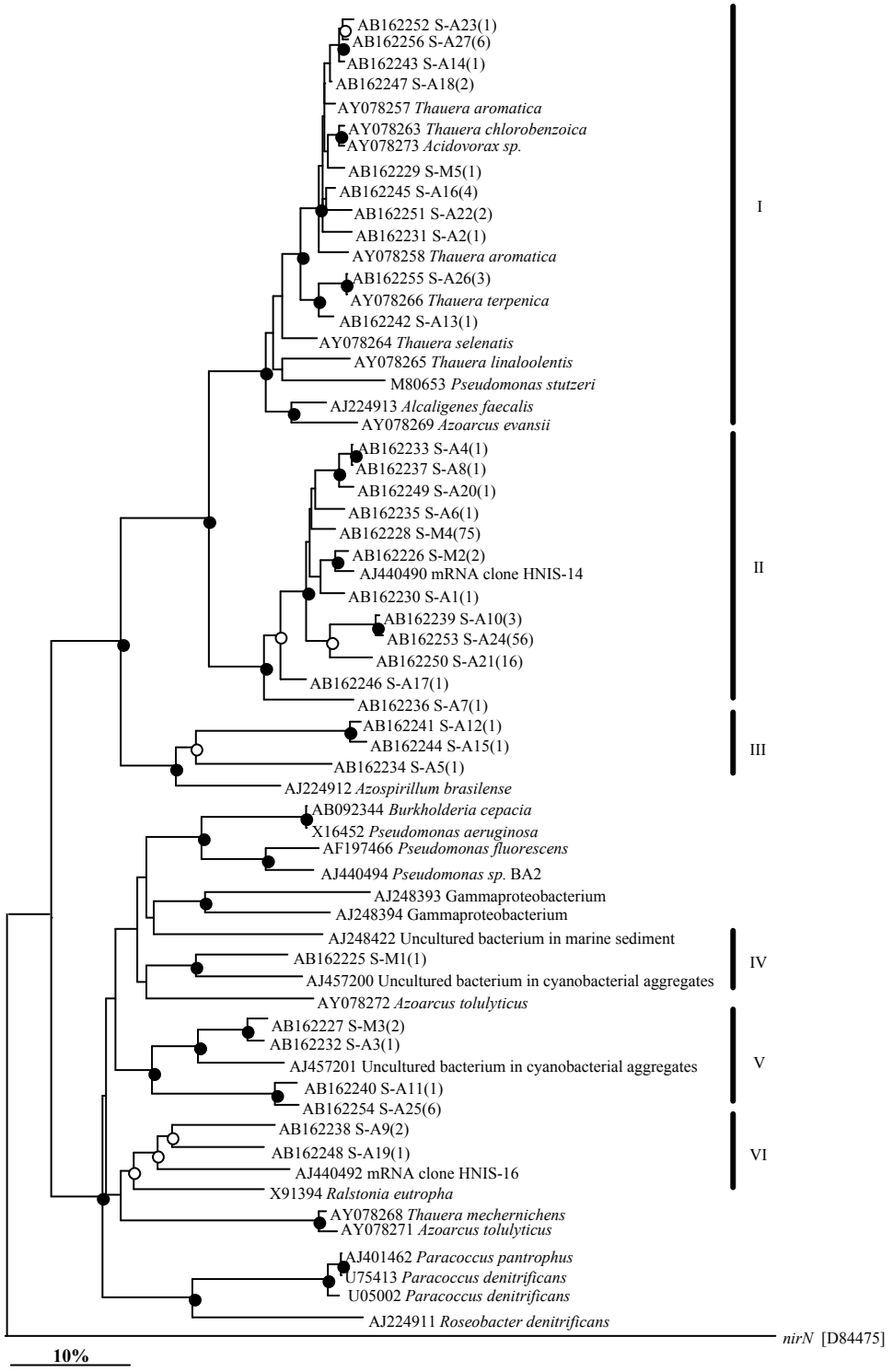

Fig. 3.2. Neighbor-joining analysis of partial *nirS* gene products (261 amino acids) from the ^{13}C-DNA retrieved from acetate samples (symbol: S-A) and methanol samples (symbol: S-M). The number of clones assigned to each *nirS* sequences with more than 97 % identity is shown in parentheses. *nirN* from *Pseudomonas aerginosa* (accession no. D84475) was 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.

3.3.2 Nitrite reductase genes (*nirK* and *nirS*) in acetate-assimilating populations

Eighty-seven *nirK* clones retrieved from the ^{13}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 to *nirK* genes of isolated denitrifiers, such as *Mesorhizobium*, *Pseudomonas*, and *Rhizobium*. OTU 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).

One hundred seventeen *nirS* clones retrieved from the ^{13}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 (Nogales *et al*., 2002). In particular, OTU S-A24 (56 clones) was the most dominant in the ^{13}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 *Azospirllum brasilense* (AJ224912) and the *nirS* clones in cluster VI were related to the *nirS* genes of *Ralstonia eutropha* (X91394).

3.3.3 Nitrite reductase genes (*nirS* and *nirK*) in methanol-assimilating populations

Eighty *nirK* clones retrieved from the ^{13}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}C-DNA clone library generated from methanol samples.

Eighty-one *nirS* clones retrieved from the ^{13}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 (Nogales *et al*., 2002). In particular, OTU S-M4 (75 clones) was the most dominant in the ^{13}C-DNA clone library generated from methanol samples. The *nirS* gene sequences of OTU S-M5 (1 clone) belonging to cluster I were related to the *nirS* genes of *Thauera aromatica* (AY078257). The remaining clones were related to the *nirS* clones detected in a cyanobacteria aggregate (AJ457201 and AJ457200).

3.3.4 Rarefaction analysis for evaluation of *nir* gene diversity

The diversity of the *nir* clones isolated from acetate and methanol samples and the original sludge sample was evaluated by rarefaction analysis (**Fig. 3.3**). 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. In comparison with the diversity of *nirS* and *nirK*, it was found that *nirK* exhibited higher diversity than *nirS* in acetate-assimilating populations. On the other hand, the diversity of *nirS* and *nirK* was almost the same in methanol-assimilating populations. The *nirS* diversity in the original sludge sample characterized without SIP treatment was about twice as high as that in acetate-assimilating populations, and about ten times as high as that in methanol-assimilating populations. These results indicated that various denitrifying bacteria possessing *nirS* were present in the municipal sewage treatment systems.

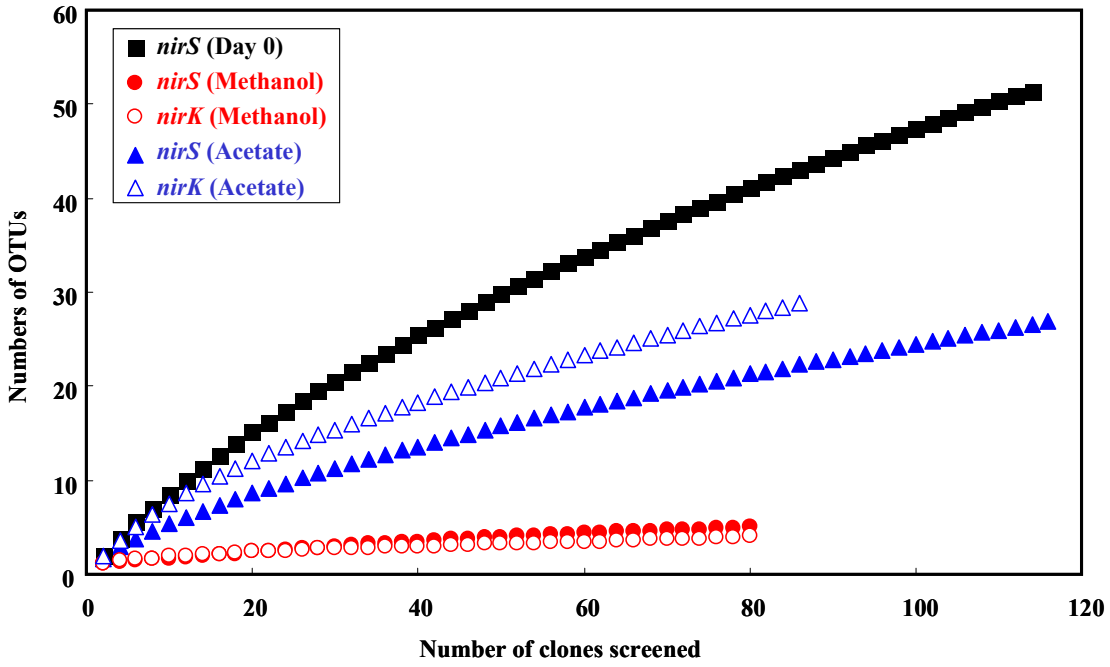

Fig. 3.3 Rarefaction curves indicating the diversity of denitrifying bacteria as OTU of nitrite reductase gene (*nirS* and *nirK*) sequences retrieved from methanol and acetate samples and the original sludge sample.

3.4 Discussion

In denitrifying functional gene analysis of methanol-assimilating bacterial populations, a *nirK* clone highly related to the *nirK* gene of *Hyphomicrobium zavarzinii* was detected only in the ^{13}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. In a previous study, the genera *Hyphomicrobium*, *Paracoccus, Hydrogenophaga*, *Rhodobacter*, and *Blastobacter* have been reported to contain methanol-assimilating denitrifying bacteria in freshwater (Lemmer *et al*., 1997a; Lemmer *et al*., 1997b; Neef *et al*., 1996). However, the active methanol-assimilating denitrifying bacteria in the actual environment might have been missed because these members were identified by culture-dependent methods. Therefore, it is necessary to characterize methanol-assimilating denitrifying bacteria by a molecular biology technique linking microbial function with taxonomic identity. Additionally, there is a lack of *nirS* and/or *nirK* gene sequence data for most of the methlyotrophs in the database because the denitrification ability of methylotrophs was not previously characterized. This might support the finding that many *nirS* and *nirK* clones detected 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}C-DNA clone library in chapter 2.

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}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}C-DNA had *nir* sequences related to the *Paracoccus* isolates, which were found to be dominant by 16S rRNA gene analysis, wheras *nirK* clones retrieved from ^{13}C-DNA were highly related to *Alcaligenes faecalis* (99% identity), which was not detected by 16S rRNA gene analysis. Although it was not necessarily appropriate to make compare results obtained by the 16S rRNA gene- and nitrite reductase genes-based approach, we discuss the possible explanations for these findings as follows. First, the 16S rRNA gene approach detects all organisms that are active under nitrate-reducing or anaerobic (e.g. fermentation) but not under only denitrifying conditions. Second, there is a lack of analysis of a statistically significant number of clones required for understanding complex communities. In this study, some rarefaction curves did not reach a plateau, which would indicate that the diversity was sufficiently analyzed. Third, there might be the bias of PCR amplification and degenerated primers. Lueders and Friedrich (2003) 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 phylogentic 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 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 might 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 one of the important members in the nitrogen removal system.

As for the horizontal gene transfer, Song and Ward (2003) reported an interesting correlation between 16S rRNA and nitrite reductase genes (*nirS*) and discussed the possibility of the horizontal gene transfer of *nirS*. 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 *Gammaproteobacteria*, although phylogenetic relationships based on 16S rRNA genes from these genera are very different. The authors discussed that this might provide evidence for the horizontal gene transfer of *nirS* among the genera *Pseudomonas*, *Thauera*, *Acidovorax* and *Azoarcus* (Song and Ward, 2003). However, it was difficult to infer the horizontal gene transfer from our data sets at the community level.

Although it has been reported that the *nirS* genes were more diverse than the *nirK* genes in a previous study (Liu *et al*., 2003; Prieme *et al*., 2002), rarefaction analysis showed that the *nirS* clones detected by the SIP technique had almost the same diversity as the *nirK* clones in both methanol-assimilating and acetate-assimilating denitrifying bacteria. This result implies that active denitrifying bacteria possessing *nirS* are as diverse as active denitrifying bacteria possessing *nirK* in municipal sewage treatment systems. Meanwhile, the *nirS* clones detected from the original sludge sample without using the SIP technique were about twice as diverse as the *nirS* clones from acetate samples. This result showed that the bacteria possessing *nirS* genes were in abundance

in sewage treatment systems. Additionally, *nirK* clones were detected from the sludge sample after SIP although *nirK* amplification was not successful before SIP (i.e. the original sludge sample), indicating that some enrichment of *nirK*-containing denitrifiers occurred.

3.5 Conclusions

Stable-isotope-probing (SIP) technique combined with functional gene analysis was applied for characterizing the active denitrifying bacterial population in activated sludge. In this study, we characterize the nitrite reductase gene in acetate-assimilating populations or methanol-assimilating populations. It is found that the diversities of *nirK* and *nirS* gene in methanol-assimilating populations are much lower than those in acetate-assimilating populations. Furthermore, phylogenetic analysis of nitrite reductase genes (*nirS* and *nirK*) suggests that the denitrifying bacterial population strongly depends on the type of organic carbon source. In acetate–assimilating denitrifying bacteria, *nirK* sequences related to those of *nirK* genes of the genus *Alcaligenes* and *nirS* sequences closely related to those of *nirS* genes of the genus *Thauera* were detected, and many *nir* sequences were related to *nir* sequences obtained from various environments. In methanol-assimilating populations, a *nirK* clone closely related to the *nirK* gene in *Hyphomicrobium zavarzinii* was detected. However, most of the *nirS* clones and the *nirK* clones were not closely related to the genes of any known cultivated denitrifying bacteria or *nir* clones observed in various environments.

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