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Molecular analysis of the nitrogen cycle in deep-sea microorganisms from the Nankai Trough: genes for nitrification and denitrification from deep-sea environmental DNA

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Abstract Nitrification and denitrification are bacterial functions, which are important for the global nitrogen cycle. Thus, it is important to study the diversity and distribution of bacteria in the environment, which are involved in the nitrogen cycle on the earth. Ammonia monooxygenase encoded by the *amoA* gene and nitrite reductase encoded by *nirK* or *nirS* are essential enzymes for nitrificaton and denitrification, respectively. These genes can be used as markers for the identification of organisms in the nitrogen cycle. In this study, we identified *amoA* (42 clones) and *nirS* (98 clones) genes in parallel from samples recovered from the deep-sea of the Nankai Trough. Genes for *nirK* could not be amplified from these samples. The obtained *amoA* sequences were not so closely related to those of *amoA* genes from previously isolated environmental organisms and those of genes from environmental DNAs. On the other hand, the *nirS* genes sequenced showed some relationship to some extent with the latter genes. However, some of the newly sequenced genes formed clusters, which contained no previously identified genes on a phylogenetic tree. These are likely present in specific denitrifiers from the deep-sea. The results of this study further suggest that nitrifiers and denitrifiers live in the same area of the Nankai Trough and the nitrogen cycle exists even in the deep-sea.

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Introduction

Nitrification (oxidation of ammonia to nitrate via nitrite) and denitrification (reduction of nitrate to N_2 via nitrite, nitric oxide and nitrous oxide) are essential functions of the global nitrogen cycle. Both processes are performed by bacteria as well as a few species of fungi and archaea. Nitrifying bacteria are found in many environments and nitrification consists of a two-step process involving two different types of bacteria. Ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite and nitrite-oxidizing bacteria oxidize nitrite to nitrate. No bacterial species which oxidizes ammonia to nitrate directly has been found to date. Bacteria require these processes for energy conservation within their lithotrophic growth. Many studies have been performed previously regarding nitrification (Hooper et al. 1997; Herbert 1999). Denitrifying bacteria have been also found in many environments. In contrast to nitrifying bacteria, individual denitrifying strains can reduce nitrate to N_2 (or to N_2O in some bacteria). Denitrifying ability is widespread among bacteria, and thus is most likely due to lateral gene transfer. Many enzymes are involved in the nitrate-reduction step (Zumft and Körner 1997; Zumft 2004). Nitrifiers and denitrifiers both strongly contribute to the maintenance of the nitrogen balance on the earth. Thus, it is important to study the distribution and diversity of these organisms in the environment. Recent technical advances for the direct extraction of DNA from soil now provide novel approaches to the efficient detection of environmental microorganisms (Rondon et al. 2000). With this strategy, many studies have now been performed to analyze

the biodiversity of microorganisms in the environment with ribosomal RNA genes (Arakawa et al. 2006a, b, c) or to detect other useful genetic resources (Nagaya et al. 2005; Tamegai et al. 2006) by using specific gene probes for each purpose.

Since the growth of nitrifiers is normally quite slow, nitrifiers cannot be the dominant species in most environments. In such cases, analysis with ribosomal RNA genes is less effective. Ammonia monooxygenase (AMO) is one of the key enzymes involved in nitrification. This enzyme catalyzes the oxygenation of ammonia to hydroxylamine and consists of three subunits (AmoA, AmoB, and AmoC). Because of its essential function in the nitrification step, AMO is likely constitutively expressed in AOB. Thus, the *amo* genes can be useful markers for the detection of AOB (Rotthauwe et al. 1997). On the other hand, denitrifiers were found in a wide range of physiological groups. However, analysis with ribosomal RNA genes could be difficult because the ability of denitrification is sometimes different between two closely related strains. For example, *Pseudomonas chloritidismutans* has no ability for denitrification though the strain is closely related with denitrifying *Pseudomonas stutzeri* by the analysis of the 16S rDNA sequences (Wolterink et al. 2002). Dissimilatory nitrite reductase (NIR) is one of the essential enzymes for denitrification. There are two types of NIR, one is a cytochrome cd_1-type NIR encoded by the *nirS* gene and the other is a Cu-containing NIR encoded by the *nirK* gene. Denitrifying bacteria express either of these and a strain, which contains both types of NIR, has not been found. Although they are different in the structure, their physiological functions are equivalent (Zumft 1997). Because this reduction step is necessary for denitrification, *nirK* and *nirS* genes can be markers for the identification of denitrifiers (Braker et al. 2000). Using *amo* and *nir* genes as markers, some molecular analyses have been performed for the detection of such organisms in natural environments (Bothe et al. 2000).

The deep sea is still a relatively unknown world because of the technical difficulties involved in its exploration. The Japan Agency for Marine-Earth Science and Technology (JAMSTEC) has operated manned and unmanned submersibles to investigate the deep-sea environment and its endogenous organisms. Many interesting organisms have been isolated from the mud of deep-sea samples collected by these submersibles. Among these, the denitrifier *Pseudomonas* sp. strain MT-1 has been isolated from the mud of the Mariana Trench collected by a sterilized mud sampler using the JAMSTEC unmanned submersible *KAIKO* in 1996 (Tamegai et al. 1997; Sikorski et al. 2005; Tamegai et al. 2005b). Earlier, the nitrogen cycle in the shallow depths of the oceans has been investigated (Ward 1996). The presence of deep-sea denitrifiers may indicate that the nitrogen cycle operates even in the depths of the deep sea. However, a nitrate supply would be required for the denitrifying growth of MT-1 and the existence of nitrifiers is therefore suggested. Furthermore, several studies have been performed for investigating the respiratory systems in the deep sea (Tamegai et al. 1998, 2002, 2004, 2005a). Thus, molecular studies of the nitrogen cycle could provide novel insights into the energy conservation systems in the deep sea.

In this study, we initiated a molecular analysis of nitrification and denitrification in the deep-sea of the Nankai Trough with *amoA*, *nirK* and *nirS* genes as markers. Both *amo* and *nir* genes were identified from the same environmental DNA samples. These results suggest the existence of the nitrogen cycle involving microorganisms in the deep sea of the Nankai Trough.

Materials and methods

Organisms

Escherichia coli DH5α was cultured in an LB medium or on LB-agar containing 50 μg/ml of ampicillin when necessary.

DNA extraction from deep-sea sediments

The deep-sea sediments were collected by the manned submersible *SHINKAI* 6500 from the Nankai Trough (Arakawa et al. 2006c). Samples 6K881-S1 (34°04.003′N, 137°47.217′E, 652 m depth, normal bottom), 6K882-S2 (34°04.509′N, 137°47.351′E, 609 m depth, chemosynthetic communites), 6K883-S1 (33°15.809′N, 136°42.993′E, 2,071 m depth, normal bottom), 6K883-S4 (33°15.782′N, 136°43.055′E, 2,046 m depth, chemosynthetic communites), 6K884-S1 (32°34.974′N, 134°41.718′E, 3,310 m depth, normal bottom), and 6K884-S2 (32°34.930′N, 134°41.682′E, 3,306 m depth, chemosynthetic communites) were used. DNA extraction was carried out with the UltraClean Soil DNA Kit (MO BIO, Carlsbad, CA) according to the supplier's protocol.

Cloning of the genes by PCR

For the amplification of *amoA*, amoA-1F and amoA-2R were used as primers (Rotthauwe et al. 1997). PCR (one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and then 72°C for 10 min) was carried out with these primers using soil DNA as templates. In order to identify *nirS* and *nirK* genes from environmental DNA, nirS1F, nirS2F, nirS3F, nirS4F, nirS3R, nirS5R nirS6R, nirK1F, nirK2F, nirK3R, nirK4R and nirK5R were used as primers (Braker et al. 1998). The primers are indicated by nirK for the *nirK* gene and nirS for the *nirS* gene. Touchdown PCR (one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, annealing for 1 min and 72°C for 1 min, and then 72°C for 10 min) was carried out with these primers using soil DNA as templates. During the first 10 cycles, the annealing

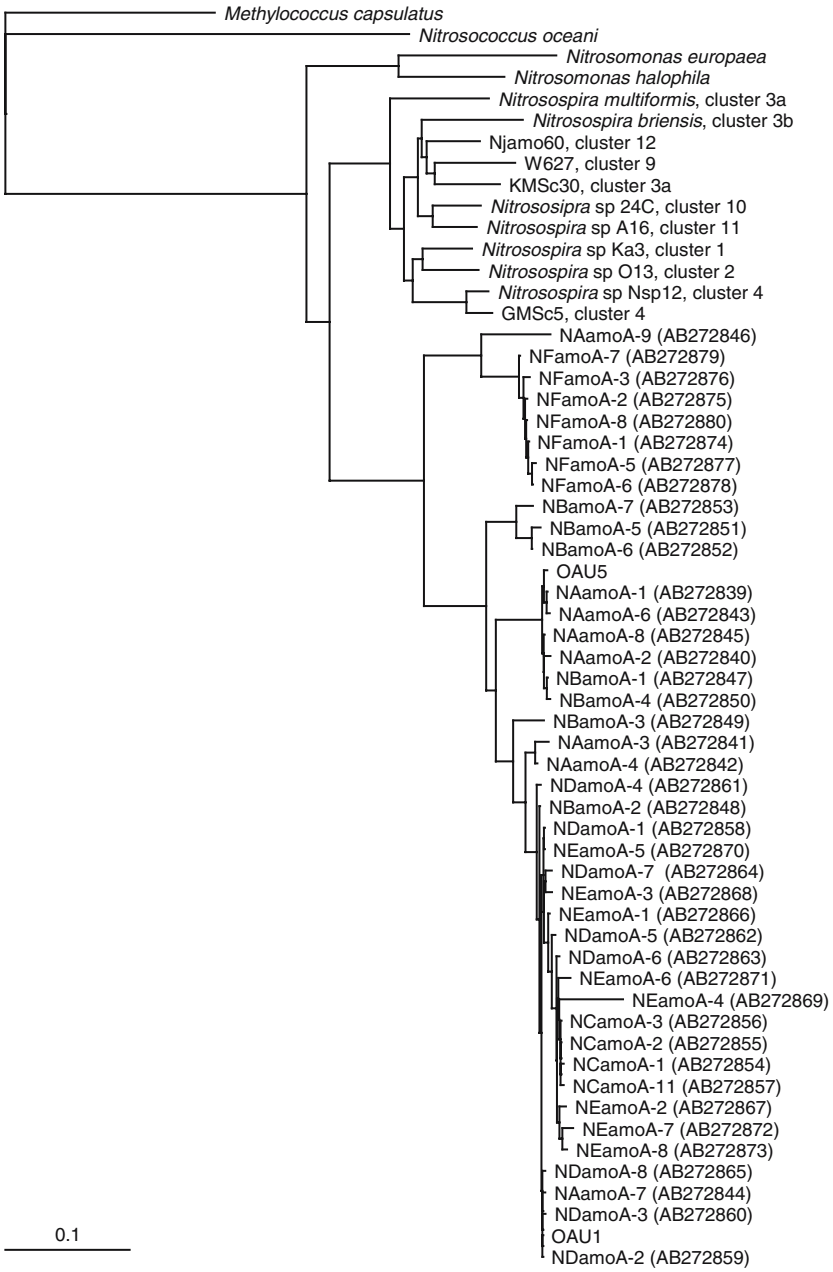

Fig. 1 Phylogenetic tree of *amoA*. *Nitrosococcus oceani*; U96611, *Nitrosomonas europaea*; L08050, *Nitrosomonas halophila*; AF272398, *Nitrosospira briensis*; U76553, *Nitrosospira multiformis*; U91603, *Nitrosospira* sp. A16; AJ298888, *Nitrosospira* sp. Ka3; AJ298696, *Nitrosospira* sp. Nsp12; AJ298716, *Nitrosospira* sp. O13; AJ298722, *Nitrosospira* sp. 24C; AJ298885, GMSc5; AY249673, KMSc30; AY249652, Njamo60; AF351560, OAU5; AB261615, OAU1; AB261611, W627; AF353250. The sequence of methane monooxygenase gene from *Methylococcus capsulatus* was used as an out group. Clones with accession numbers in the tree were obtained in the present study. The numbers of cluster were based on the classification by Avrahami and Conrad (Avrahami and Conrad 2003). The bar indicates 0.1 nucleotide substitutions per site

temperature was decreased 0.5°C every cycle, starting at 45°C until it reached touchdown at 40°C. The additional 30 cycles were performed at an annealing temperature of 43°C. All relevant combinations of primers were tested to eliminate false-positive results. The obtained PCR product was subcloned into pT7-blue T vector (Novagen, San Diego, CA), and the resulting plasmid was sequenced randomly.

Molecular biological procedures

The DNA manipulations were performed as described in the literature (Sambrook et al. 1989). The DNA sequencing was carried out by Bio Matrix Research (Chiba, Japan). PCR was performed with MyCycler (Bio-Rad, Hercules, CA) using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Oligo DNA for PCR primers was synthesized by Sigma Genosys (Hokkaido, Japan). The purification of plasmids was carried out with a GFX MicroPlasmid Prep Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). Restriction enzymes and modification enzymes were purchased from Takara Bio (Shiga, Japan). All other reagents were of the highest grade commercially available. The genetic analysis was performed with GENETYX-WIN ver.5.00 (Software Development, Tokyo) and a database search was carried out with BLAST (Tatusova and Madden 1999) on the Internet (http://www.ddbj.nig.ac.jp/search/blast-j.html). An evolution-

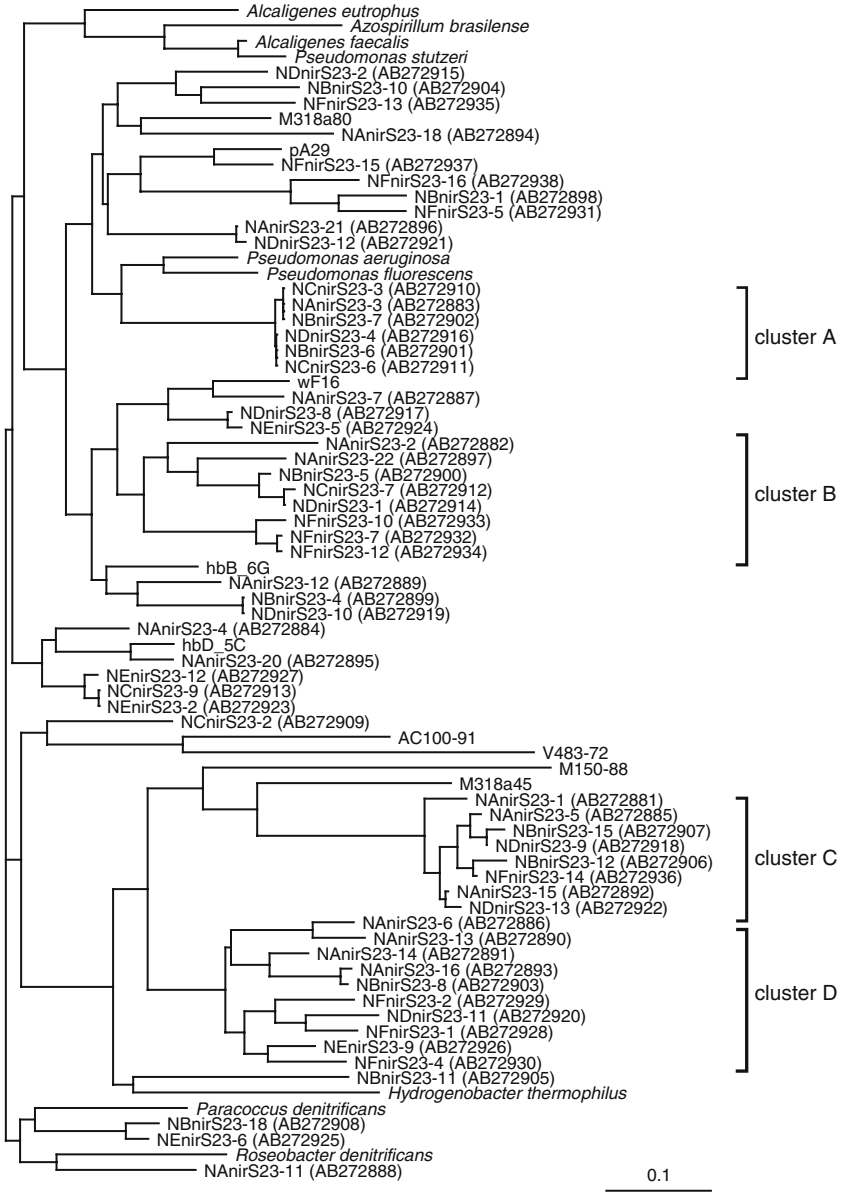

Fig. 2 Phylogenetic tree of *nirS* (nirS2F-nirS3R region). *Azospirillum brasilense*; AJ224912, *Alcaligenes eutrophus*; X91394, *Alcaligenes faecalis*; AJ224913, *Hydrogenobacter thermophilus*; AB210046, *Pseudomonas aeruginosa*; AM230891, *Paracoccus denitrificans*; U75413, *Pseudomonas fluorescens*; AF197466, *Pseudomonas stutzeri*; X56813, *Roseobacter denitrificans*; AJ224911, AC100-91; AJ811509, hbB_6G; DQ159547, hbD_5C; DQ159613, M318a45; AY195897, M318a80; AY195932, M150-88; DQ072184, pA29; AJ248410, V483-7C; AY336909, wF16; AJ248437. Clones with accession numbers in the tree were obtained in the present study. The bar indicates 0.1 nucleotide substitutions per site

ary tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL_W program (Thompson et al. 1994) on the Internet (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). The sequences determined in the present study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers as shown in Figs. 1, 2 and 3.

Results and discussion

Identification of amo genes

For the analysis of *amo* genes, 42 individual clones (452 bp) were obtained from six deep-sea samples (Table 1; Fig. 1) and depth-dependent diversity was not observed. Avrahami and Conrad have suggested that *amoA* genes can be classified into 9 clusters (Avrahami and Conrad 2003). Figure 1 showed that GMSc5 and KMSc30 (Avrahami and Conrad 2003), which were obtained from the soils of Germany and Israel, respectively, fell into this classification. However, *amoA* genes from the Nankai Trough cannot be classified accordingly. The clones obtained from deep-sea sediment cores (OAU1 and OAU5, Hayashi et al. unpublished) were closely related with those from the Nankai Trough and classified into several novel clusters. These clones seem to be specific genes for the AOBs in deep-sea and these findings clearly indicated that there are many unknown AOBs in the deep-sea. Freitag and Prosser have reported that a novel cluster of AOB was dominant in anoxic marine sediments (Freitag and Prosser 2003). There might be some relationship between our and their results though the detail of it was

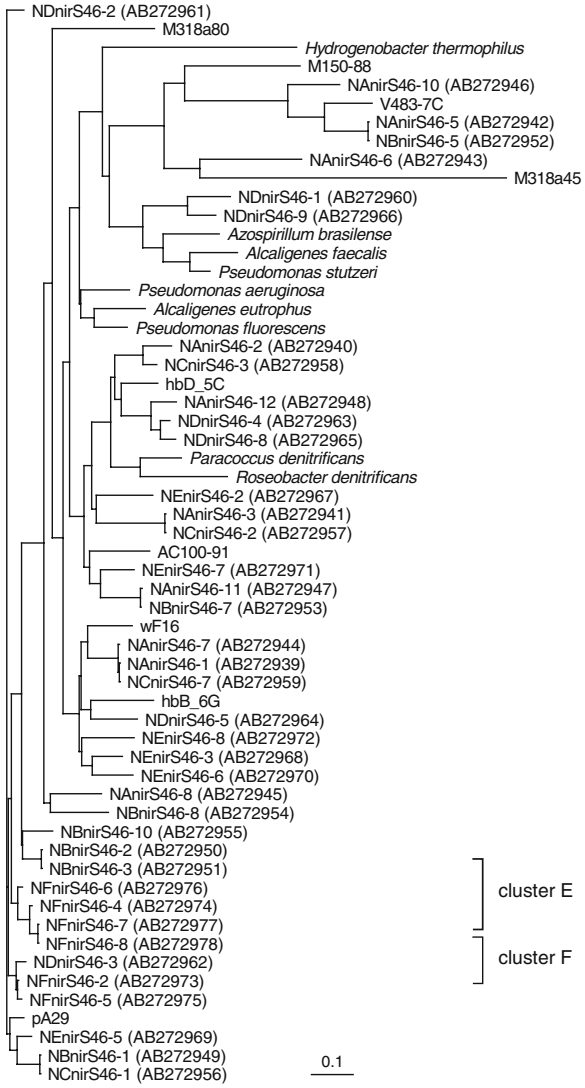

Fig. 3 Phylogenetic tree of *nirS* (nirS4F-nirS6R region). Clones with accession numbers in the tree were isolated in the present study. The bar indicates 0.1 nucleotide substitutions per site

unclear because their results were based on the 16S rDNA sequences. The diversity of AOB appears to be greater than originally anticipated.

Identification of nir genes

As described in "Materials and methods", all the relevant combinations of primers for *nir* gene amplification were tested. Although many *nirS* genes were identified, amplification of *nirK* genes was not observed in all samples. However, there are many reports of isolating *nirK* genes from other sea samples (Braker et al. 2000; Liu et al. 2003; Catstro-González et al. 2005; Hannig et al. 2006; Santoro et al. 2006). Therefore, the absence of *nirK*-containing organisms seems to be specific in the Nankai Trough area.

Table 1 Soil samples and their isolated clones

Soil samples (depths)	Clones (primers)		
	amoA (amoA-1F-amoA-2R)	*nirS* (nirS2F-nirS3R)	*nirS* (nirS4F-nirS6R)
6K881-S1 (652 m)	NAamoA-1, -2, -3, -4, -6, -7, -8, -9 (8 clones)	NAnirS23-1, -2, -3, -4, -5, -6, -7, -11, -12, -13, -14, -15, -16, -18, -20, -21, -22 (17 clones)	NAnirS46-1, -2, -3, -5, -6, -7, -8, -10, -11, -12 (10 clones)
6K882-S2 (609 m)	NBamoA-1, -2, -3, -4, -5, -6, -7 (7 clones)	NBnirS23-1, -4, -5, -6, -7, -8, -10, -11, -12, -15, -18 (11 clones)	NBnirS46-1, -2, -3, -5, -7, -8, -10 (7 clones)
6K883-S1 (2,071 m)	NCamoA-1, -2, -3, -11 (4 clones)	NCnirS23-2, -3, -6, -7, -9 (5 clones)	NCnirS46-1, -2, -3, -7 (4 clones)
6K883-S4 (2,046 m)	NDamoA-1, -2, -3, -4, -5, -6, -7, -8 (8 clones)	NDnirS23-1, -2, -4, -8, -9, -10, -11, -12, -13 (9 clones)	NDnirS46-1, -2, -3, -4, -5, -8, -9 (7 clones)
6K884-S1 (3,310 m)	NEamoA-1, -2, -3, -4, -5, -6, -7, -8 (8 clones)	NEnirS23-2, -5, -6, -9, -12 (5 clones)	NEnirS46-2, -3, -5, -6, -7, -8 (6 clones)
6K884-S2 (3,306 m)	NFamoA-1, -2, -3, -5, -6, -7, -8 (7 clones)	NFnirS23-1, -2, -4, -5, -7, -10, -12, -13, -14, -15, -16 (11 clones)	NFnirS46-2, -4, -5, -6, -7, -8 (6 clones)

From all samples, *nirS* genes were obtained by PCR using the primers nirS2F-nirS3R (58 clones, 127 bp, Fig. 2) and nirS4F-nirS6R (40 clones, 247–298 bp, Fig. 3) as summarized in Table 1. No amplification was observed with the other combination of primers. Depth-dependent diversity also was not observed. In contrast to *amoA*, the *nirS* genes identified showed some similarity to some extent with those of previously isolated bacteria. This difference might be due to the fact that normally denitrifiers can be isolated more readily than nitrifiers and greater numbers of denitrifiers have been isolated than that of nitrifiers. For example, clone NBnirS23-11 showed close relationship with the *nirS* of *Hydrogenobacter thermophilus*. Therefore, the organisms containing the former clone in its genome may be a thermophile. Furthermore, some of the *nirS* genes identified here also showed a similarity with the clones obtained from other environments (AC100-91 from the seawater off the eastern-southeast Pacific Ocean (Catstro-González et al. 2005), hbB_6G and hbD_5C from sediments from a beach in California (Santoro et al. 2006), M318a45 and M318a80 from sediments from the Pacific coast off Mexico, M150-88 from the Baltic Sea (Hannig et al. 2006), pA29 and wF16 from sediments isolated in the northwest Pacific Ocean (Braker et al. 2000), and V483-7C from the coastal seawater off the Arabian Sea (Jayakumar et al. 2004). The results of this study suggested that denitrifiers can move around in the oceans. However, some clusters contained no previously identified clones, indicated in Fig. 2 (cluster A, B, C, and D) and Fig. 3 (cluster E and F). Therefore, these genes may be from previously uncharacterized organisms in this area. We have previously suggested that deep-sea denitrifiers appeared to have evolved independently from those in other environments (Tamegai et al. 2002, 2004). The results of the present study further support such a suggestion.

Conclusions

In the present study, we identified genes for nitrification and denitrification in the same environmental DNA samples extracted from the mud of the Nankai Trough. Depth-dependent diversity was not observed in the present study. However, the sample 6K883-S1 showed low diversity both in *amoA* and *nirS* genes. Thus suggests that the nitrogen cycle in such areas is not very active.

Although many studies have been performed on each system, this is the first report that found genes for both systems in the same deep-sea samples. This result suggests that nitrifiers and denitrifiers live in the same area in the Nankai Trough and the nitrogen cycle is operative even in the deep-sea.

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