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Original

Construction of a Consortium Comprising Ammonia-oxidizing Bacteria and Denitrifying Bacteria Isolated from Marine Sediment

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A unique consortium of ammonia-oxidizing bacteria (AOB) and denitrifying bacteria was obtained via a long-term, 3-step cultivation of isolates from organically-enriched marine sediment. We developed this microbial consortium for possible applications in the remediation of degraded habitats in closed aquaculture or other aquatic environments via microbial degradation. Analysis of media components found definitive evidence of nitrogen removal via the coupling of ammonia-oxidation and denitrification. The phylogenetic diversity of the consortium was investigated by performing polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) that targeted the 16S rRNA gene, and the functional genes involved in ammonia-oxidation (*amoA*) and denitrification (*nirS*, *nirK*, *norB* and *nosZ*). Consequently, no significant divergence was observed, and thus it is suggested microbial populations were selected via a long-term, 3-step incubation process. All of the 16S rRNA clones fell into three phylogenetic groups, namely, *γ-proteobacteria*, *Actinobacteria* and *Flavobacteria*. For almost half of the clones, the closest relatives in the database were identified as *Alcanivorax* spp. and these clones were present at all cultivation stages. The presence of these species as the dominant clones is significant since these bacterial species are known to reduce nitrate to nitrite. Accordingly, their abundance in our microbial consortium may have been responsible for the observed stepwise denitrification. All sequences of the *amoA* gene were identified to be *Nitrosomonas* lineage. Half of the *nirS* clones were identified to be from one major group of well-known denitrifying bacteria, *Pseudomonas* sp. Furthermore, 70% of the *nirK* clones were closely related to the *nirK* sequences of uncultured bacterial clones isolated from arable soil. The *qnorB* clones consisted of clusters exclusively, and formed a distinct cluster from the novel sequences of cultivated species. The *nosZ* clones also were not found in any of the closest relatives in the database including the uncultured bacterium from marine sediment. The unique clones obtained from the functional genes were related to each denitrification step.

Key words : Consortium/Ammonia-oxidizing bacteria/Denitrifying bacteria/Marine sediment/
PCR-DGGE.

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INTRODUCTION

Marine aquaculture produces a large amount of effluent, which is often characterized by high concentrations of nitrogen, phosphorus and organic matter and such effluent increases the biochemical oxygen demand (BOD) and contributes to the deterioration of the water quality (Wu et al., 1995, Mazzola et al., 1999). Some substances are readily labile and undergo spontaneous degradation in the water column, but many are highly recalcitrant and accumulate in the sediment, which functions as a reservoir that continually reintroduces chemicals into the water. With the development of the aquaculture industry, efficient, cost-effective and environmentally-preserving bioremediation methods are desirable for improving the quality of effluent prior to its discharge. Microbial degradation is a potential tool for the treatment of effluent, since microbes have long been used for the effective degradation of both conventional and toxic pollutants.

Microbial nitrification and denitrification play an important role in the global nitrogen cycle, and these processes are essential for converting combined nitrogen into dinitrogen gas. In the first step of the nitrification process, chemolithoautotrophic ammonia-oxidizing bacteria (AOB) are primarily responsible for the oxidation of ammonia to nitrite. Nitrification occurs aerobically and produces the substrate needed for subsequent denitrification (Casciotti et al., 2005). Denitrification, a multi-step process whereby nitrate is reduced to dinitrogen gas (N_2), is mediated by a phylogenetically diverse group of bacteria, some archaea and fungi (Zumft, W.G., 1997), and mainly occurs under anaerobic conditions. In marine environments, denitrification can eliminate more than 50% of all the nitrogen present in the oceans (Christensen et al., 1987), and it is assumed to occur on the sea floor, particularly in organic-rich continental margin sediment.

Nitrification (ammonia-oxidation) and denitrification have traditionally been considered as spatially distinct processes since the former is aerobic and the latter occurs under anaerobic conditions. Furthermore, the energy acquirement systems of AOB and denitrifying bacteria are quite distinct from each other. Therefore, the wastewater treatment system for nitrogen removal requires one aerobic tank and one anaerobic tank. However, the coupled processes of benthic nitrification and denitrification are generally assumed to follow a strict sequence of vertically stratified diagenetic reactions with nitrification occurring in the oxic layer of the sediment, and denitrification in the slightly lower suboxic or anoxic

sediment layer (Henriksen et al., 1988). Although these 2 processes appear to be mutually exclusive with respect to the oxygen requirements, the sharp gradients of O_2 , NH_4^+ , and NO_3^- often present in the surface sediment enable them to occur within close proximity of each other (Hulth et al., 2005). From a practical viewpoint, these reactions should be considered to form a sequence in the N-transforming metabolic pathway since only when both occur can bound nitrogen be completely removed from the effluent in natural environments. This study was undertaken to exploit the bacterial consortium involved in both the ammonia-oxidation and denitrification processes for possible use in the remediation of dredged habitats wherein nitrogenous materials have accumulated in the bottom sediment. As an initial step in this direction, we attempted to develop a consortium comprising both AOB and denitrifying bacteria via a long-term 3-step cultivation of bacteria isolated from marine sediment. PCR-DGGE analysis was applied to assess the community structure and variations in the enrichment culture.

MATERIALS AND METHODS

Sampling of sediment and an enrichment culture of AOB and denitrifying bacteria

Sediment was collected at an underwater depth of 10 m from an organically-enriched enclosed coast located inside Ago Bay in the Mie prefecture of Japan. This organically-enriched sediment has accumulated on the sea bottom for many years as a consequence of pearl and oyster culturing. Characterization of the sediment yielded the following information for COD, acid volatile sulfides (AVS), total nitrogen (T-N) and total organic carbon (TOC) (per g dry sediment): 55.8 mg O_2 , 1.57 mg, 3.77 mg and 21.77 mg, respectively. Sediment water content and the oxidation-reduction potential (ORP) value were 72.4 % and -109.3 mV, respectively (Nakanishi et al., 2001).

The procedure for the acquisition of an enrichment culture was divided into three main steps: 1) obtaining and enhancing the nitrifying bacteria; 2) activating the denitrifying bacteria; 3) starving the heterotrophs and recovering the nitrifying bacteria. The medium composition and schematic outlines for obtaining a consortium are described in Tables 1 and 2. In step 1, a sediment sample was inoculated (3 % wt/vol) into a 500-ml glass bottle containing 400 ml of Nm medium (Table 1). The general medium was selected for obtaining one of the universal AOB members (Sato et al., 1985), and $(NH_4)_2SO_4$ at 1.5 g was added to enhance the ammonia-oxidation. The sample was processed on a rotary shaker at 95 rpm in the

TABLE 1. Composition of three types media used in this study for obtaining the consortium of AOB combined with denitrifying bacteria and ANA3 medium used for analytical measurement.

Composition/ Medium type	Medium type			
	Nm* ¹	NiD-d* ¹	NiD-a* ¹	ANA3* ¹
Artificial sea water				
NaCl	30g	5.84g	5.84g	5.84g
KCl	0.7g	0.66g	0.66g	0.66g
MgCl ₂ · 6H ₂ O	10.8g	10.8g	10.8g	10.8g
MgSO ₄ · 7H ₂ O	5.4g	5.4g	5.4g	5.4g
CaCl ₂ · 2H ₂ O	1g	1g	1g	1g
Na ₂ MoO ₄ · 2H ₂ O	1mg			
FeSO ₄ · 7H ₂ O	1mg			
MnSO ₄ · 4H ₂ O	1mg			
Others				
(NH ₄) ₂ SO ₄	1.5g	0.33g	0.33g	0.264g
NaNO ₃				0.344g
carbonate buffer* ²	10ml	3ml	3ml	3ml
phosphate buffer* ²	10ml	0.5ml	0.5ml	0.5ml
phenol red			0.5mg	
phenol red solution* ³		1ml		1ml
CaCO ₃ * ⁴	1g			
Trace mineral solution				
EDTA		50mg	50mg	6mg
FeSO ₄ · 7H ₂ O		5mg	5mg	
FeCl ₃ · 6H ₂ O				1mg
CuSO ₄ · 5H ₂ O		1.6mg	1.6mg	4 μg
MnSO ₄ · 4H ₂ O		5mg	5mg	0.6mg
(NH ₄) ₂ MoO ₄ · 4H ₂ O		1.1mg	1.1mg	
Na ₂ MoO ₄ · 2H ₂ O				0.15mg
H ₃ BO ₃		50 μg	50 μg	
KI		10 μg	10 μg	
CoCl ₂ · 6H ₂ O		50 μg	50 μg	4 μg
ZnSO ₄ · 7H ₂ O				0.3mg
pH	7.5-8.0	7.5-8.0	7.5-8.0	7.5-8.0

*¹ Measured per 1000 ml distilled water.

*² Referred to the report (Sato et al., 1985).

*³ Phenol red solution containing 25 mg of phenol red was dissolved in 10 ml of ethanol and 50 ml of distilled water to detect denitrifying bacteria presented.

*⁴ Added for ammonia-oxidizers.

dark at 20 °C, and a silicone[®] (plug silicone) cap was placed at the top to make contact with oxygen. Concentrations of NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were measured every 2 weeks with a Merckoquant test strip (Merck, Germany). Each culture that met the following criteria was inoculated at 5 % (vol/vol) into a new medium and incubated 7 to 8 times repeatedly: 1) more than 40-80 mg/l of nitrite production, 2) the reduction of ammonium ions, 3) nitrate formation, and 4) the decrease in pH. In step 2, a subculture from the first step was inoculated at 1 to 5 % (vol/vol) onto NiD-d medium (Table 1) at room temperature in the dark without shaking. In this step a carbon source (99.5 % ethanol) was added as a

catalyst for denitrification with the phenol red (pH indicator) solution, which included 0.2 ml of ethanol (Table 1). In this study ethanol was used as the carbon source, and a low C/N ratio (C/N ratio; 1) was applied in consideration for the growth effect on AOB because autotrophs are vulnerable to high loads of ammonium ions and organic matter even though high C/N ratios favor denitrifiers. The incubation was continued until bubbles formed, and the color of the medium changed from pink to yellow. The concentrations of NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were also monitored. In step 3, a subculture from the second step was inoculated at 10 % (vol/vol) onto NiD-a medium (Table 1) at room temperature in the dark without shaking to recover nitrifying bacteria and starve heterotrophs. The incubation was carried out 2 to 3 times using the criteria for step 1, and the final culture consortium yielded was then stored at -80 °C. The frozen stock was then used according to every analytical measurement.

Chemical analysis of the consortium

To examine the activities of AOB combined with denitrifying bacteria in a consortium, a subculture from the third stage (step 3) was inoculated at 10 % (vol/vol) onto ANA3 medium (Table 1) at room temperature, in the dark and without shaking. The pH of the culture and the concentrations of NH₄⁺-N, NO₂⁻-N, NO₃⁻-N and T-N were measured in order to monitor the ammonia-oxidation and denitrification pathway (s): the samples were harvested at 0, 7, 14, 21 and 37 days. Each sample was mixed well before being harvested, thus oxygen was newly supplied. The concentrations of NH₄⁺-N, NO₂⁻-N and NO₃⁻-N in the culture sample were examined using a Traacs 2000 (BRAN+LUEBBE, Norderstedt, Germany). A TOC analyzer was used for measuring T-N (an optional system installed in a TOC analyzer, TOC-Vc series, Shimadzu, Japan); pH was analyzed with the D-52 pH meter (HORIBA Ltd, Kyoto, Japan). In addition, bacterial cells were collected for subsequent DGGE.

Enumeration of AOB and denitrifying bacteria

The most probable number (MPN) technique was used for enumerating AOB using microtitre plates (Rowe et al., 1977). The NiD-a medium without phenol red was used for the culture medium, and the pH was adjusted to 8.0 by adding sterile 5 % Na₂CO₃. An aliquot (150 μl) of the enrichment culture, taken from the diluted serial samples, was dispensed into each well of a 96-well microtitre plate. Eight twofold serial dilutions of the enrichment culture were performed. The inoculated MPN plates were incubated in the dark for three weeks at 20 °C. Following

incubation, the wells were assayed for nitrite and nitrate by adding Griess Ilosvay reagent (GR reagent) 1 and 2 (Schmidt and Belser, 1982), and through the development of a blue colour after the addition of one or two drops of 0.2 % diphenylamine in concentrated H_2SO_4 (Schmidt and Belser, 1982). They were scored as positive for ammonia oxidizers via the detection of nitrite. The presence of heterotrophic denitrifying bacteria was verified by the formation of gas inside the Durham tube and the reduction of nitrate by the GR-reagent after four weeks. One litre of nutrient broth, which consisted of 900 ml seawater that had the same composition as the Nm medium (Table 1) and 100 ml of distilled water, contained $NaNO_3$, 1 g; yeast extract, 3 g; and polypepton, 5 g. Serial dilutions of the sample were prepared with sterilized seawater as the diluents. Five tubes were inoculated with decimal dilutions to 10^{-9} , as is usual for MPN that is determined according to the standard methods for the examination of water and wastewater published by the American Public Health Association (1975).

DNA extraction

Samples (18 ml) of the enrichment culture were harvested at 0, 7, 14, 21 and 37 days, and cells were collected by centrifugation ($14,000 \times g$ for 5 min). Bacterial cells were resuspended, rinsed with phosphate-buffered saline (PBS) buffer twice, and centri-

fuged ($14,000 \times g$ for 5min). Total genomic DNA was extracted using a G-MOME kit (BIO101, Inc. La Jolla, CA, USA) following the manufacturer's instructions.

PCR amplification of 16S rRNA and the functional genes *amoA*, *nirS*, *nirK*, *norB*, and *nosZ*

To examine the change in microbial diversity and characterize the microbial community structure, samples of DNA extracted at different stages were amplified, targeting the 16S rRNA gene, and subjected to DGGE. Subsequently, a DGGE analysis of the functional diversity was performed based on the functional genes *amoA*, *nirS*, *nirK*, *norB* and *nosZ*, which are involved in ammonia-oxidation and each step of denitrification. The bands on the gels were cloned and sequenced, and members involved in AOB and denitrifying pathways were classified phylogenetically. PCR primer sequences and positions were used to amplify fragments from the 16S rRNA gene, and the functional genes of *amoA*, *nirS*, *nirK*, *nosZ* and *norB* are shown in Table 3. PCR amplification was performed by preparing the following solution: 3 μ l of the DNA sample (extracted at 0, 7, 14, 21 and 37 days), 0.6 μ l of 25 pmol of each of the primers and 25 μ l of AmpliTaq gold PCR master mix (Applied Biosystems, Weiterstadt, Germany). The total volume was adjusted to 50 μ l with sterile deionized water. The PCR thermocycling profile is

TABLE 2. Specifications for obtaining the consortium of AOB for combination with denitrifying bacteria from marine sediment.

Culture Specifications	Incubation step		
	Step 1	Step 2	Step 3
Media type	Nm* ¹	NiD-d* ¹	NiD-a* ¹
Incubation temperature (°C)	20	room temperature	room temperature
Light/dark condition	dark	dark	dark
Oxygen contact	yes, with rotary shaker at 95rpm via silicone cap	no, without shaking	no, without shaking
Criteria for transfer to new media	>40 to 80 mg/l nitrite and nitrate production, reduction of ammonium ion* ² and decreasing pH	added phenol red solution* ¹ to activate denitrification, and checked the concentration* ² , bubble formation and media color change from pink to yellow	incubated until nitrite production, reduction of ammonium ion and pH decrease was observed* ²
Time interval for checking the medium	2 weeks	2 weeks	2 weeks
Frequency of transfer to new medium	7 times	once	incubated several times and stored at -80°C
Inoculation volume transferred to new medium	3 % (wt/vol) with initial sediment, and 5 % (vol/vol) with next step	1 to 5 %	10%

*¹ Refer to Table 1.

*² Check the conc. of nitrite, nitrate and ammonium ion with commercial kit, merckoquant test strip (Merck, Germany).

TABLE 3. Primer sequences and positions used to amplify fragments from 16S rRNA, *amoA*, *nirS*, *nirK*, *norB* and *nosZ* genes in the enrichment culture sample.

Genes	Primer	Positions* ³	Primer sequence (5'-3')
16S rRNA* ¹	GM5F	341-357	CC TAC GGG AGG CAG CAG
	907R	907-927	CCG TCA ATT CCT TTG AGT TT
	907RA	907-927	CCG TCA ATT CAT TTG AGT TT
<i>amoA</i> * ²	amoA-1F	332-349	GGG GTT TCT ACT GGT GGT
	amoA-2R	802-822	CCC CTC KGS AAA GCC TTC TTC
<i>nirS</i> * ²	cd3aF	916-935	GTR AAC GTS AAG GAR ACS GG
	R3cd	1322-1341	GAS TTC GGR TGS GTC TTG A
<i>nirK</i> * ²	FlaCu	568-584	ATC ATG GTS CTG CCG CG
	R3Cu	1021-1040	GCC TCG ATC AGR TTG TGG TT
<i>norB</i> * ² (<i>qnorB</i>)	qnorB2	1204-1220	GGN CAY CAR GGN TAY GA
	qnorB5	1466-1444	ACC CAN AGR TGN CAN ACC CAC CA
<i>norB</i> * ² (<i>cnorB</i>)	cnorB2F	553-571	GAC AAG NNN TAC TGG TGG T
	cnorB6R	942-925	GAA NCC CCA NAC NCC NGC
<i>nosZ</i> * ²	NosZ-F	1169-1188	CGY TGT TCM TCG ACA GCC AG
	nosZ1622R	1603-1622	CGC RAS GGC AAS AAG GTS CG

*¹ A 40-bp GC-clump (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was added to the 5' end of the primer GM5F for separation of the DNA fragment on DGGE (Muyzer et al., 1998).

*² A 33-bp GC-clamp (5'-GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC-3') was added to the reverse primer R3cd for *nirS*, R3Cu for *nirK*, and nosZ1622R for *nosZ* (Throback et al., 2004).

*³ Positions in the 16S rRNA of *E. coli* and a mixture of two reverse primers, 907R and 907RA were used, since primer 907R may not yield efficient results with some marine representatives of the δ -proteobacteria due to a mismatch at the target site (Schafer et al., 2001). Positions in the *amoA* gene of *Nitrosomonas europaea*, a specific primer set for ammonia-oxidizing bacteria from the β -subdivision of the *Proteobacteria*, [K=G or T; S=G or C] (Rhtthauwe et al., 1997). Positions in the *nirS* gene of *Pseudomonas stutzeri* Zobell ATCC 14405 (X56813), [R=A or G; S=G or C] to amplify *nirS* (Throback et al., 2004). Position in the *nirK* gene of *Alcaligenes faecalis* S-6 (D13155) (Hallin et al., 1999). Positions in the *qnorB* gene of *Ralstonia eutropha* H16 (AF002661) and *Paracoccus denitrificans* Pd1222 (U28078), [N=A, C, G, or T; Y=C or T; R=A or G] were used for *cnorB* gene. Position in the *nosZ* gene of *Pseudomonas aeruginosa* DSM 50071 (X65277), [Y=C or T; M=A or C] was used for *nosZ* gene (Throback et al., 2004).

TABLE 4. Summary of the PCR conditions used in this study.

Primer set	Thermocycling program
GM5F - 907R, 907RA	95 °C for 10 min; 10 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min; 10 cycles each at annealing temperature of 60 °C and 53 °C; and a 5-min final extension.
amoA-1F - amoA-2R	95 °C for 10 min; 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 2 min; and a 7-min final extension.
cd3aF - R3cd	95 °C for 10 min; 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 2 min; and a 7-min final extension.
FlaCu - R3Cu	95 °C for 10 min; 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 2 min; and a 7-min final extension.
qnorB2 - qnorB5	95 °C for 10 min; 10 cycles of 94 °C for 30s, 57 °C for 1 min, and 72 °C for 1 min; 30 cycles of 94 °C for 30s, 55 °C for 1 min, and 72 °C for 1 min; and a 7-min final extension.
nosZ-F - nosZ1622R	95 °C for 10 min; 13 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min; 13 cycles each at an annealing temperature of 60 °C and 56 °C; and a 7-min final extension.

shown in Table 4. Amplification was performed with a Takara PCR thermal cycler MP (TaKaRa Shuzou, Shiga, Japan). Functional genes such as *nirS*, *nirK* and *nosZ* have high sequence variation, and melting profiles obtained in WinMelt showed that all three denitrifying genes also had multiple melting domains (Throback et al., 2004). To minimize the effect of these domains and avoid the complete denaturing of

the PCR-amplified fragment, a 33-bp GC-clamp was attached to the three reverse primers R3cd, R3Cu and nosZ1622R (Throback et al., 2004).

DGGE profiling

DGGE was performed as described by Muyzer et al. (1998) using a Bio-Rad DCcode universal mutation detection system (Bio-Rad Laboratories, Munich,

Germany). A 6 to 12% polyacrylamide (acrylamide/N, N'-methylene bisacrylamide; 37.5:1) gradient was introduced together with a 20 to 60% denaturant (100% denaturant is 7 M urea and 40% (vol/vol) formamide) to allow for more effective band distinction. Electrophoresis was performed at a constant voltage of 200V at 61°C for 5h. The gel was then stained with SYBR Gold (Molecular Probes, Eugene, Canada) and visualized with UV transillumination. Individual excised DGGE bands were purified with a QIAEX DNA extraction kit (Qiagen, Chatworth, CA, USA) for further analysis, unless a second DGGE application was employed.

Cloning, sequencing, and phylogenetic analysis

The eluted fragments were reamplified with primers without a GC-clamp using 4-7 μ l of DNA in a total reaction volume of 55 μ l. PCR products were ligated into a pMD18-T vector (TaKaRa Shuzou, Shiga, Japan), and subsequently transformed into JM109 competent cells. Transformed colonies were screened for inserts of the correct size by PCR amplification with the vector-specific primers M13-47 and RV-M (TaKaRa). At least four clones with the correct fragment size insert were subjected to amplification for sequencing using a TempliPhi DNA amplification kit (GE Healthcare Bio-Science Corp., NJ, USA) according to the manufacturer's instructions. Cycle-sequencing reactions were conducted with an ABI 310 DNA sequencer (Applied Biosystems).

Sequences were aligned with the same region of the closest relatives available using the BLAST search option of the DDBJ database (DNA Data Bank of Japan). Sequence alignments were achieved using Clustal X (Thompson et al., 1994) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis with 1000 replicates was performed to check the robustness of the tree.

Nucleotide sequence accession numbers

The gene sequences obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession no. AB261431 through AB261511.

RESULTS AND DISCUSSION

Developing a bacterial consortium from marine sediment samples

During the initial incubation step 1 (Table 2), the time intervals maintained for transferring the nitrite- and nitrate-generating subcultures to new media exceeded 1 month. As the cultivation proceeded,

however, we reduced the intervals. The cultivation process during step 1 counted for approximately 6 months of the project time. Overall, both nitrite and nitrate were accumulated and accompanied by a noticeable pH decrease during ammonia-oxidation and the subsequent nitrite-oxidation.

In step 2 (Table 2) gaseous production was initially detected at the surface of the medium, and both nitrite and nitrate immediately disappeared with the addition of a carbon source. This almost complete reduction of the total nitrogen was probably the result of the rapid conversion of bound, inorganic forms to gaseous nitrogen. The concentration and type of carbon source used may have selectively affected certain denitrifying members of the populations in step 1 of the cultivation. The decrease in pH (pink-to-yellow change in the medium) reflected the growth of AOB, suggesting that these bacteria are capable of sustaining their metabolic activity even in the presence of organic compounds. Some organic compounds have been observed to increase the growth and cell yield of AOB cultured in the presence of an inorganic energy source (Krummel et al., 1982). However, the precise influence of the organic carbon source on the nitrification has not been well understood.

In step 3 (Table 2) the culture medium became less cloudy as the cultivation proceeded. The heterotrophs in the culture may have undergone stress since incubation was carried out with NiD-a medium comprising inorganic compounds. Furthermore, the second selection may have occurred among the heterotrophs and other denitrifying bacteria due to the deletion of the organic substrate. Moreover, the nitrifying bacteria could be recovered and their activity sustained.

Chemical analysis of the consortium

The subculture obtained at the end of step 3 was inoculated into ANA3 medium (Table 1) for performing analytical measurements. The ammonia and nitrate were added at an initial concentration of 4 mM each, and these decreased after 37 days: nitrite accumulated in the ANA3 medium increased to 5.3 mM at 37 days post-inoculation (Fig.1). The accumulation of nitrite in the medium suggests the following possibilities. (1) Nitrite-oxidizers had been lost after the second incubation step due to substrate depletion and competition for oxygen via the aerobic pathways. (2) The rapid decrease in nitrate observed during the first 7 days reflects the occurrence of denitrification and probably depended on the availability of organic carbon. This suggests that the carbon source provided was exhausted by heterotrophs competing for growth and by other denitrifying bacteria; thus no

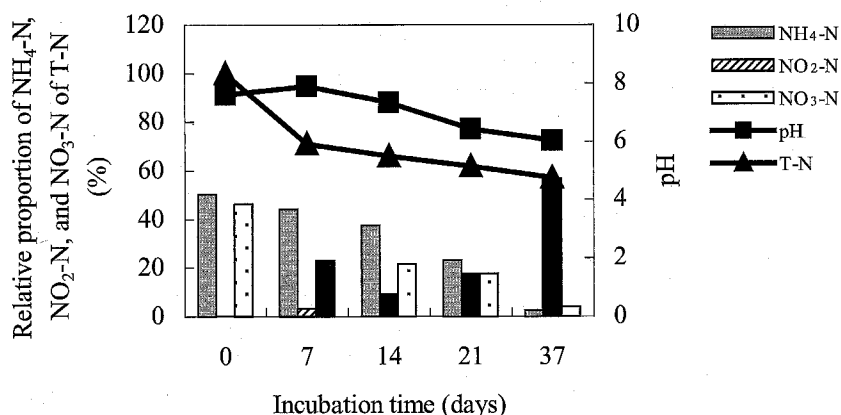


FIG. 1. Relative proportion of NH_4^+ -N, NO_2^- -N, and NO_3^- -N among T-N. The new culture sample was inoculated at 10 % into a new ANA3 medium and incubated for 37 days. Initial concentrations of NH_4^+ -N and NO_3^- -N added to the ANA3 medium were both 4 mM. Concentrations of NH_4^+ -N, NO_2^- -N, NO_3^- -N, T-N and pH values were obtained throughout the incubation.

further nitrite reduction was observed. (3) Nitrite oxidizing activity was inhibited due to the presence of ammonia and organic carbon under the static culture conditions. The activities of both AOB and denitrifying bacteria were observed to vary with time; however, the AOB population increased from 2.5×10^4 (day 0) to 5.1×10^5 (day 37) cells/ml, while the denitrifying bacterial population decreased from 7.0×10^8 (day 0) to 2.0×10^9 (day 37) cells/ml as measured by the most-probable-number (MPN) method.

Analytical measurements demonstrated the occurrence of the coupled processes of nitrification (ammonia-oxidation) and denitrification due to the consortium of AOB and denitrifying bacteria. Consequently nitrite accumulated in the medium and was not reduced further. For the process of denitrification, heterotrophic denitrifiers require organic carbon compounds such as carbohydrates, organic alcohols, amino acids, and fatty acids. In addition, denitrification rates depend on the type of carbon source. In this study, ethanol was used as carbon source; however, the impact on denitrifying bacterial growth by using a different type of carbon source was not investigated. Probably the dominant type of denitrifiers and the denitrification rate depends on the type of carbon source. In general, ethanol, methanol, and glucose are used as electron donors for denitrification in recirculating aquaculture systems (van Rijn et al., 2006). Regarding the C/N ratio, for the most readily available organic carbon source, a COD/ NO_3^- -N (w/w) ratio of 3.0-6.0 enables the complete nitrate reduction of nitrate to elemental nitrogen (Skindes and Bhagat, 1982). In this experiment the limited carbon source, a low C/N ratio of 1.6 (NH_4^+ -N, 4mM; NO_3^- -N, 4mM; ethanol, 5mM)

was employed in consideration of the effect of the growth of AOB because autotrophs are vulnerable to high loads of ammonium ions and organic matter, therefore, the energy source for denitrification might be exhausted. As mentioned in a report (van Rijn et al., 2006), carbon limitation will result in the accumulation of intermediate products, such as NO_2 and N_2O , while excess carbon will promote the dissimilatory nitrate reduction to ammonia. Recently, using the same consortium in this study, Nakahama et al. (2008) reported that the dissolved inorganic nitrogen (DIN: sum of the NH_4^+ -N, NO_2^- -N, and NO_3^- -N concentrations) decreased by more than 10-fold after an operation time of 126 h when compared with the initial value (C/N ratio: 10). Their study revealed that this consortium could achieve almost complete nitrogen removal; furthermore, a high C/N ratio enabled the acceleration of the nitrogen removal rate. The timing of the catalyst addition may be a key parameter for improving the efficiency of nitrogen removal without nitrite accumulation in the medium. A system wherein the nitrite produced by AOB can be immediately reduced by denitrifying bacteria in the presence of adequate energy could be more effective for nitrogen removal.

The reproducibility of the ammonia-oxidation/denitrification activities in this consortium was observed when using the new, stocked inoculum for incubation. To obtain a stable partial nitrification combined with denitrification, a long-term and repeated incubation is not recommended due to the transition in the microbial components of the consortium.

DGGE for 16S rRNA fragments obtained at different culture stages

To visualize changes in the phylogenetic diversity of the bacterial assemblage, DGGE was performed for PCR-amplified 16S rRNA fragments, derived from DNA samples that were collected from bacteria in the consortium at the different growth stages. The banding patterns obtained indicated that no remarkable changes occurred during the incubation except from days 0 to 7, although a few dissimilarities were observed among the band intensities for days 7 to 37 (Fig.2). Twelve bands were obtained on the gel (Fig.2). The structure of the microbial community of the consortium did not vary drastically during the analytical experiment in comparison with the variation derived from the chemical analysis.

In total, 29 positive clones were obtained and were found to have close relatives in the database. These clones belonged to 3 phylogenetic groups, namely, *γ-proteobacteria*, *Actinobacteria* and *Flavobacteria*, as shown in Fig.3. Consequently, no significant divergence was observed, suggesting that the microbial population type is selected via the long-term, 3-step incubation process. For the samples corresponding to 2 bands, 2d and 2g, the cloning procedure was unsuccessful. For 15 clones, the closest relatives in the

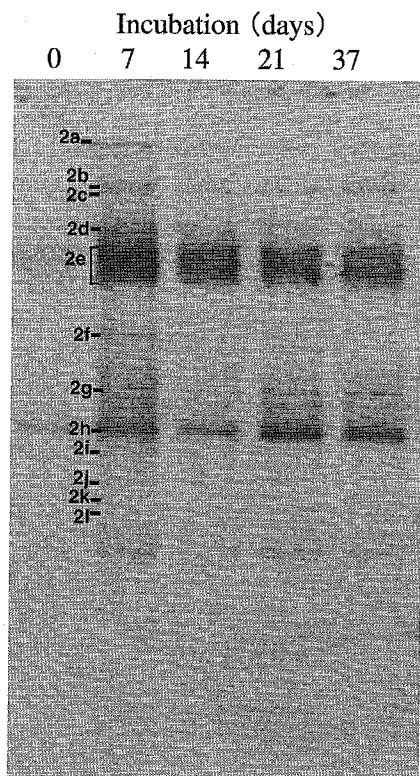


FIG. 2. DGGE profiles targeting amplified 16S rRNA gene fragments at different stages of the culture enrichment.

database were identified as *Alcanivorax* spp. and these clones were present at all cultivation stages. In addition, 11 clones were matched to *Alcanivorax* sp. EPR 6 (550/550, 100% identity) that was originally isolated from a deep-sea hydrothermal vent (Vetriani et al., 2005). Clone v1 corresponding to band 2a was matched to *A. dieselolei* B-5 (550/550, 100 % identity), which has recently been recognized as a novel alkane-degrading bacterium isolated from seawater and deep-sea sediment (Liu et al., 2005). Interestingly, they became a predominant part of the microbial community in crude oil-contaminated seawater when nitrogen and phosphorus are supplemented (Harayama et al., 1999). The presence of *Alcanivorax* spp. as the dominant clones was significant since these bacterial species are known to reduce nitrate to nitrite (Yakimov et al., 1998). In addition, the genomic sequence of the marine hydrocarbonoclastic bacterium *A. borkumensis* SK2 is reported as a type strain. Genome analysis yielded unprecedented insights into the bacterium's capacity for 1) n-alkane degradation, including metabolism, biosurfactant production and biofilm formation, 2) scavenging nutrients and cofactors in the oligotrophic marine environment and 3) coping with various habitat-specific stress factors (Schneiker et al., 2006). Furthermore, *A. borkumensis* SK2 expressed *nrrS* genes including *nirK* and *nor* (Schneiker et al., 2006), implying that the species performs nitrite and nitrate reduction sequentially.

In this experiment, three types of species, which exhibit denitrifying activities, were isolated from this consortia. Two of them were identified as belonging to *Alcanivorax* sp. and *Pseudomonas* sp. using 16S rRNA analysis (data not shown). Accordingly, considering their abundance in our microbial consortium, the dominant bacteria may have been responsible for the observed nitrate and nitrite reduction. The other dominant clones were identified as *Dietzia* spp. These were originally isolated from coastal sub-seafloor sediment (Rainay et al., 1995), and belong to group *Actinobacteria*. Three clones, v26, v27 and v28, corresponding to band 21 which was a weak band, were identified as *Vitellibacter vladivostokensis* (547/547, 100 % identity), a member of the family *Flavobacteriaceae* that was discovered in 2003 and that can be found in marine environments (Nedsahkovskaya et al., 2003). Band 2c represented a complex mixture beyond the genus level, as shown in Fig.3. The closest relatives of clone v9 matched *Pseudomonas stutzeri* (532/550, 96 % identity) a denitrifying bacteria belonging to the phylogenetic group *γ-proteobacteria*. Clone v8 was included in an actinobacterial cluster and v7 in the *Alcanivorax* sp.

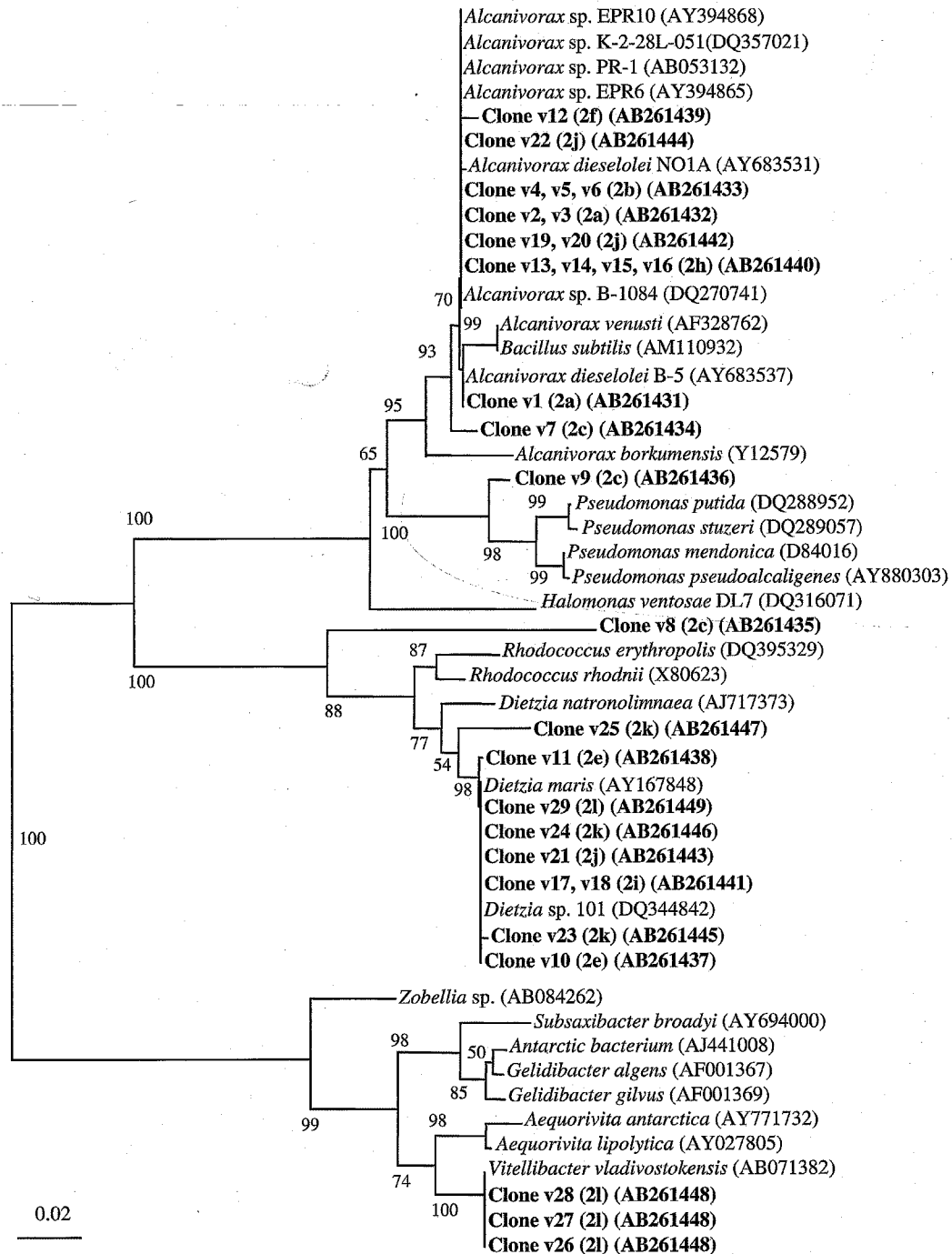


FIG. 3. Phylogenetic analysis based on partial 16 S rRNA gene fragments. The tree was constructed by the neighbor-joining method. The scale bar indicates 2% nucleotides substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 replicates: only values greater than 50 are expressed. Nucleotide sequence database accession numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

cluster. This type of diverse assemblage can be expected in environmental samples that contain complex mixtures of microorganisms of various origins, and it would not allow discrimination among any co-

migrating bands having different sequences. The performance of the cloning procedure did not improve when targeting 16S rRNA genes as compared to that of functional gene analysis.

DGGE profiles of the functional gene *amoA* in AOB

DGGE analysis for all the functional genes *amoA*, *nirS*, *nirK*, *norB* and *nosZ* performed at 0, 7, 14, 21 and 37 days post-inoculation revealed no remarkable differences in the banding patterns among the cultivation stages although some dissimilarities were obtained in the band intensity. Therefore, the DGGE variations at 37 days of incubation for *amoA*, and at 21 days of incubation for *nirS*, *nirK*, *norB* and *nosZ* were further assessed.

Seven bands were obtained on the gel (Fig. 4(a)), and the cloning efficiency was ideal for the *amoA* gene. We then examined the sequences for 28 positive clones corresponding to those 7 bands. All the retrieved sequences were among previously published *amoA* sequences belonging to the β -subdivision of *Proteobacteria*. Almost one-third of the clones corresponding to bands a1 to a4 were identified as *Nitrosomonas* sp. Nm107 (453/453 bp, 100% identity), and 50% of those corresponding to bands a5 to a7 matched *Nitrosomonas* sp. Nm148 (434/452 bp,

96% identity). Figure 5 illustrates a phylogenetic tree constructed based on the *amoA* sequence recovered from the enrichment culture as compared to certain reference sequences selected from the DDBJ database. Of 28 clones, 14 (14/28, 50%) were mutually identical and their closest pure-culture representative was *Nitrosomonas nitrosa* with high bootstrap values, resulting in clustering within the *N. communis* lineage. The remaining 14 clones were related to *Nitrosomonas* sp. strain Nm107, and exhibited mutations in several nucleotides. The findings of a previously reported 16S rRNA-based phylogenetic analysis (Purkhold et al., 2000) suggest that this organism is most probably a strain of the *N. europaea-Nitrosococcus mobilis*.

Nitrosomonas spp. are observed to be dominant in aquatic environments and are often found in wastewater treatment plants: this is not surprising considering the high ammonia levels often present in municipal effluent. The *Nitrosomonas* spp. and *N. communis* clusters that were identified in this study are often found in environments with high substrate concentrations (Koops et al., 2001). These organisms exhibit higher K_s (lower substrate affinity) values and higher growth rates than members of the genus *Nitrosospiras* (Pommerening-Roser et al., 1996; Scharamm et al., 2000). In this experiment, the AOB related to *Nitrosomonas* spp. were maintained in the population under high-ammonia culture conditions. The δ -subclass of *Proteobacteria* was originally discovered in a marine environment; however, there is no evidence that these types of AOB were absent. A functional gene would be a better target for fine-scale resolution with regard to the functional and ecological aspects of the microbial phylogenetic variations.

In this study, a homoduplex was produced for an *amoA*-based analysis of gene fragments, and several mutations were observed among the 4 clones corresponding to each band. As a speculation, these mutations could be caused by having used the degenerated primers. Another is that the AOB in the enrichment culture could have exhibited a point mutation in multiple copies of *amoA*. In a previous DGGE-based study, *N. multiformis* was found to yield 4 bands, whereas that of *N. europaea* yielded only a single band (Nicolaisen et al., 2002). This discrepancy may also have been due to misinterpretation and misreading of the results of the PCR and sequencing procedures. The existence of identical copies of some genes or of several mutations in certain gene sequences is not expected to have any significant effect on the melting behaviour of these genes. Mendum et al. (1999) reported that no remarkable

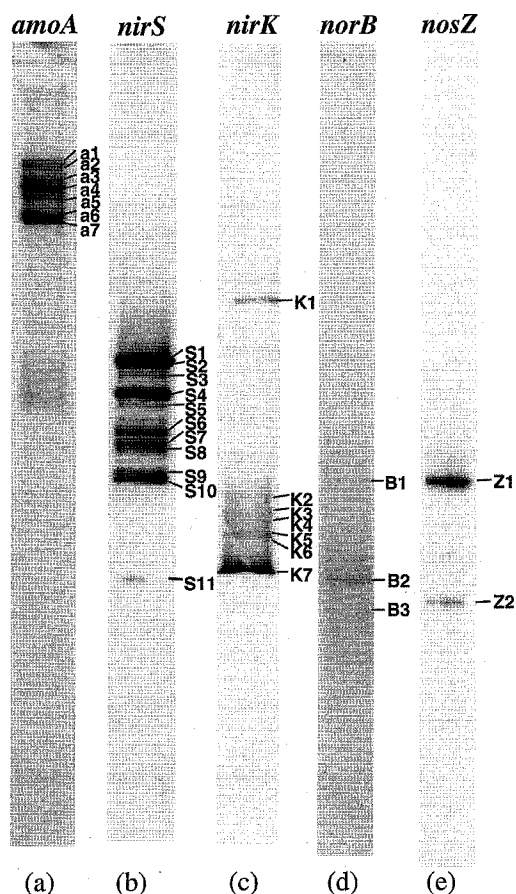


FIG. 4. DGGE profiles of dominating ammonia-oxidizers and denitrifying communities from enrichment culture samples.

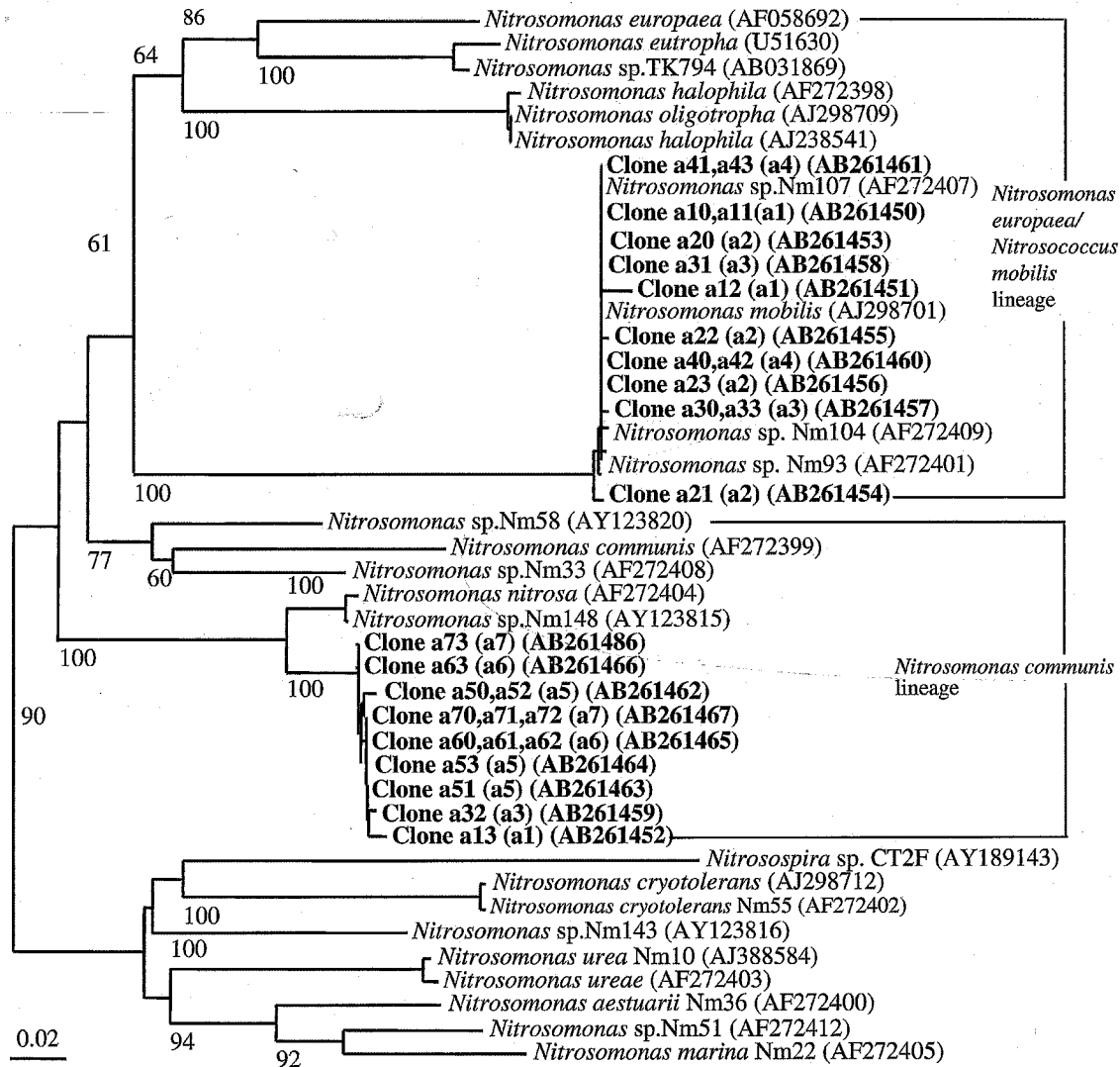


FIG. 5. Phylogenetic analysis based on partial *amoA* gene fragments. The tree was constructed with by the neighbor-joining method. The scale bar indicated 2% nucleotide substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replicates: only values greater than 50 are shown. Nucleotide sequence database accession numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

changes were observed in AOB populations despite significant alterations in their metabolic activity. Certain transient markers, such as mRNA, can be suitable for determining particular *in situ* activities of microorganisms while investigating the community structure in microbial habitats.

DGGE for the functional genes *nirS*, *nirK*, *norB* and *nosZ* of denitrifiers

DGGE performed for a part of the *nirS* gene produced a total of 11 bands for the enrichment culture samples (Fig.4-(b)). A total of 44 clones were sequenced and compared with sequences from the

database; of those 22/44 (50%) exhibited homology with the *nirS* gene of *Pseudomonas* sp. R125 (318/319, 99% identity). In addition, all 4 clones corresponding to bands S2, S3, S5, S6 and S11 were identical, and 1 or 2 nucleotide mutations were observed among the 4 clones corresponding to the other bands. Fig.6 illustrates a phylogenetic tree based on the *nirS* sequence as compared to certain reference sequences selected from the database. One major cluster was observed, accompanied by a subcluster of closely related *nirS* sequences of *Pseudomonas* sp. and related isolates (cluster A). Since PCR-DGGE analysis targeting the 16S rRNA

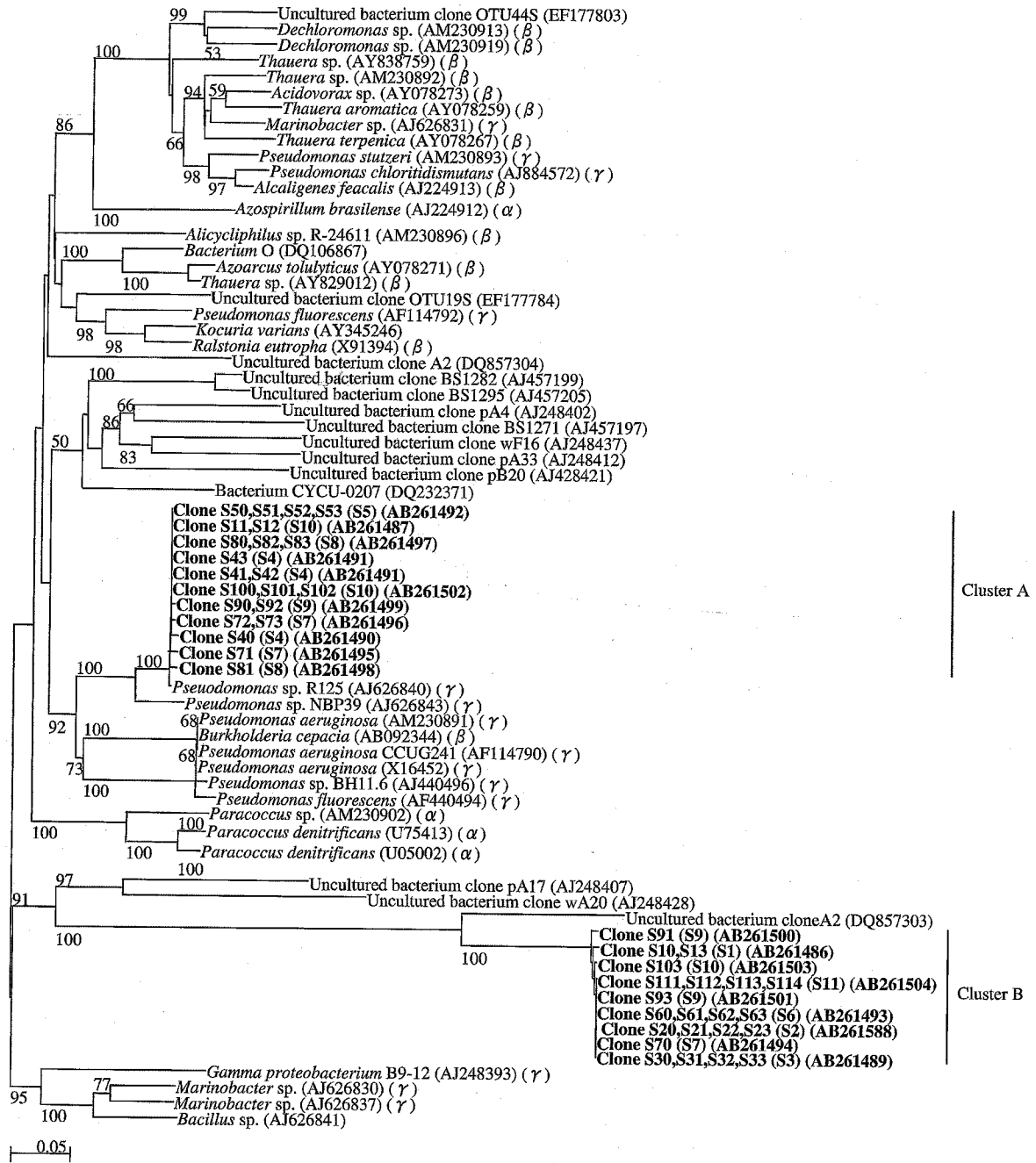


FIG. 6. Phylogenetic analysis based on partial *nirS* gene fragments. The tree was constructed by the neighbor-joining method. The scale bar indicates 5% nucleotide substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replicates: only values greater than 50 are expressed. Nucleotide sequence database accession numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

genes revealed clone v9 to be most closely related to *Pseudomonas stutzeri*, we expected that the *Pseudomonas* sp. was involved in denitrification in the enrichment culture. Interestingly, the other subcluster comprised sequences that were very distantly related to the *nirS* sequences of major cultured

strains (cluster B), which are closely related to the *nirS* genes of the uncultured bacterial clone A2 that was originally isolated from activated sludge (Geets et al., 2007). The other subcluster within this branch that included isolates from marine clones was found due to high bootstrap values (Liu et al., 2003).

Ultimately the clones from cluster B were classified under group *Proteobacteria*.

DGGE performed for a part of the *nirK* gene by using the enrichment culture yielded a total of 7 bands (Fig.4-(c)). A total of 28 clones were sequenced and the sequences were compared with those in the data-

base. The *nirK* gene sequences did not suggest this gene to be strongly related to any of the known *nirK* sequences considered in this study. Fig.7 presents the phylogenetic tree based on the *nirK* sequence in the database. The major cluster (cluster A) comprised 19 of 28 clones (68%) closely related to *nirK*

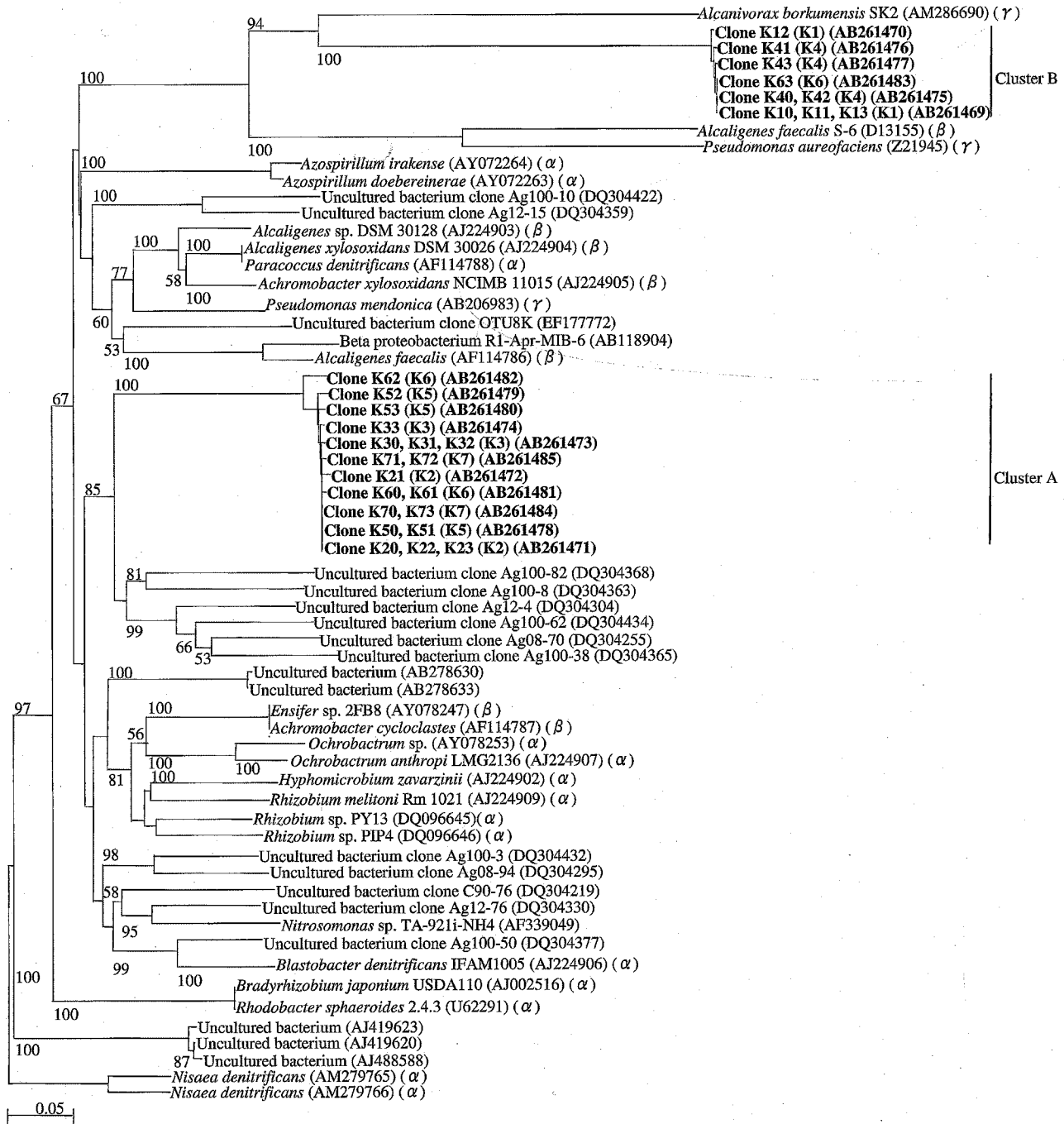


FIG. 7. Phylogenetic analysis based on partial *nirK* gene fragments. The tree was constructed using the neighbor-joining method. The scale bar indicates 5% nucleotide substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replicates: only values greater than 50 are shown. Nucleotide sequence database numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

sequences of the uncultured bacterial clone Ag100-3 with high bootstrap values (Throback et al., 2007), which has been isolated from arable soil containing the heavy metal silver. Thus, cluster A was considered the dominant clone comprising novel species of denitrifiers. The other subcluster (cluster B) comprised of 9 of 28 clones (32%) and included the well-known denitrifying bacteria *Alcaligenes faecalis* S-6 (β -subclass), *Pseudomonas aureofaciens* (γ -subclass), and *Alcanivorax borkumensis* SK2 (γ -subclass). As mentioned above, PCR-DGGE analysis targeting the 16S rRNA genes of *A. borkumensis* SK2 indicated that this organism performs nitrite reduction followed by nitrate reduction. Furthermore, based on the PCR-DGGE results that more than 50 % of the clones were identified as close relatives of *Alcanivorax* spp., it is likely that certain *Alcanivorax* sp. strains in the consortium were predominantly denitrifiers. Also, these groups probably belonged to group *Proteobacteria*.

The molecular diversity of the *nirS* and *nirK* genes has previously been investigated to elucidate the composition and structure of denitrifying communities in the oxygen-deficient zone off the Pacific coast of Mexico. It was observed that geographic location and biochemical properties (particularly nitrate and oxygen profiles) affect the structure of denitrifying communities in marine sediment (Braker and Tiedje, 2003). In our study the high nitrate concentration, oxygen level and organic matter (ethanol concentration) throughout the cultivation period may have been responsible for the temporal changing in the microbial community composition and could be a key parameter for identifying denitrifying bacteria among the members of consortium.

In a recent report, Braker and Tiedje (2003) proposed that *norB* which encodes nitric oxide reductase may be used as a functional marker for investigating the distribution of denitrifying bacteria in environmental samples. One class of *norB* encodes the quinol-oxidizing single-subunit class (qNorB), while the other encodes a cytochrome *bc*-type complex (cNorB). Here, 2 primer sets were tested, namely, qnorB2F-5R targeting quinol-oxidizing nitric oxide reductase, and cnorB2F-6R targeting cytochrome-oxidizing nitric oxide reductase. The PCR amplification was satisfactory with the qnorB2F-5R primer, but not with cnorB2F-6R. The partial *norB* genes yielded 3 bands; however the cloning results for band B1 were unsatisfactory (Fig. 4-(d)). A total of 8 clones were obtained of which 5 (63%) exhibited homology with the *Alcaligenes xylosoxidans* nitric oxide reductase gene *norB* (187/222, 84% identity). Of 3 clones corresponding to band B3, 2 did not match

norB in terms of size, while the third, i.e. clone B30, did. The results of the phylogenetic analysis are shown in Fig.8. All of 5 clones that demonstrated homology with *A. xylosoxidans* were clustered within a branch of the *qnorB* gene, corresponded to other cultured organisms including *Alcaligenes* sp., *A. faecalis*, *A. xylosoxidans*, *Ralstonia eutropha*, and some nondenitrifying pathogenic microorganisms such as *Neisseria meningitidis*. In contrast to the *nirS* and *nirK* genes, *qnorB* clones from the enrichment culture were not found to be closely related to any of environmental clones. A previous study used restriction fragment length polymorphism (RFLP) to screen a total of 130 cloned *norB* PCR products from sediment samples (Braker and Tiedje, 2003). The clones of the environmental *qnorB* genes were present on separate branches within clusters and exhibited the differences typically observed in genes from cultures versus those from natural environmental sources. Our data support the possibility that the clones obtained from the enrichment culture originated from different bacteria that were not determined to possess the *qnorB* gene. These clones are possibly from members of the phylum *Proteobacteria*.

Furthermore, the *nosZ* gene was also not found to be very closely related to the genes in the database. DGGE produced one dense band and one weak band, i.e., Z1 and Z2 respectively (Fig.4-(e)). The clones obtained did not match any known *nosZ* gene sequence (Fig.9). The 7 clones obtained were divided into 2 groups due to a high bootstrap value of 100. Both clusters were well separated from the cultured group that included the isolates from marine sediment (AF016058 and AF016059). In addition, the cluster diagram illustrated that these clones were significantly separated from the uncultured clones derived from marine sediment (AF119919, AF119944, AF119918, AF016057, AF119950, and AF119937) (Scala and Kerkhof, 1998). A possible reason for this distinct separation is that these organisms are novel denitrifiers, and were previously cultivated from marine sediment but not identified to possess the *nosZ* gene. Scala and Kerkhof (1998) reported that the sequences of environmentally derived isolates were not closely related to those found from cultured ones and that the former exhibit substantial diversity with regard to *nosZ*, which is not true for the latter. In our study, no candidates were found in databases representing cultivated collections also in uncultured clones. These clones must also be members of *Proteobacteria* phylum.

A unique consortium of AOB and denitrifying bacteria was obtained from organic enriched marine sediment. The high nitrate concentration and organic

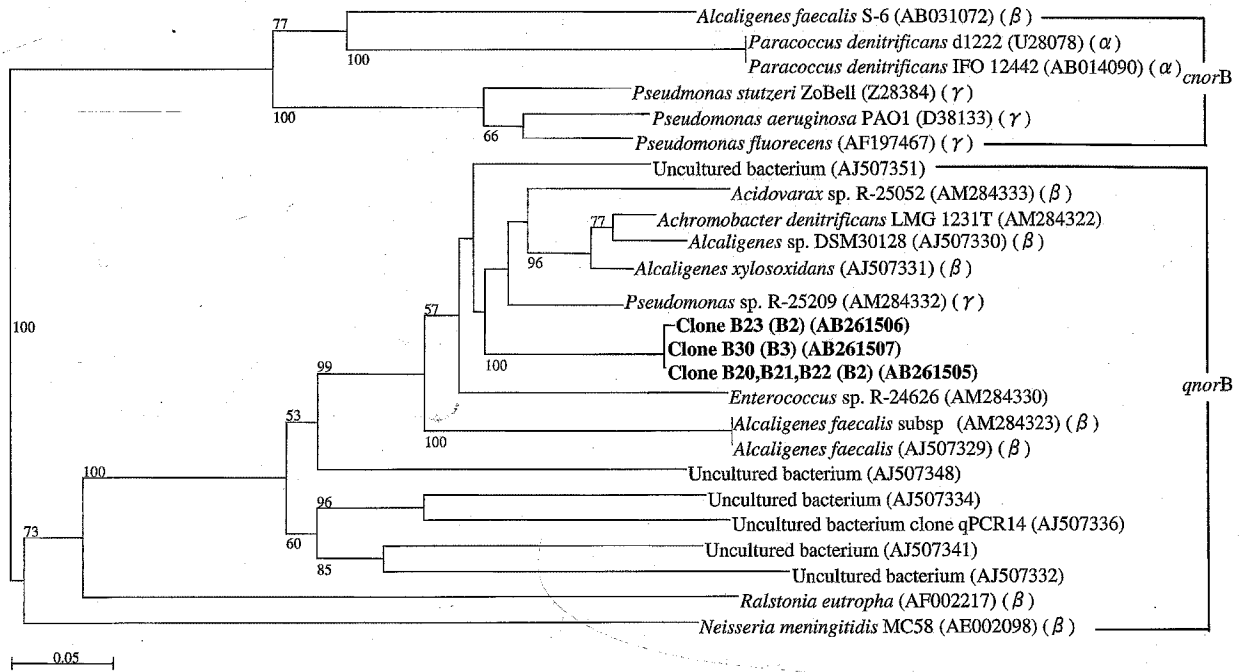


FIG. 8. Phylogenetic analysis based on partial *norB* gene fragments. The tree was constructed by the neighbor-joining method. The scale bar indicates 5% nucleotide substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replicates: only values greater than 50 are expressed. Nucleotide sequence database accession numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

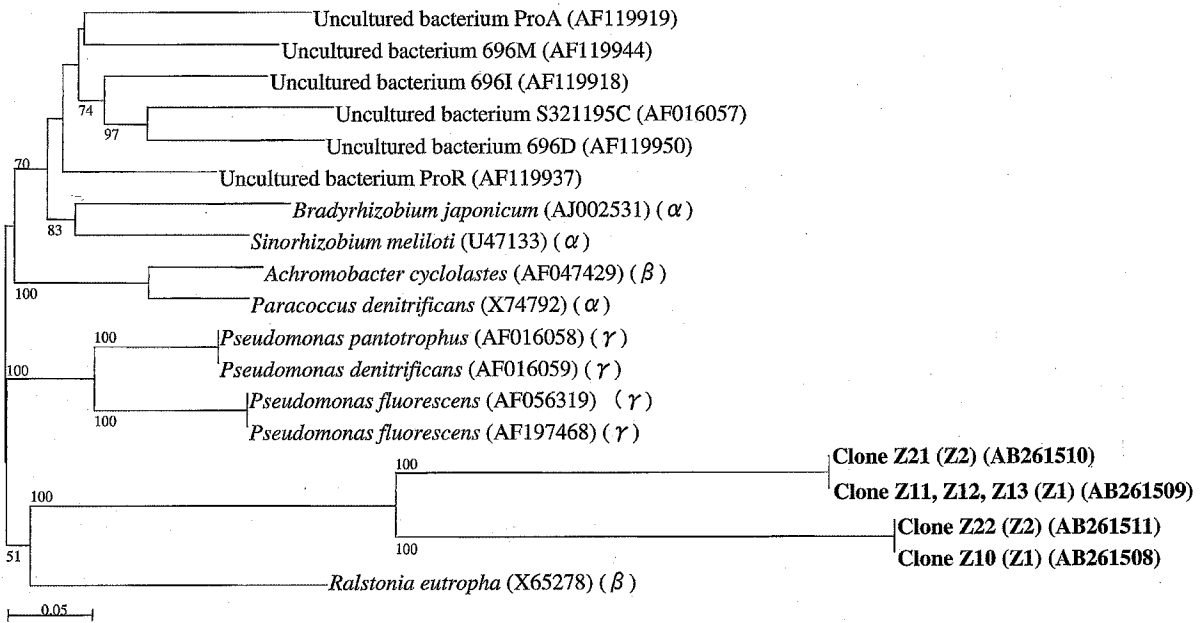


FIG. 9. Phylogenetic analysis based on partial *nosZ* gene fragments. The tree was constructed with the neighbor-joining method. The scale bar indicates 5% nucleotide substitutions. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replicates: only values greater than 50 are shown. Nucleotide sequence database accession numbers are shown in parentheses. The band number is indicated followed by the number of each clone in parentheses. The clones obtained in this study are given in bold letters.

compounds via the cultivation procedure may have been responsible for determination in the microbial community composition. Optimizing the different environmental factors could be a key parameter for identifying the denitrifying bacteria in the consortium. The unique clones obtained from the functional genes related to each denitrification step were observed to be of the following types: 1) closely related to uncultured bacterial clones, and 2) exclusively comprising clusters of novel sequences determined for cultivated species. The data presented here provided a substantive basis for assessing the use of a bacterial consortium for the biodegradation of environmental contaminants via nitrogen removal.

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