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New *nirS*-Harboring Denitrifying Bacteria Isolated from Activated Sludge and Their Denitrifying Functions in Various Cultures

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Abstract By using PCR with *nirS* gene primers, three *nirS*-harboring denitrifying bacteria (strain N6, strain N23, and strain R13) were newly isolated from activated sludge of a weak municipal wastewater treatment plant. Small-subunit rRNA gene-based analysis indicated that strain N6, strain N23, and strain R13 were closely related to *Arthrobacter* sp., *Staphylococcus* sp., and *Bacillus* sp., respectively. In an attempt to identify their roles in biological nitrate and nitrite removal from sewage, we investigated their specific denitrification rates (SDNRs) for NO_3^- and NO_2^- in various cultures. All pure-cultures of each isolated *nirS*-harboring bacterial strain could remove NO_3^- and NO_2^- simultaneously in high efficiency, and the carbon requirements for NO_3^- removal of strain N6 and strain R13 were effectively low at 3.1 and 4.1 g COD/g NO_3N, respectively. In the case of mix-cultures of the strains (N6+N23, N6+R13, N23+R13, and N6+N23+R13), their SDNRs for NO_3^- were also effective, and their carbon requirements for NO_3^- removal were also effective at 3.0–3.8 g COD/g NO_3N. However, all tested mix-cultures accumulated NO_2^- in their culture media. On the other hand, the continuous culture of activated sludge mixed with strain N6 showed no significant increase of NO_3^- removal in comparison with strain N6's pure culture. These results suggest that nitrate and nitrite removal in biological wastewater treatment might be dependent on complicated bacterial interactions, including several effective denitrifying bacteria isolated in this study, rather than the specific bacterial types present and the number of bacterial types in activated sludge.

Key words: *nirS*-Harboring denitrifying bacteria, weak municipal wastewater, SDNRs

Denitrification has been recognized for more than a century as a key process in the nitrogen cycle. Denitrification has taken on an added importance due to its role as a major source for NO and N_2O gases, which are major contributors to atmospheric ozone destruction and global warming [17, 28]. In addition, denitrification is important in wastewater treatment, as a means of both removing excess nitrate and stimulating carbon removal when aeration is difficult. In the latter case, there is an increased interest in using nitrate to drive pollutant bioremediation in aquifers [15, 18]. In wastewater treatment, the removal of nitrogen compounds from wastewaters can be accomplished by a combination of nitrification (ammonia oxidation to nitrate or nitrite) and denitrification (nitrate reduction to N_2 or N_2O) [11, 17]. This requires the combined or sequential actions of various groups of microorganisms, including nitrifying bacteria and denitrifying bacteria. The denitrification processes are therefore difficult to control, and may affect the reactor performance. For stable operation of denitrifying reactors, a better understanding of denitrifying microorganisms in activated sludge is required.

The key step of the denitrification pathway is the nitrite reduction by nitrite reductase (Nir) [14]. This reaction distinguishes denitrifiers from nitrate respires. Denitrification includes two well-known and distinct types of Nir enzymes: one with heme *c* and heme *d*1 (*cd*1-Nir) and the other containing copper (Cu-Nir). Denitrifying bacteria possess either a cytocrome-*cd*1 emzyme (*cd*1-Nir) encoded by the *nirS* gene or a Cu-containing enzyme for nitrite reduction (Cu-Nir) encoded by the *nirK* gene. It is believed that *cd*1-Nir is the more widespread and predominant nitrite reductase in denitrifying bacteria, but Cu-Nir shows greater variation in molecular weight and immunological reactions, and is present in more taxonomically unrelated strains [4]. Therefore, the *nirS* gene is more useful than the *nirK* gene as a molecular marker for PCR to detect denitrifying bacteria.

The goal of this work was to elucidate the role of denitrifying bacteria and to investigate the relationships between denitrifying bacteria and environmental factors, which can change the bacterial population dynamics in an activated

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sludge process and consequently lead its denitrifying efficiency to come down. In this direction, three *nirS*-harboring bacteria were newly isolated from the activated sludge of a weak municipal wastewater treatment plant, by using PCR with *nirS* gene primers, and then we investigated their specific denitrification rates (SDNRs) for NO_3^- and NO_2^- in various cultures treating sewage, using laboratory-scale bioreactors.

MATERIALS AND METHODS

Environmental Samples

Sewage taken from the G wastewater treatment plant (WWTP; Sung Nam City, Korea) was fed to a laboratory-scale reactor system. The sediment samples of sewage were used for the isolation of *nirS*-harboring bacteria. All samples were maintained on ice during transport to the laboratory. The sediment samples were divided into aliquots and immediately frozen at -80°C for further analysis. The characteristics of the sewage are shown in Table 1.

Laboratory Experiments

The purpose of laboratory experiments was to investigate the denitrifying functions of the *nirS*-harboring bacteria isolated in this study (see below). The sewage sample was filtered through 0.45-μm pore-size acetate cellulose filters (Advantec, Japan), and then the filtrate was used as a medium for laboratory-scale reactor systems. The laboratory-scale reactor systems were operated in two distinctively different modes: (1) batch reactor (for pure cultures and mix-cultures of the isolated bacteria: strain N6, strain N23, and strain R13), and (2) continuous reactor (for sediment swwage mixed with strain N6) as shown in Fig. 1. All the conditions of batch and continuous cultures are described in Table 2.

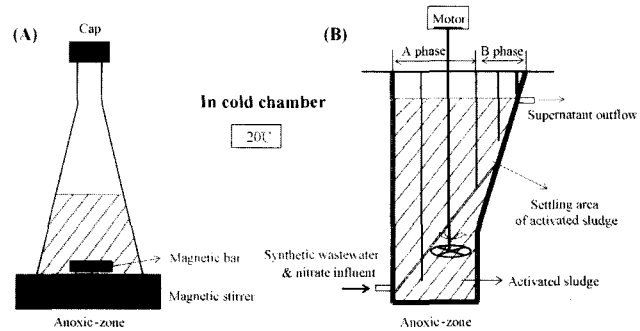

Fig. 1. Schematic diagrams of anoxic batch (A) and anoxic continuous (B) reactors.
The A phase is the denitrifying-reaction phase and the B phase is the setting phase of the activated sludge.

At first, all the bacterial strains used in this study were adapted to 500 mg NO_3N/l of KNO_3 in LB broth, and then used as inocula for the laboratory-scale reactor systems. Each well-grown bacterial strain in LB broth containing 500 mg NO_3N/l of KNO_3 was centrifuged (4,500 rpm, 20 min), and the precipitate was inoculated into each batch reactor (capped 5-l glass flask; anoxic condition) as to be 50 mg VSS/l, respectively. Inoculated cell mass of the individual strain in mix-cultures was equal to each other (to be total 50 mg VSS/l). The batch reactors of the sediment sewage sample and *Pseudomonas aeruginosa* ATCC 10145, which were used to optimize the amplification of denitrification genes [5, 6], were also conducted as a control, respectively. All of the batch reactors were initially supplemented by approximately 100 mg NO_3N/l of KNO_3. In the case of continuous reactor, sediment sewage sample and strain N6 were mixed [5% of strain N6 (w/w as mg VSS/l) to total sediment sample], and then used as inoculum. The culture periods of batch reactor and continuous reactor were 30 h

Table 1. Characteristics of the sewage.

Constituents	Values	
	Range	Average
pH	7.1-7.5	-
Alkalinity as $CaCO_3$	70-95	-
Total suspended solid	74-110	80
Volatile suspended solid	45-70	-
Total COD_{Cr}[a]	150-200	150.0
Soluble COD_{Cr}	23-40	30.0
BOD[b]	-	103.0
Volatile fatty acid	-	16.3
Total Kjeldahl-N	11-25	20.0
NH_4N	8-17	-
Total phosphate	4.5-6.6	5.0
PO_4P	3.5-4.7	-

[a]COD, chemical oxygen demand as chromium.
[b]BOD, biological oxygen demand.
*All values are expressed as mg/l, except pH.

Table 2. The entire conditions of batch and continuous cultures.

Parameters	Conditions	
	Batch	Continuous
Initial conc. of KNO_3 as N (mg/l)	120-160	180-200
Initial TCOD (mg/l)[a]	370-420	870
Agitating speed (rpm)	80	80
pH	6.7-7.2	6.7-7.2
Temperature (°C)	20±1.0	20±1.0
Working volume (l)	2	2.4
HRT (h)[b]	-	4
SRT (day)[c]	-	10
Flow rate ratio (V_N/V_W)[d]	-	1/8.6
Inoculums (mg VSS/l), Inocula/Sludge (w/w)	50	1/20
Average bacterial mass (mg/l) during culture	100-200	1,500-2,000

[a]Total chemical oxygen demand including COD value of sewage.
[b]Hydrolic retention time.
[c]Sludge retention time.
[d]V_N, volume of nitrate solution (960 mg NO_3N/l); V_W, volume of sewage.

and 10 days as solid retention time (SRT), respectively. The experimental temperature of 20°C was automatically controlled in a cold chamber.

Chemical Analysis

The liquid samples (25 ml) were centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatants were diluted as required by the relevant analytical methods. NO_3N and NO_2N were analyzed by ion chromatography [DIONEX (Sunnyvale, CA, U.S.A.) model DX-500]. The precipitate was measured for volatile suspended solid (VSS) of bacteria. Specific denitrification rates (SDNRs) were calculated by the following equation: SDNR=mg (ΔNO_3N or ΔNO_2N)/(g VSS·h) [10], where ΔNO_3N and ΔNO_2N are the amounts of NO_3^- and NO_2^- removed in the culture, respectively; VSS is an average bacterial cell mass; h is the interval decreasing the most of nitrate or nitrite (12 h for ΔNO_3N or 10 h for ΔNO_2N), during the culture period. The ratio of COD consumed to nitrate reduced [(ΔCOD)/(ΔNO_3N)] was calculated as carbon (C) requirement for NO_3^- removal. $SDNR_{NO3}$, $SDNR_{NO2}$, and SDNRs are the SDNR for NO_3N, NO_2N, and $SDNR_{NO3}$ and $SDNR_{NO2}$, respectively. All other wastewater analyses for the examinations of water and wastewater were conducted according to the standard methods [3]. All analyses were conducted in triplicates.

Isolation of *nirS*-Harboring Denitrifying Bacteria

Two grams of activated sludge obtained from the G wastewater treatment plant were washed twice with phosphate-buffered saline [135 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 (pH 7.4)], and suspended and then serially diluted in sterile distilled water (D.W.). Denitrifying bacteria were attempted to isolate by plating each sludge dilution on two types of agar media: the modified nutrient medium [NM; prepared by mixing 0.03% beef extract, 0.05% peptone, and 1.5% agar per liter of D.W. (pH 7.0)], and the sludge medium (SM; prepared by mixing 15 g of agar per liter of sewage supplement). The plates were incubated at 20°C in an anaerobic jar for 10 days and, based on the basis of pigment, shape, size, surface texture, and opacity, the morphologically distinct colonies were isolated by transferring single colonies on SM. This stage was repeated three times. Among isolates, *nirS*-harboring denitrifying bacteria were selected by using PCR with *nirS* primers [5] (see below). In order to select *nirS*-harboring denitrifying bacteria possessing effective nitrate removal ability, all of the isolates were first examined for their denitrifying function by a nitrate reduction test with Griess Ilosvary reagent and zinc powder in liquid NM [13]. The purity of three selected isolates was examined by Gram staining and microscopic observation, and their identity was confirmed by 16S rDNA sequence analysis (see below). *Pseudomonas aeruginosa* ATCC 10145 was used as a control strain for *nirS*-harboring denitrifying bacteria [5, 6].

DNA Extraction

Chromosomal DNA of the isolates was extracted and purified, as described by Lee *et al.* [20] and Park *et al.* [25]. The purity and concentration of DNA extracted were analyzed by standard agarose electrophoresis and by reading absorbance at 260 nm.

PCR Amplification of *nirS* Gene from DNA

Amplification of the *nirS* gene from the chromosomal DNA extracted was carried out in a DNA thermal cycler (Model 480; Perkin-Elmer, Norwalk, Conn., U.S.A.) with *nirS* gene primers, S1F (5'-CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T-3') and S6R (5'-CGTTGAACTT(A/G)CCGGT-3') [5]. The PCR temperature program began with an initial 5-min denaturation step at 94°C, 30 cycles of 94°C for 40 sec, 57°C for 40 sec, and 72°C for 45 sec, and a final 10-min extension step at 72°C. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany), and then cloned using pGEM-T Easy vector system I (Promega, Madison, WI, U.S.A.), according to the manufacturer's instructions. The clones containing appropriate-sized inserts were identified by agarose gel electrophoresis of the PCR products obtained from the system's host lysates, by using PCR with the vector primers, T7 and SP6, complementary to the vector sites flanking the insertion site. The nucleotide sequences of the cloned products were determined using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and ABI310 Sequencers (Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacturer's instructions.

For confirmation, the partial sequences of *nirS* genes of the isolates were compared with those on publicly accessible databases, using the Basic Local Alignment Search Tool (BLAST) program. The accession numbers for the partial *nirS* gene sequences of strain N6, strain N23, and strain R13 are AF335922, AF335923, and AF335924 in the GenBank nucleotide sequence database, respectively.

DNA Preparation, Sequencing, and Analysis of 16S rDNA Sequencing Data

Amplification of 16S rDNA from the chromosomal DNA was carried out in the DNA thermal cycler (Model 480; Perkin-Elmer, Norwalk, Conn., U.S.A.) with universal bacterial 16S rDNA primers, 27F (5'-AGAGTTTGATCMTGCTCAG-3') and 1492R (5'-GGTTACCTTTGTTACGACTT-3') [7]. The PCR temperature program began with an initial 5-min denaturation step at 94°C, 30 cycles of 94°C for 1 min, 57°C for 2 min, and 72°C for 2 min, with a final 10-min extension step at 72°C. The cloning and sequencing of 16S rDNA fragments were also performed in the same procedure described above. The sequences obtained from 16S rDNA libraries were checked for chimeras using the CHECK-CHIMERA software of the Ribosomal Database Project [21], and compared with those at the GenBank nucleotide

sequence database by using the BLAST and FASTA programs (Wisconsin Package version 9.1 of the Genetics Computer Group). The sequences were aligned and analyzed by using CLUSTAL W enclosed within the Lasergene software package (DNASTAR Inc., Madison, WI, U.S.A.). The partial 16S rDNA sequences of strain N6, strain N23, and strain R13 are available in the GenBank nucleotide sequence database under the accession numbers AF335919, AF335920, and AF335921, respectively.

RESULTS

Isolation and Identification of *nirS*-Harboring Bacteria

Nine *nirS*-harboring denitrifying bacteria were selected among 78 bacterial isolates from the activated sludge by using PCR with *nirS* primers [5]. Among them, three *nirS*-harboring denitrifying bacteria, strain N6, strain N23, and strain R13 (Fig. 2), were selected as the strains possessing effective nitrate removal ability from the nitrate reduction test with Griess Ilosvary reagent and zinc powder (data not shown) [13]. Results for the partial 16S rDNA sequences and identities for the three selected *nirS*-harboring denitrifying bacteria are shown in Table 3, showing that strain N6, strain N23, and strain R13 are closely related to *Arthrobacter* sp., *Staphylococcus* sp., and *Bacillus* sp., respectively. All partial *nirS* gene sequences of the three selected denitrifying bacteria showed 83–85% identity to the *nirS* gene of *Psedomonas stutzeri* in the homology search in BLAST (Table 3), confirming that these newly isolated denitrifying bacteria are parentally harboring the *nirS* gene.

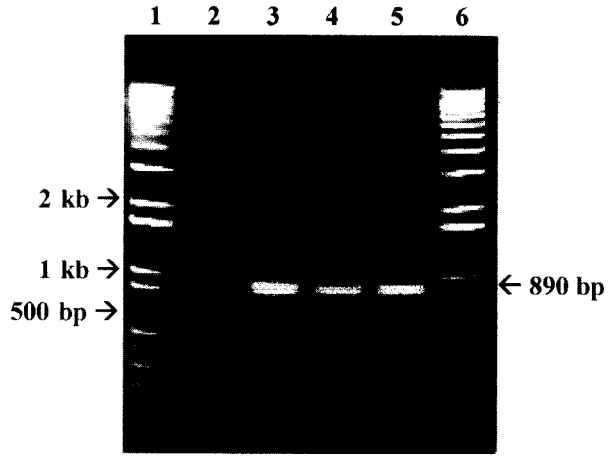

Fig. 2. Gel electrophoresis pattern of *nirS* genes from the three isolated *nirS*-harboring bacteria.
nirS genes, from *P. aeruginosa* ATCC 10145 (lane 2); from strain N6 (lane 3); from strain N23 (lane 4); from strain R13 (lane 5). Lanes 1 and 6, 1 kb ladder DNA marker. The positions of molecular weight standards are indicated on both side of the gel.

Denitrifying Functions of *nirS*-Harboring Isolates in Pure Culture

In order to investigate whether it is possible to achieve significant nitrogen removal using each pure culture of the three selected *nirS*-harboring denitrifying bacteria in a laboratory-scale reactor, each pure culture's specific denitrification rates (SDNRs) for NO_3^- and NO_2^- were examined (Table 4). The SDNRs for the control activated sludge and *P. aeruginosa* ATCC10145 were also examined as controls, respectively. The SDNRs of all three selected

Table 3. Homology search based on partial 16S rRNA and *nirS* gene sequences of the selected *nirS*-harboring bacteria in BLAST*.

Strain	Partial 16S rDNA sequences			
	Closest match (Accession No.)	No. of nucleotides compared	% Similarity with closest match	Sequenced bps & Access. No.
N6	*Arthrobacter polychromogenes* (X80741)	407	95	557 bp AF335919
	Arthrobacter oxydans (X83408)	407	95	
N23	*Staphylococcus succinus* (AF004219)	462	95	472 bp AF335920
	Staphylococcus succinus SB72 (AJ320272)	462	95	
R13	*Bacillus* sp. VAN26 (AF286485)	88	97	134 bp AF335921
	Bacillus pumilus (AF288735)	88	97	
Strain	Partial *nirS* gene sequences			
	Closest match (Accession No.)	No. of nucleotides compared	% Similarity with closest match	Sequenced bps & Access. No.
N6	*Pseudomonas stutzeri nirS, nirT, nirB, nirM* genes (X56813)	322	83	458 bp AF335922
N23	*Pseudomonas stutzeri* nitrite reductase gene (M80653)	267	85	551 bp AF335923
R13	*Pseudomonas stutzeri nirS, nirT, nirB, nirM* genes (X56813)	126	84	546 bp AF335924

*The Basic Local Alignment Search Tool program.

Table 4. The SDNRs for NO_3^- and NO_2^-, and C requirement for NO_3^- removal of the various cultures.

Cultures	[a]COD	[a]VSS	[a]NO_3N	[b]NO_2N	[c]SDNR for NO_3^-	[d]SDNR for NO_2^-	[e]COD/NO_3N
Pure-culture							
Activated sludge	-63	175	-9.6	NC	4.6	>4.6*	6.6
P. aeruginosa ATCC 10145	-324	144	-50.4	NC	29.2	>29.2*	6.4
N6	-317	111	-103.1	-61.0	51.1	30.3	3.1
N23	-335	116	-51.2	-60.8	36.8	43.7	6.6
R13	-356	137	-86.4	-32.1	52.6	19.5	4.1
Mix-culture							
N6+N23	-312	150	-82.3	+62.2	45.7	NC	3.8
N6+R13	-409	178	-141.5	+122.5	66.3	NC	3.0
N23+R13	-415	180	-128.0	+128.4	59.3	NC	3.2
N6+N23+R13	-404	198	-125.2	+128.5	52.7	NC	3.2
Continuous culture							
Control	-763	1,092	-122.0	NC	4.6	NC	6.3
Modified**	-767	1,239	-143.0	NC	4.8	NC	5.4

Strain N6, *Arthrobacter* sp. N6; strain N23, *Staphylococcus* sp. N23; strain R13, *Bacillus* sp. R13. NC, could not calculated; -, consumed or removed; +, accumulated.

The units were [a]mg/l for 12 h, [b]mg/l for 10 h, [c]mg NO_3N/(g VSS·h), [d]mg NO_2N/(g VSS·h), and [e]g COD/g NO_3N, respectively.

*Because the concentration of NO_2N was almost undetectable but the concentration of NO_3N was still decreased during the same period, respectively.

**The modified sludge was mixed with strain N6 [5% (w/w) to total sludge]. All values were averaged from triplicate measurements.

isolates and *P. aeruginosa* ATCC 10145 were remarkably high compared with that of the control activated sludge (Table 4). Specifically, the $SDNR_{NO3}$ of strain N6 and strain R13 were 51.1 and 52.6 mg NO_3N/(g VSS·h), respectively. These values are over 10 times of the average $SDNR_{NO3}$ of general wastewater treatment plants (WWTPs) [3.5-5 mg NO_3N/(g VSS·h)] [8, 22]. In addition, the C requirements for NO_3^- removal of strain N6 and R13 were practically low at 3.1 and 4.1 g COD/g NO_3N, respectively, compared with that of the control activated sludge (6.6 g COD/g NO_3N) and *P. aeruginosa* ATCC10145 (6.4 g COD/g NO_3N) (Table 4). The average C requirements for NO_3^- removal of general WWTPs have been reported to be over 5 g COD/g NO_3N [8, 22]. However, in the case of $SDNR_{NO2}$, the $SDNR_{NO2}$ of strain R13 [19.5 mg NO_2N/(g VSS·h)] was not so effective even compared with that of *P. aeruginosa* ATCC10145 [>29.2 mg NO_2N/(g VSS·h)] (Table 4). In the case of strain R13, NO_2^- was removed very slowly (Fig. 3), however, the nitrite concentration decreased to 0 after day 5 of the reactor operation (data not shown). This kind of aspect by denitrifying bacteria has been also reported by Almeida *et al.* [2] and Park *et al.* [24]. The SDNRs of strain N23 were effectively high at 36.8 mg NO_3N/(g VSS·h) and 43.7 mg NO_2N/(g VSS·h), whereas its C requirements for NO_3^- was not effective, at 6.6 g COD/g NO_3N, compared with those of the control activated sludge and *P. aeruginosa* ATCC10145 (Table 4).

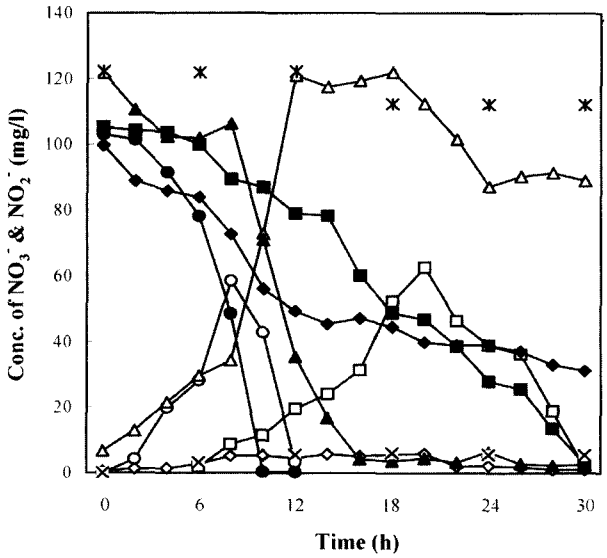

Fig. 3. NO_3^- and NO_2^- removal by pure-cultures of the isolated *nirS*-harboring denitrifying bacteria.

NO_3^- (✳, close symbol), NO_2^- (×, open symbol); Activated sludge (✳, ×); *P. aeruginosa* ATCC 10145 (◆, ◇); strain N6 (●, ○); strain N23 (■, □); strain R13 (▲, △).

Denitrifying Functions of *nirS*-Harboring Isolates in Mix-Culture

The mix-culture of strain N6 and strain N23 removed NO_3^- to the extent of an undetectable level with 45.7 mg NO_3N/(g VSS·h) (Table 4). However, its NO_2^- removal was not carried out in the same operating period (Fig. 4). The mix-culture of strain N6 and strain R13 performed a

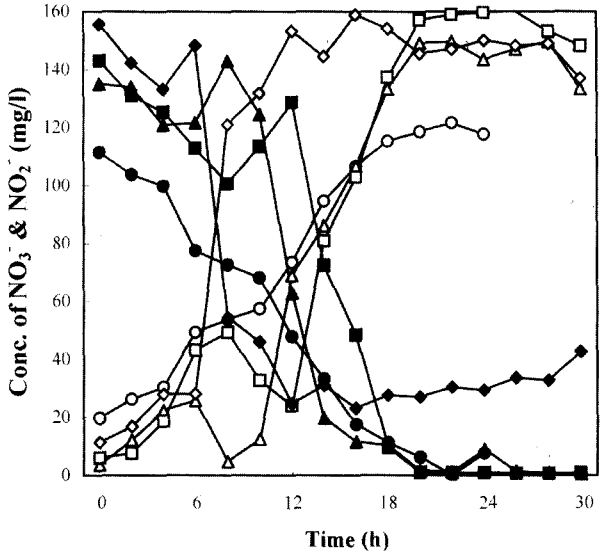

Fig. 4. NO_3^- and NO_2^- removal by mix-cultures of the isolated *nirS*-harboring denitrifying bacteria.
NO_3^- (close symbol), NO_2^- (open symbol); N6+N23 (●, ○); N6+R13 (■, □); N23+R13 (▲, △); N6+N23+R13 (◆, ◇).

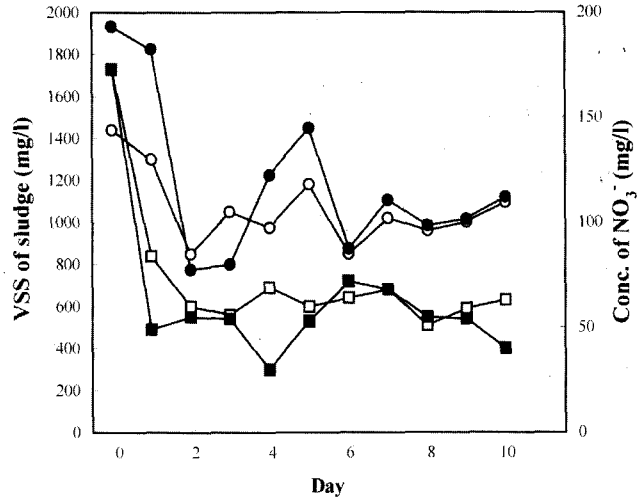

Fig. 5. NO_3^- removal by continuous cultures of the modified sludge and the control sludge.
■, NO_3^- in the modified sludge; ●, modified sludge mass; □, NO_3^- in the control sludge; ○, control sludge mass.

great NO_3^- removal at 66.3 mg NO_3N/(g VSS·h) (Table 4), and its $SDNR_{NO3}$ was the highest one compared with those of all the cultures examined in this study. However, it did not perform any NO_2^- removal either during the experimental period (Fig. 4). The $SDNR_{NO3}$ of the mix-culture of strain N23 and strain R13 was determined as 59.3 mg NO_3N/(g VSS·h), and it was relatively high even compared with that of strain N6's pure-culture, but its NO_2^- removal was not performed either in the same operating period. The NO_3^- removal by the mix-culture of strain N6, strain N23, and strain R13 was performed with 52.7 mg NO_3N/(g VSS·h), and it was similar to those by other mix-cultures examined in this study. But its NO_2^- removal was not performed either during the experimental period.

The mix-cultures of all three selected *nirS*-harboring isolates demonstrated more effective NO_3^- removal compared with a pure culture [12, 26] and an activated sludge [8, 22], and their C requirements for NO_3^- removal were also very low at approximately 3.4 g COD/g NO_3N. However, NO_2^- reduced from influent NO_3^- could not be effectively transformed to the next form of nitrogen oxides in any mix-cultures, even in the mix-culture of strain N6 and strain N23, which effectively removed nitrite in their pure cultures (Fig. 3). This NO_2^- accumulation is similar to that aspect of complex bacterial community reported by Krishnamachari and Clarkson [19]. The nitrite build-up could have occurred due to a limitation of organic carbon and deficiency of nitrite reductase activity [24]. However, the accumulation of NO_2^- in all the mix-cultures remains unclear.

Denitrifying Function of An *nirS*-Harboring Isolate in Continuous Culture

To investigate the effect of one of the selected *nirS*-harboring isolates in real denitrifying activated sludge, the modified sludge was constructed by mixing 5% (w/w as mg VSS/l) of strain N6 and 95% of the control sludge in a continuous reactor. Two bioreactors of modified sludge and control sludge were operated with sewage for 10 days at the same time. Until day 3 of the reactor operation, nitrate concentration and bacterial mass were rapidly decreased in both bioreactors, simultaneously. After day 3, nitrate concentration and bacterial mass were not significantly changed in both bioreactors, except for a small decrease of nitrate concentration according to an increase of bacterial mass in the bioreactor of modified sludge (Fig. 5). The $SDNR_{NO3}$ were 4.8 mg NO_3N/(g VSS·h) for modified sludge and 4.6 mg NO_3N/(g VSS·h) for control sludge (Table 4). Between both the continuous bioreactors treating sewage, there were no noticeable differences for NO_3^- removal throughout the operating period.

DISCUSSION

The selected *nirS*-harboring denitrifying isolates are all Gram-positive bacteria according to the Gram staining (data not shown) and the homology search based on their partial 16S rRNA sequences in BLAST (Table 3). However, many denitrifiers reported are Gram-negative strains [1, 16, 23]. For Gram-positive denitrifying bacteria, it has been reported that *Bacillus* sp. is contributed by the *nirK* gene, and *Erythrobacter* sp. and *Thiobacillus* sp. are by the *nirS* gene [27]. Although Piñar *et al.* [26] had employed *Arthrobacter*

globiformis CECT 4500 in an industrial wastewater pilot plant for nitrate removal, there are, however, few reports on *nirS*-harboring Gram-positive denitrifying bacteria such as *Arthrobacter* sp., *Staphylococcus* sp., and *Bacillus* sp. The partial *nirS* gene sequences of the selected denitrifying isolates showed 83-85% identity to *Pseudomonas stutzeri's nirS* gene (Table 3), suggesting that the high similarity of *nirS* genes exists between Gram-positive and Gram-negative denitrifying bacteria. So, it is roughly said that the *nirS* gene, as a functional gene, also could be horizontally transferred among bacteria which compose the bacterial ecosystem and survive together for a long time under the same environment.

All of the three newly isolated *nirS*-harboring denitrifying bacteria could be effective to rapidly remove NO_3^- from sewage that is contaminated with a high concentration of nitrate. The $SDNR_{NO3}$ of strain N6, 51.1 mg NO_3N/(g VSS·h), was arithmetically over 50-folds of approximately 0.92 mg NO_3N/(g VSS·h) of *A. globiformis* CECT 4500 [26], showing that strain N6 possess a high potential of effective biological dissimilatory denitrification. All pure-cultures of the three selected *nirS*-harboring denitrifying bacteria could remove NO_3^- and NO_2^- simultaneously with relatively high efficiency and low C requirement for NO_3^- removal. However, the NO_2^- accumulation in all the mix-cultures was absolutely unexpectedm because all the mix-cultures were composed of the three selected *nirS*-harboring denitrifying bacteria only, which already showed effective NO_2^- removal activity in their pure-cultures. One of the reasons for the NO_2^- accumulation in the mix-cultures is thought to be similar to the report of Wilderer *et al.* [29], that it might be due to the limitation of denitrifying nitrite reductase activity by environments. The further study on the reasons of NO_2^- accumulation still remains to be done.

The continuous culture of the modified sludge [mixed with strain N6; 5% (w/w) to the total sludge] showed no significant differences for nitrate removal, compared with the continuous culture of the control sludge. Although the portion change of strain N6 in the modified sludge must be monitored to precisely understand the effect of strain N6 on the $SDNR_{NO3}$ of the modified sludge, our results indirectly support the suggestion of Dunbar *et al.* [9], that biological nitrate and nitrite removal from sewage is dependent on the frequency distribution or relative abundances of bacterial structure in activated sludge rather than the bacterial composition and richness. From the results obtained in the present study, it may be inferred that the other factors, which can change bacterial population dynamics and affect the denitrifying efficiency of activated sludge process, are needed to construct an effective denitrifying activated sludge, especially viewed in bacterial ecology, rather than bacteria and carbon concentration. Therefore, further research is required to define the effect of the relationship between denitrifying bacteria and other indigenous bacteria, and the relationship between complex bacterial interaction and various environmental factors on denitrification.

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