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Bacterial Community and Biological Nitrate Removal: Comparisons of Autotrophic and Heterotrophic Reactors for Denitrification with Raw Sewage

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An autotrophic denitrification reactor (ADR-1) and a heterotrophic denitrification reactor (HDR-2) were operated to remove nitrate and nitrite in an anoxic environment in raw sewage. The NO_3-N removal rate of ADR-1 was shown to range from 52.8% to 78.7%, which was higher than the NO_3-N removal rate of HDR-2. Specific denitrification rates (SDNR) of ADR-1 and HDR-2 were 3.0 to 4.0 and 1.1 to 1.2 mgNO_3-N/gVSS/h, respectively. From results of restriction fragment length polymorphism (RFLP) of the 16S rRNA gene, *Aquaspirillum metamorphum, Alcaligenes defragrans,* and *Azoarcus* sp. were β-*Proteobacteria* that are affiliated with denitrifying bacteria in the ADR-1. Specifically, *Thiobacillus denitrificans* was detected as an autotrophic denitrification bacteria. In HDR-2, the β-*Proteobacteria* such as Denitrifying-Fe-oxidizing bacteria, *Alcaligenes defragrans, Acidovorax* sp., *Azoarcus denitrificans*, and *Aquaspirillum metamorphum* were the main bacteria related to denitrifying bacteria. The β-and α-*Proteobacteria* were the important bacterial groups in ADR-1, whereas the β-*Proteobacteria* were the main bacterial group in HDR-2 based on results of fluorescent *in situ* hybridization (FISH). The number of *Thiobacillus denitrificans* increased in ADR-1 during the operation period but not in HRD-2. Overall, the data presented here demonstrate that many heterotrophic denitrifying bacteria coexisted with autotrophic denitrifying bacteria such as *Thiobacillus denitrificans* for nitrate removal in ADR-1. On the other hand, only heterotrophic denitrifying bacteria were identified as dominant bacterial groups in HDR-2. Our research may provide a foundation for the complete nitrate removal in raw sewage of low-COD concentration under anoxic condition without any external organic carbon or the requirement of post-treatment.

Keywords: Bacterial communities, autotrophic denitrification, heterotrophic denitrification

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Simultaneous biological nutrient removal has been observed in numerous bioreactors under anaerobic, anoxic, and oxic conditions [13, 18, 24, 31, 32, 47]. The denitrifiers are mostly heterotrophic and need chemical oxygen demand (COD) for their energy and carbon supply [24]. However, many wastewaters do not contain enough COD, and the addition of an exogenous carbon source, such as methanol and acetate, is often necessary to achieve complete denitrification [13, 17, 20, 53, 54]. Many heterotrophic bacteria can reduce nitrate and nitrite by utilizing organic substrates such as methanol, ethanol, and acetate for the conversion of nitrate to nitrogen gas under anoxic conditions [12, 27]. Denitrification can also be carried out by autotrophic sulfur bacteria, which use a variety of reduced sulfur compounds (S^{2-}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}), instead of organic compounds, while reducing nitrate [5, 26, 28, 29, 37]. The sulfur-utilizing autotrophic denitrification process (SUADP) has several advantages over the heterotrophic denitrification process (HDP). First, elemental sulfur is cheaper than methanol or ethanol. Second, it minimizes the handling cost of sludge, as less sludge is produced [30]. Third, as bacteria grow on the surface of sulfur particles, and because sulfur particles act as filter media, a secondary settling tank may not be needed for the sulfur-packed bed reactor [28, 29].

SUADPs have been applied for the treatment of wastewater with low NO_3-N (less than 100 mg/l) such as groundwater [29] and surface water [30]. Koenig and Liu [28] applied SUDNR for the treatment of leachate, which contained about 160–800 mg/l NO_3-N. moreover, many researchers have studied autotrophic denitrification in the presence of elemental sulfur or thiosulfate as electron donors to denitrify nitrate using pure cultures of *Thiobacillus denitrificans*, *Thiomicrospira denitrificans*, *Thiobacillus versutus*, *Thiosphaera pantotropha*, and *Paracoccus denitrificans* [5, 19, 48, 51, 52]. They have reported that a pathway of the nitrogen cycle, anammox, allowed ammonia to be oxidized by nitrate and nitrite under anoxic conditions [47] and was performed by autotrophic bacteria that are

members of the order *Planctomycetales* [49, 50]. However, the bacterial community of the biological autotrophic denitrification process with raw sewage is not well understood owing to a lack of knowledge regarding the bacteria that are responsible and the factors regulating the process. Therefore, there is a need to investigate the autotrophic denitrifying consortia consisting within a complex bacterial community as well as single taxa in raw sewage treatment systems.

Recently, we designed and operated two autotrophic denitrification sequence batch reactors (ADR-1) and two heterotrophic denitrification sequence bath reactors (HDR-2) for nitrate removal in raw sewage. As for the nitrate dosage, the ADR-I was operated as an autotrophic and heterotrophic denitrification process, whereas the HDR-2 was operated only as a heterotrophic denitrification process. To investigate the relationship between the denitrification rate and the relative abundances of the denitrifying genera, the composition of bacterial communities from nitrate-removing activated sludge was analyzed by using culture-independent techniques (FISH and RFLP).

MATERIALS AND METHODS

Experimental Design

Laboratory-scale anoxic reactors used in this study are shown in Fig. 1. ADR-1 treated raw sewage supplied with about 10 ppm sulfide, whereas HDR-2 was designed for heterotrophic denitrification with raw sewage without any supplements. Two replicate reactors were operated under the same condition. Raw sewage taken from Joograng' sewage treatment plant in Korea was used as feed for the reactors.

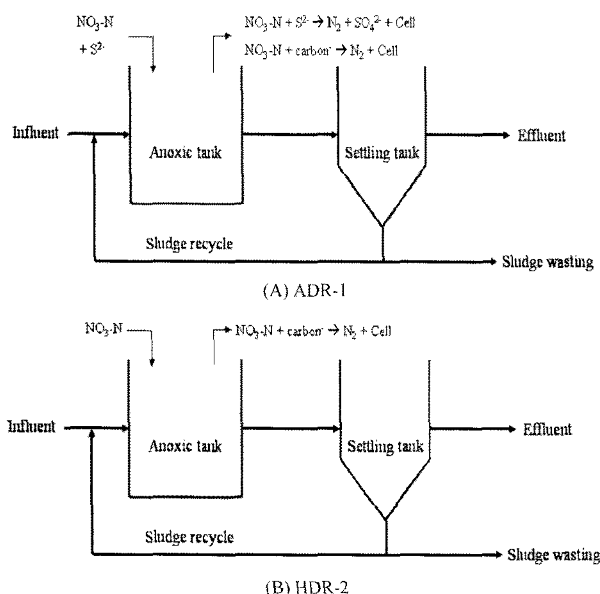

Fig. 1. Schematic diagram of ADR-1 and HDR-2 **A**, autotrophic and heterotrophic denitrification reactor (ADR-1); **B**, heterotrophic denitrification reactor (HDR-2).

For the purpose of studying denitrification, nitrate and/or sulfide were added in the diluted anoxic influent to prevent the deterioration of nutrients (Table 1). These results were average values of two replicate reactors.

The reactors were operated at 2 h of hydraulic retention time (HRT) and 10 days of solids retention time (SRT). Average mixed-liquor suspended-solids (MLSS) concentration was maintained at 2,500 mg/l. The operating temperature was 20°C controlled with a water bath. From the reactors, MLSS was sampled for analysis of denitrification rate and microbial community. The reactors, ADR-1 and HDR-2, were operated over 1 year to highly enrich the denitrifying bacteria. The performance of the reactors fluctuated over a 12-month period, and the sludge was collected at regular stable operating times for 3 months and used in the study. In order to examine the denitrification rate, batch test experiments were used. All water quality parameters were measured in accordance with the Standard Methods [3].

DNA Extraction

Genomic DNA extraction from the activated sludge was performed using a modification of the method of Lee *et al.* [33]. The modified step involved extraction of the lysate with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). The quality of extracted DNA was checked by standard agarose electrophoresis. DNA concentration was measured by absorbance at 260 nm.

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

Amplification of the 16S rRNA gene from chromosomal DNA was carried out in a DNA thermal cycler model 480 (Perkin-Elmer, Norwalk, CT, U.S.A.) with universal bacterial primers, 27F (5'-AGAGTTTGATC-MTGCTCAG-3') and 1492R (5'-GGTTACCTTTGTTACGACTT-3') [6, 23]. The PCR amplification program consisted of an initial 5 min denaturation step at 94°C followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final 10 min extension step at 72°C.

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) [39], and cloned into pGEM-T Easy vector system I (Promega, Madison, WI, U.S.A.) [21], according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Clones containing appropriate-sized inserts were identified by agarose gel electrophoresis of PCR products obtained from host lysates with primers complementary to the vector's flanking insertion sites. Approximately 100 ng of purified PCR product of re-amplified insertion site from each clone was digested overnight at 36°C, with 5 U of RSA I and HaeIII (Promega, Madis on, WI, U.S.A.). Operational taxonomic units (OTU) for unique clones were identified by RFLP analysis using the Gel Doc 2000 system (BioRad Laboratories, Hercules, CA, U.S.A.) and Quantity One software (BioRad Laboratories, Hercules, CA, U.S.A.).

The nucleotide sequences were determined from plasmid DNA preparations using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI310 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. Vector primers T7 and SP6 were used for the sequencing reactions [31].

Sequences were compared with the GenBank database using the BLAST programs. DNA sequences were checked for chimera formation using the Check Chimera program of the Ribosomal Database Project. Sequences were manually compiled and aligned using Phydit software [8]. The Jukes-Cantor evolutionary distance matrix for the

Table 1. Summary of operating conditions and performance results of ADR-1 and HDR-2.

Parameters	Influent		Effluent of ADR-1		Effluent of HDR-2	
	Average	Range	Average	Range	Average	Range
pH	7.4	6.8–8	7.2	6.8–7.7	7.3	7.1–7.8
Alkalinity	77	33–106	62.1	17.5–162.5	72.4	25–125
TSS	36	11–148	4.9	1–20	5.0	1–16
VSS	28	9–106	3.8	0.5–1.6	3.4	0.7–12
$TCOD_{Cr}$	57	32–106	16	4.8–30	16.5	6.8–42
$SCOD_{Cr}$	28	18–50	9.7	2–18	9.4	1.2–20
$TBOD_5$	21.7	18–28	5.6	2–9.7	3.7	1.8–6.5
$SBOD_5$	7.7	4.5–12	2.6	1–4.1	2.5	1.2–3.5
NO_3-N	21.2	20–22.6	4.5	0–20.9	9.1	0.6–20.5
NH_4-N	14.7	2–27	6.6	1–9.6	8.3	2–16.5
TKN	25.6	25–40	12.0	8–21	13.6	8–22.4
T-P	8.7	4.6–14.5	8.5	4.5–10.4	8.4	4.1–14.6
PO_4-P	8.3	4.1–13.6	8.3	0.2–8.6	8.0	4.0–8.9
Sulfate (SO_4^{2-})	54.8	18.5–121	82.9	22.4–188	53.8	18.1–82.8
Sulfide (S^{2-})	10	5–12	0.005	0.003–0.008	–	–

TSS, total suspended solids; VSS, volatile suspended solids; TCOD, total chemical oxygen demand; SCOD, soluble chemical oxygen demand; SBOD, soluble biochemical oxygen demand; TKN, total Kjeldahl nitrogen; T-P, total phosphorus. All values are expressed as mg/l except pH.

neighbor-joining method was generated [22]. Evolutionary trees were constructed using the neighbor-joining method [43]. The robustness of inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values of >50% were shown on the trees.

Nucleotide Sequence Accession Numbers

The 16S rDNA partial sequences obtained in this study are available from the Genbank nucleotide sequence database under accession numbers ranging from AY253426 to AY253442.

Oligonucleotide Probes

The following oligonucleotide probes were used to evaluate the microbial population in sludge: EUB338, a probe designed to detect most bacteria [2]; and ALF1b, BET42a, and GAM42a, specifically for the α-, β-, and γ-*Proteobacteria*, respectively [1, 2, 34]. CF319a, specific for the *Cytophaga-Flexibacter* cluster [34]; HGC69a, specific for *Actinobacteria* [42]; and Ps, a probe specific for most group I pseudomonads were also used [44]. To quantify *Thiobacillus denitrificans*, Betthio1001 for detection of *Thiobacillus denitrificans*-like bacterium was used in this study [15]. The 16S- and 23S-targeted oligonucleotide probes were labeled with tetramethylrhodamine-5-isothiocyanate (TRITC) at the 5' end (Takara Biochemicals, Japan).

4,6-Diamidino-2-Phenylindole (DAPI) Staining and FISH

Staining with DAPI was used to determine the total number of cells in the samples [40]. FISH of whole cells was performed using the methods reported by Snaidr *et al.* [46]. Samples were fixed with paraformaldehyde and immobilized by air-drying on glass slides with a heavy Teflon coating forming 9-mm-diameter wells. The samples were hybridized with probes as described by Amann *et al.* [2]. Probes BET42a and GAM42a were used with competitor oligonucleotides [35]. Hybridization was carried out for 90 min at 46°C in a sealed moisture chamber. Washing was conducted under conditions appropriate for each probe [15, 34, 35, 44, 46]. The hybridized and DAPI-stained samples were examined with an epifluorescence microscope (Axioplan; Zeiss, Axiophot 2) with filter sets 01 (DAPI staining) and 15 (TRITC-labeled probe). For counting each probe, activated sludge in 10 ml of distilled water was sonicated (40 to 50 Hz, 117 V, 1.0 A; Branson Ultrasonics, Danbury, CT, U.S.A.) for 30 s to separate bacterial cells and flocs. About 1,000 cells stained with DAPI were enumerated. Two replicate samples were counted for each hybridization [32].

RESULTS AND DISCUSSION

Steady-State Characteristics of Nitrate-Removing Reactors

Table 1 shows the measured chemical concentrations of influent and effluent wastewaters from the reactors. The average reduction NO_3-N concentrations in ADR-1 and HDR-2 were 16.7 and 12.1 mg/l, respectively. The average NO_3-N removal rates of ADR-1 and HDR-2 were 78.8% and 57.1%, respectively. These results indicate that the removal efficiency of the ADR-1 was 21.5% higher than that of the HDR-2 during the regular stable operating period. It can be deduced that nitrate and nitrite could be removed by heterotrophic denitrification by heterotrophic denitrifying bacteria, cell synthesis, and endogenous denitrification in HDR-2. Nitrogen removal in ADR-1 could be attributed to autotrophic denitrification by autotrophic denitrifying bacteria, heterotrophic, denitrification by heterotrophic denitrifying bacteria and cell synthesis and endogenous denitrification. Generally, the disadvantage of autotrophic denitrification is the decreased alkalinity due to the formation of sulfate from sulfide [28]. However, alkalinity had increased but was offset as oxidation of sulfide and autotrophic denitrification. This result shows that while alkalinity decreased slightly, the pH did not drop markedly (Table 1).

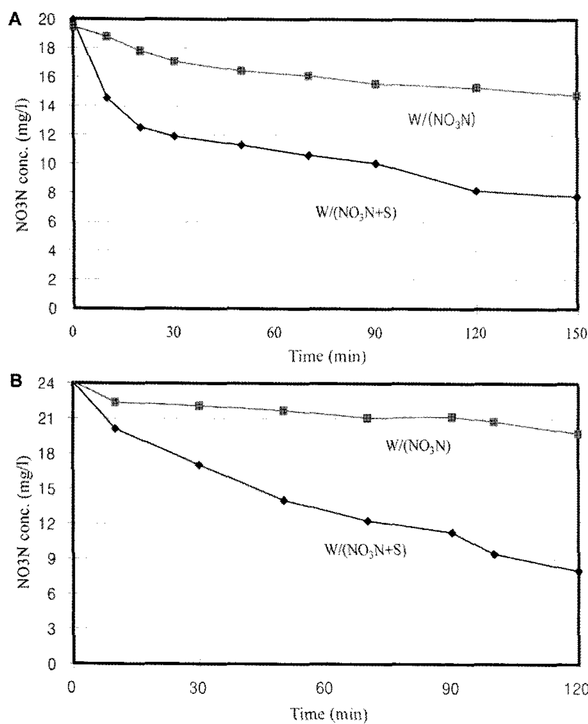

Fig. 2. Typical anoxic batch assay monitoring to measure the specific denitrification rate (SDNR).
A Sulfide (10 mg/l) and NO_3-N (10 mg/l) added. **B** Sulfide (20 mg/L) and NO_3-N (10 mg/l) added. W/(NO_3N+S) is the bath test result of ADR-1 sludge and W/(NO_3N) is the bath test result of HDR-2 sludge.

To confirm the nitrogen removal efficiency of autotrophic denitrification and heterotrophic denitrification reactors, we performed batch tests with sludge from ADR-1 and HDR-2. The results of the batch test are shown in Fig. 2. The specific denitrification rates (SDNR) of ADR-1 and HDR-2 were 3.0–4.0 mgNO_3-N/gVSS/h and 1.1–1.2 mg NO_3-N/g VSS/h, respectively. The SDNR of ADR-1 with regards considering autotrophic denitrification was about 3-fold higher than that of HDR-2. This result indicates that it is possible to denitrify by autotrophs within the anoxic HRT.

Community Structure Based on 16S rDNA Clone Libraries

16S rRNA genes amplified from the two reactors were cloned and identified to enable detailed characterization of the bacterial community. Cloned 16S rRNA gene was grouped by restriction fragment length polymorphism (RFLP) analysis. A total of 74 clones were analyzed by RFLP analysis, and then 8 OTUs were classified as dominant OTUs in ADR-1 (Table 2). In this study, each OTU was grouped by at least two clones. We selected 3 clones from many clones of each OUT and then sequenced them for identifying bacterial phyla and species of each OUT. The bacterial phyla and species of each OUT are presented in Table 2.

The β-*Proteobacteria* represented a minimum of 50% of total clones, the α-*Proteobacteria* were at least 14.9%, the

Table 2. Summary of phylogenetic diversity of the domain clone OTUs-based 16S rRNA gene sequences identified in the BLAST database from ADR-1 and HDR-2.

OTUs	16S rRNA gene sequencing			Putative taxon
	Closest match	No. of nucleotides compared	(%)Similarity with closest match	
ADR-1				
AC1	*Aquaspirillum metamorphum* (6)[f]	406	97	β[b]
AC2	*Bosea thiooxidans* (11)	423	96	α[a]
AC3	*Alcaligenes defragrans* (9)	443	97	β
AC4	*Flavobacterium indologenes* (8)	426	94	CF[c]
AC5	*Thiothrix unzii* (3)	369	88	γ[d]
AC6	*Azoarcus denitrificans* (14)	418	98	β
AC7	*Thiobacillus denitrificans* (8)	442	98	β
AC8	*Arcobacter nitrofigilis* (6)	550	95	ep[e]
HDR-2				
HC1	Denitrifying Fe-oxidizing bacteria (9)	489	97	β
HC2	*Flavobacterium ferrugineum* (7)	439	97	CF
HC3	*Pseudomonas aeruginosa* (3)	404	98	γ
HC4	*Alcaligenes defragrans* (8)	443	98	β
HC5	*Acidovorax* sp. (9)	412	99	β
HC6	*Azoarcus denitrificans* (15)	436	97	β
HC7	*Aquaspirillum metamorphum* (7)	439	98	β
HC8	*Bradyrhizobium japonicum* (4)	591	98	α
HC9	Uncultured sludge bacterium (4)	422	96	β

[a]Alpha subclass of Proteobacteria; [b]Beta subclass of Proteobacteria; [c]*Cytophaga-Flexibacter* cluster; [d]Gamma subclass of Proteobacteria; [e]Epsilon subclass of Proteobacteria; [f]Number in parenthesis is the number of clones. OUT, operational taxonomic unit.

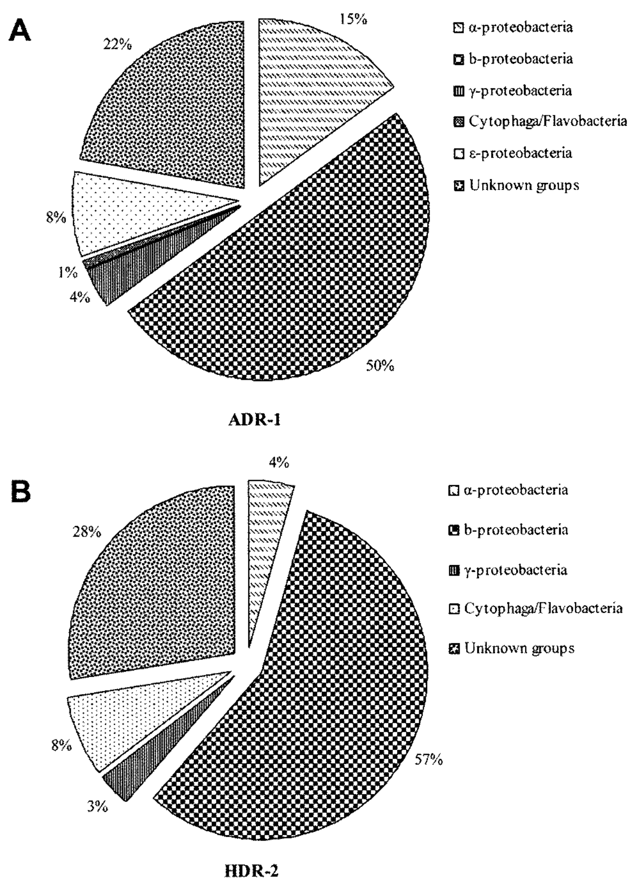

Fig. 3. Comparison of putative bacterial phylotype distribution for 16S rRNA gene libraries by RFLP analysis in activated sludge samples from the ADR-1 (**A**) and HDR-2 (**B**).

γ-*Proteobacteria* were at least 4.1%, the CF group were at least 0.8%, and the ε-*Proteobacteria* were at least 8.1% of the total 74 clones in ADR-1 (Fig. 3A). Groups AC1, AC3, AC6, and AC7 are affiliated with denitrifying bacteria or bacterial genera harboring denitrifying species [27, 38, 58]. AC1 is related to *Aquaspirillum metamorphum* (Y18618) that reduces nitrate [45]. AC3 is most closely related to *Alcaligenes defragrans* (AJ005450) that can use nitrate as an electron acceptor [11]. AC6 is closely related to *Azoarcus* sp. (L33693), which is a denitrifying bacterium [57]. Group AC7 most closely matches *Thiobacillus denitrificans* (AJ243144), which is physiologically similar to the type species of the genus *Thiobacillus* and *Thiobacillus thioparus* that belong to the β-*Proteobacteria* group. *T. denitrificans* is capable of growing as a facultative anaerobic chemolithotroph, coupling the oxidation of inorganic sulfur compounds to reduce nitrate, nitrite and, other oxidized nitrogen compounds to dinitrogen [25]. These groups belong to the β-*Proteobacteria* group. Group AC2 is phylogenetically related to *Bosea thiooxidans* (AF508803) that is capable of oxidizing reduced inorganic sulfur compounds from agricultural soil. Group AC5 is most closely related to *Thiothrix unzii* (L79961), an autotrophic *Thiothrix* species that is more morphologically similar to chemoorganotrophic bacteria than filamentous sulfide-oxidizing bacteria. The sequence of group AC8 is most similar to that of *Candidatus Arcobacter sulfidicus* (95% similarity). *Candidatus Arcobacter sulfidicus* achieved sulfide-oxidizing and production in purified cultures [55]. The phylogenetic positions of the domain OTUs in ADR-1 based on 16S rRNA gene sequences are shown in Fig. 4.

In the HDR-2, a total 91 clones were isolated from select medium and classified into 9 OTUs by RFLP analysis (Table 2). The β-*Proteobacteria* made up at least 57.1% of the clones. The α-*Proteobacteria* were at least 4.4%, the γ-*Proteobacteria* were at least 3.3%, and the CF group was at least 7.7% of total 91 clones in HDR-2 (Fig. 3B). Groups HC1, HC4, HC5, HC6, and HC7 are affiliated with denitrifying bacteria or bacterial genera harboring denitrifying species [27, 38, 58]. HC1 is most closely related to Denitrifying Fe-oxidizing bacteria (U51101). HC5 is phylogenetically related to *Alcaligenes defragrans* (AF508101). These are nitrate-reducing bacteria capable of degrading phenol that have been isolated from natural and contaminated environments under low-oxygen conditions [4]. HC5 is most closely related to *Acidovorax* sp. (AF457653). HC6 is most closely related to *Azoarcus denitrificans* (L33694), a toluene-degrading denitrifier capable of degrading toluene under denitrifying conditions [57]. HC7 is phylogenetically related to *Aquaspirillum metamorphum* (Y18618), the majority of whose strains reduces nitrate under denitrifying condition. Group HC9 is most closely related to the uncultured sludge bacterium S21 (AF234738) that was identified from a nitrifying-denitrifying activated sludge, which was taken from an industrial sewage treatment plant and analyzed by the full-cycle rRNA approach [24]. These groups belong to the β-*Proteobacteria.* Group HC3 most closely matches *Pseudomonas aeruginosa* PAO1 (AE004949), which is capable of reducing all three inorganic N compounds to N_2 gas [56]. The sequence of group HC8 was most similar to that of *Bradyrhizobium japonicum* (98% similarity). Among the diazotrophic bacteria, *Bradyrhizobium japonicum* and a considerable number of other rhizobia are able to denitrify [36]. The phylogenetic positions of the domain clone OTUs in HDR-2 based on 16S rRNA gene sequences are shown in Fig. 4. Lee *et al.* [32] reported that among *Proteobacteria*, the β-*Proteobacteria* such as *Alcaligenes defragrans*, *Dechloromonas* sp., *Zoogloea ramigera*, and Denitrifying Fe-oxidizing bacteria play important roles to remove nitrate in nitrate-removing activated sludge. In the case of HDR-2, the bacterial community including nitrate-reducing and denitrifying bacteria was similar to the composition found in a nitrate-removing reactor [32]. These results imply that the β-*Proteobacteria* play an important role in the removal of nitrate in heterotrophic denitrification reactors.

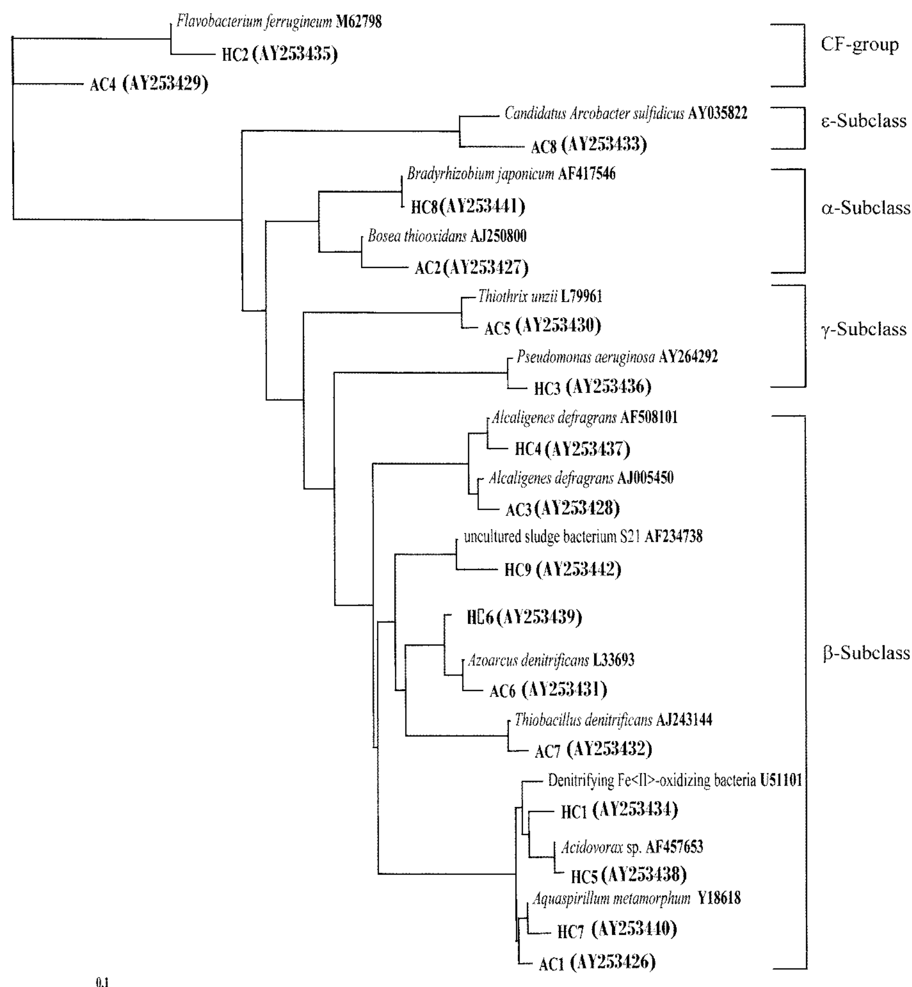

Fig. 4. Phylogenetic tree of sequences of bacteria 16S rDNA. *Flavobacterium ferrugineum* (M62798) is included as an outgroup. The phylogenetic tree was constructed using neighbor-joining analysis with 1,000 bootstrap replicates. The scale bar indicates 0.10 nucleotide substitutions per site.

Quantification of Bacteria and *Thiobacillus denitrificans*-Like Bacterium by Using FISH

To quantify the total cell number, subclass of *Proteobacteria,* and specific bacteria in sludge, FISH analysis was performed. Total cell numbers were determined by DAPI staining. The total cell number was 6.4×10^8 cells per ml of suspended activated sludge sample (standard deviation [SD]=2.64×10^8 cells per ml of suspended activated sludge sample) in ADR-1 and was 8.4×10^9 cells per ml of suspended activated sludge sample ([SD]=1.02×10^8 cells per ml of suspended activated sludge sample) in HDR-2. Bacteria were quantified by FISH with domain-specific probes. It was found that 72% (ADR-1) and 81% (HDR-2) of total DAPI cell counts hybridized to the bacterial-specific probe EUB338, respectively. EUB338 counts of the ADR-1 were slightly hybridized in comparison with that of the HDR-2. It can be argued that the change in bacterial community was caused by adding sulfide, which changed the environmental habitat of the domain bacteria.

The subclass probe ALF1b, BET42a, GAM42a, and CF319a counts for the activated sludge samples are given in Fig. 5. In samples from ADR-1, ALF1b hybridized to 19.8% of the cells hybridized with probe EUB338; probe BET42a hybridized to 21.9%; GAM42a hybridized to 7.8%; CF391a hybridized to 8.6%; and HGC69a hybridized to 3.7%. In samples of HDR-2, probe BET42a hybridized to 39%, ALF1b to 10.2%, GAM42a to 12.7%, CF391a to 6.7%, and HGC69a to 5.4%. The results of FISH analysis indicate that the β-*Proteobacteria* were the predominant group in the two reactors, but the community structures of the other bacterial groups were quite different in the two differently loaded denitrifying reactors. More specifically, there was no significant difference between ALF1b and BET42a counts in ADR-1. However, the probe BET42a count was approximately four times that of ALF1b counts in HDR-2. It can be concluded that β- and α-*Proteobacteria* in ADR-1 and β-*Proteobacteria* in HDR-2 play a key role in the removal of nitrate. We suggest that the β- and α-*Proteobacteria*

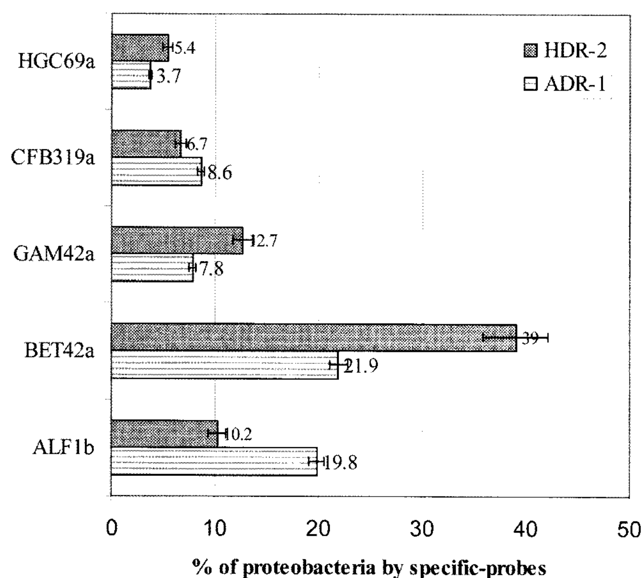

Fig. 5. Comparison of bacterial community in activated sludge samples obtained from the ADR-1 and HDR-2 sludges as determined by FISH. ALF1b, probe for α-Proteobacteria; BET42a, probe for β-Proteobacteria; GAM42a, probe for γ-Proteobacteria; CF319a, probe for *Cytophaga-Flexibacter* cluster; HGC69a, probe for the class *Actinobacteria.*

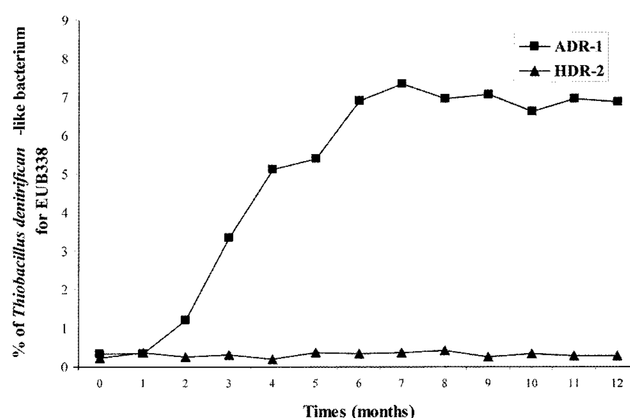

Fig. 6. Quantification of *Thiobacillus denitrificans*-like bacterium by using probe Betthio1001.
Values represent the average from triplicate experiments.

likely were important denitrifiers under autotrophic condition (low COD with sulfate) for nitrogen removal in the raw sewage studied.

There are various reports on bacteria capable of denitrification under different oxygen partial pressures [7, 9, 41], and rRNA-group I pseudomonads contain many denitrifying species of *Pseudomonas* [58]. These bacteria were quantified by rRNA-group I pseudomonads-specific probe (Ps) in both ADR-1 and HDR-2. Approximately 3.2×10^4 in ADR-1 ([SD]=4.6×10^3 cells per ml of suspended activated sludge sample) and 2.1×10^5 ([SD]=6.6×10^3 cells per ml of suspended activated sludge sample) in HDR-2 were detected with this probe, respectively. Members of the *Pseudomonas* genus accounted for less than 0.01% of those detected by the bacterial-specific probe EUB338 in both ADR-1 and HDR-2. From this result, the rRNA-group I pseudomonads were unlikely to play an important role in denitrification or were not the dominant bacterial group in our reactors.

Notably, *Thiobacillus denitrificans* was identified as autotrophic denitrifying bacteria in ADR-1 by using RFLP analysis (Table 2). Based on results of RFLP analysis, probe Betthio1001 was used to quantify *Thiobacillus denitrificans*-like bacterium in both reactors. In the first stage (0 time), the number of ADR-1 and HDR-2 was 0.32% and 0.23% of the number of EUB338, respectively. The number of Betthio1001 increased after 2 months of initial operation, and then the percentage of Betthio1001 was approximately 7.0% of EUB338 after 6 months during the operation period. However, the percentages of Betthio1001 were almost the same in HDR-2 during the study (Fig. 6). These results suggest that *Thiobacillus denitrificans* could directly operate and function as autotrophic denitrifying bacteria in ADR-1 after 2 months of initial operation, but could not do the same in HDR-2 during the operation period. These results also suggest that the ADR-1 is a useful system that can remove nitrate and nitrite without any supplements such as carbon sources under anoxic condition in raw sewage.

Generally, methanol, ethanol, and acetate have been used to increase denitrification rates, and the use of external carbon sources are three times higher than the rates for waste organic matter alone in raw wastewater [16, 18]. According to Ginige *et al.* [13, 14], the culture-independent rRNA-based molecular methods, including stable-isotope probing (SIP), were used to identify the dominant denitrifer in a laboratory-scale activated sludge process fed with methanol and acetate and operated with only nitrate as the terminal electron acceptor. In the studies of Ginige *et al.* [13, 14], the β-*Proteobacteria* was the dominant bacterial group based on FISH analysis in methanol-fed SBR [14] and acetate-fed denitrifying SBR [13], when high denitrification rates were exhibited. In our studies, the dominant denitrifier or nitrate-reducing bacteria were autotrophic denitrifying bacteria such as *Thiobacillus denitrificans*, heterotrophic denitrifying bacteria such as *Aquaspirillum metamorphum*, *Alcaligenes defragrans*, and *Azoarcus* sp., of the β-*Proteobacteria* in ADR-1. The dominant denitrifying bacterial groups were Denitrifying Fe-oxidizing bacteria, *Pseudomonas aeruginosa*, *Aquaspirillum metamorphum*, *Alcaligenes defragrans*, *Azoarcus denitrificans*, and *Azoarcus* sp., of the β-*Proteobacteria* group in HDR-2. Analysis of RFLP screening and sequencing cannot completely explain the bacterial community and functional groups in denitrifying activated sludge of reactors; however, this analysis revealed dominant bacterial groups and bacterial diversity related to denitrification in autotrophic and heterotrophic denitrification reactors. It is possible that other bacterial

groups in the reactors were also able to remove nitrogen in raw sewage with sulfur or without sulfur, but these were not investigated in this study.

Results of FISH, 16S rRNA gene cloning, and RFLP indicated that β-*Proteobacteria* were the dominant *Proteobacteria* in ADR-1 and HDR-2 (Figs. 3 and 5). Autotrophic bacteria capable of denitrification such as *Thiobacillus denitrificans*, and heterotrophic denitrifying bacteria such as *Aquaspirillum metamorphum*, *Alcaligenes defragrans*, and *Azoarcus* sp., were detected by molecular techniques in ADR-1. In HDR-2, autotrophic denitrifying bacteria were not detected by molecular techniques, but many heterotrophic denitrifying bacteria such as Denitrifying Fe-oxidizing bacteria, *Pseudomonas aeruginosa*, *Aquaspirillum metamorphum*, *Alcaligenes defragrans*, *Azoarcus denitrificans*, and *Azoarcus* sp. were detected in the activated sludge of the heterotrophic denitrification process. The patterns of bacterial distribution of ADR-1 based on an analysis of FISH were similar to that of RFLP analysis. moreover, the bacterial patterns obtained from FISH analysis of HDR-2 showed a pattern similar to that of RFLP analysis. These data suggest that β- and α-*Proteobacteria* were important denitrifiers in ADR-1 whereas β-*Proteobacteria* were the main denitrifiers in the heterotrophic denitrification reactor (Figs. 3 and 5). Although many researchers have isolated *Thiobacillus denitrificans*, *Thiomicrospira denitrificans*, and *Thiosphaera pantotropha* [5, 19, 48, 51, 52] as autotrophic denitrifying bacteria in various environments, not much is known about the bacterial community in an autotrophic denitrification reactor under anoxic condition. From results of FISH analysis by using Betthio1001 and RFLP analyses, we know that *Thiobacillus denitrificans* as autotrophic denitrifying bacteria were directly related to the autotrophic denitrification in the biological autotrophic denitrification reactor under low COD conditions (ADR-1) (Fig. 6).

Although the same amount and composition of inoculum (original seed sludge) was used for all treatments (data not shown), after operation for 12 months, microbial communities of the original seed sludge shifted to a very different bacterial structure (Table 2, Figs. 2 and 5). These changes may be due to different reactor conditions, since ADR-1 was anoxic under the added sulfide and low COD concentration, whereas HDR-2 was in an anoxic condition at only low COD concentration. In ADR-1, heterotrophs coexisted with autotrophs; on the other hand, only heterotrophs harboring denitrifying bacteria were dominant *Proteobacteria* in HDR-2. In comparison with a heterotrophic denitrification system (HDR-2) without the addition of a great amount organic substrate, this sulfide-using denitrification system (ADR-1) can effectively remove nitrate and nitrite in raw sewage at low COD by correlation between autotrophic and heterotrophic denitrifying bacteria. Therefore, we suggest that the ADR-1 system has a significant advantage in that the cost of external organic carbon and the requirement of post-treatment are not necessary for removal of nitrate from the wastewater.

Overall results of the bacterial community show that sulfur denitrifying, heterotrophic denitrifying, and autotrophic bacteria (sulfide-oxidizing bacteria and sulfur-reducing bacteria) coexisted in the activated sludge of ADR-1. This phenomenon by the bacterial community indicates that biological nitrate and nitrite removal in raw sewage is dependent on the frequency distribution or relative abundance of bacteria in the activated sludge, rather than the types of bacterial composition and the number of bacteria. This study empirically supports the suggestion of Dunbar *et al.* [10] and Lee *et al.* [32]. It can be argued that to enable coexistence of sulfur denitrification and heterotrophic denitrification bacteria, a sulfate adaptation-step (adding sulfate) is necessary to change the microbial structures. However, this study could not establish a period of the sulfate adaptation-step time since the type of influent raw sewage and initial microbial communities in the activated sludge varied significantly. Therefore, for stable operation of nitrate-removing reactors, more information about the bacterial communities in the various activated sludge batches used as inocula and the optimum conditions are needed. Further research is required to define the effect of the relationships among *Proteobacteria* in the sulfur cycle and heterotrophic denitrifying bacteria under anoxic conditions.

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