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Denaturing Gradient Gel Electrophoresis Analysis of Bacterial Populations in 5-Stage Biological Nutrient Removal Process with Step Feed System for Wastewater Treatment

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Changes in the bacterial populations of a 5-stage biological nutrient removal (BNR) process, with a step feed system for wastewater treatment, were monitored by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA fragments. DGGE analysis indicated seasonal community changes were observed, however, community profiles of the total bacteria of each reactor showed only minor differences in the samples obtained from the same season. The number of major bands was higher in the summer samples, and decreased during the winter period, indicating that the microbial community structure became simpler at low temperatures. Since the nitrogen and phosphate removal efficiencies were highly maintained throughout the winter operation period, the bacteria which still remaining in the winter sample can be considered important, playing a key role in the present 5-stage BNR sludge. The prominent DGGE bands were excised, and sequenced to gain insight into the identities of the predominant bacterial populations present, and most were found to not be closely related to previously characterized bacteria. These data suggest the importance of culture-independent methods for the quality control of wastewater treatment.

Key words: Biological nutrient removal (BNR), 16S rDNA, denaturing gradient gel electrophoresis

Enhanced biological phosphorus removal (EBPR) is an activated sludge process modification that allows for a high degree of phosphate removal from wastewater. This process is based on the enrichment of the so-called polyphosphate-accumulating organisms (PAOs) carrying out anaerobic phosphate release and aerobic intracellular accumulation of polyphosphate in excess of normal metabolic requirements. Under anaerobic conditions PAOs take up organic substrates, volatile fatty acids (VFA) preferably, and store them as poly-hydroxyalcanoates (PHA), while the reducing equivalents are provided by the degradation of internally stored glycogen. The energy is generated by the conversions of glycogen and polyphosphate, resulting in phosphate release into the solution. In the subsequent aerobic phase, the internal pool of PHA is oxidized, and used for growth, phosphate uptake, glycogen synthesis and maintenance (Mino *et al.*, 1998). This results in a net uptake of phosphate during the cycle. Although activated sludge systems are designed, and operated globally, for the microbiological removal of phosphate, the ecology of EBPR processes, the microbes involved, and their biological functions and possible reasons for performance unreliably are still unknown. Recently, it was demonstrated that, not only under aerobic conditions, but also under anoxic conditions, i.e., with nitrate as the electron acceptor, some PAOs are capable of polyphosphate accumulation (Kuba *et al.*, 1993; Jorgensen and Pauli, 1995; Barker and Dold, 1996). The attractions of exploiting such denitrifying PAOs (dPAOs) in wastewater treatment include: the possibility of achieving simultaneous removal of nitrogen and phosphate, producing less sludge, with no need for aeration and having a process that is cheaper to run. Furthermore, a lower requirement for a metabolizable substrate than in conventional EBPR systems overcomes a major problem associated with the treating of municipal wastes using EBPR (Seviour *et al.*, 2003). The most promising field of research at present is considered to be that of the development of an activated sludge process with dPAOs (Bortone *et al.*, 1996; Shoji *et al.*, 2003). For the purpose of the stimulation of simultaneous nitrogen and phosphate removals, the 5-stage BNR process, with a step feed system, named PADDO, was especially developed and successfully operated with high removal efficiencies (Park *et al.*, 2002). This process consists of pre-anoxic, anaerobic, anoxic-1, anoxic-2, oxic and secondary clarifier. The step feed system to the pre-anoxic, anaerobic and anoxic-2

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increases the phosphorus release and denitrification rates. This process reduced the requirement of oxygen, and could be operated with long sludge retention times (SRT). The sludge production was less than with other BNR processes. The nitrogen and phosphate removal efficiencies for a winter period were 79 and 87%, respectively, which were much higher than any other system (Ydstebo *et al.*, 2000), and maintained more stably than those previously reported (McClintock *et al.*, 1992; Mamais and Jenkins, 1992; Brdjanovic *et al.*, 1997), where partial or complete loss of the EBPR performance was shown under downward temperature shifts. Thus, a series of laboratory experiments were aimed at investigating the microbial community of the 5-stage BNR system, in relation to seasonal changes, to obtain information on those exhibiting denitrifying phosphorus accumulating organism (dPAO) and playing a key role in the present 5-stage BNR sludge.

In this study, activated sludge samples, obtained from a PADDO process, were analyzed by denaturing gradient gel electrophoresis (DGGE) analysis, in order to directly determine the seasonal changes of the complex microbial population.

Materials and Methods

5-Stage BNR process with step feed system and sludge samples

The flow diagram of the 5-stage BNR process (PADDO) is shown in Fig. 1. The pilot plant (50 m^3/day) consisted of pre-anoxic, anaerobic, anoxic-1, anoxic-2 and oxic tanks, operating at the S-city sewage treatment plant for 2 years (Kyunggi-do, Korea). The ammonium ions were nitrified in the oxic tank, and then denitrified in the anoxic-1 and anoxic-2 tanks. The phosphate to be released in the anaerobic tank was accumulated as the intracellular poly-p level under the anoxic/aerobic conditions of the anoxic-1 and oxic tanks. The excess accumulated phosphate was designated to be removed through wasting after settling in the clarifier. The concentration of the mixed liquor suspended solids (MLSS) in the tank was maintained by return activated sludge (RAS) to the pre-anoxic tank. The step feed system, which properly distributes raw water containing the organics required to remove the nitrogen and phosphorus in the pre-anoxic, anaerobic and anoxic-2 stages, can maximize the phosphorus release and denitrification rates. The operation conditions of the 5-Stage BNR process are shown in Table 1. The SRT (sludge retention time), NRCY (nitrified recycle) and RAS ratio of the system were 12~33 d (average 22 d), 200~243 (average 230%) and 36~67% (average 40%, based on influent flow rate), respectively. To investigate the temporal variations in the microbial populations, sludge samples were collected from the anaerobic, anoxic-1, anoxic-2 and oxic tanks, at different times (April, August, October, 2002 and January 2003). After the sludge

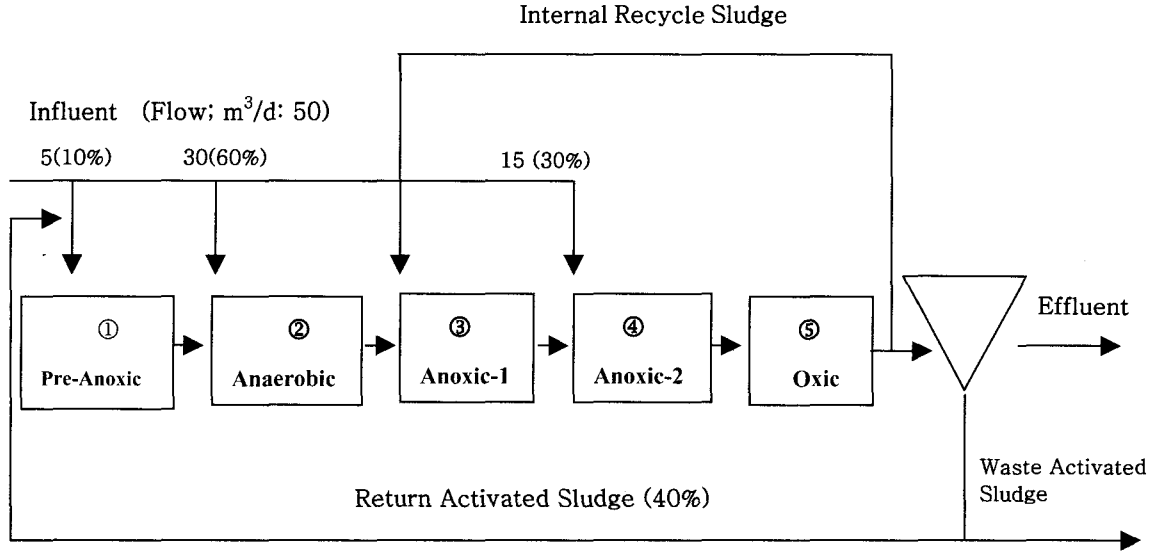

Fig. 1. Flow schematic of the 5-stage BNR system (PADDO).

Table 1. Operating conditions of the 5-stage BNR system

Items		① Pre-Ax	② An	③ Ax-1	④ Ax-2	⑤ Ox	IR(%)	RAS(%)	T(°C)
HRT	hr	0.5	1.0	0.5	2.0	3.5	230	40	11-28
MLSS	mg/l	10,840	5,320	4,730	4,430	4,450			
Volume	m^3	1.1	2.1	1.1	4.2	7.7			

*The volatile portion of the MLSS was 72~78%; An: anaerobic, Ax: anoxic, Ox: oxic, IR: internal recycle, RAS: return activated sludge, T: temperature, HRT: hydraulic retention time, MLSS: mixed liquor suspended solids.

had settled, the supernatant was removed, and the pellet immediately frozen at -70°C prior to analysis.

Chemical analysis

The liquid samples were centrifuged at 12,000 rpm (16,000 ×g) and 4°C, for 15 min. The supernatants were diluted, as required, for the relevant analytical methods. The nitrate and phosphate were analyzed by ion chromatography, DIONEX; model DX-500 (Sunnyvale, USA). All other measurements were performed according to *the Standard Methods for the examination of water and wastewater* (APHA, 1995). All chemical analyses were performed on triplicate samples.

DNA extraction, purification, and quantification

The DNA was directly extracted from the activated sludge taken from each tank (anaerobic, anoxic-1, anoxic-2 and oxic tanks) and purified, as described by Lee *et al.* (1996). The quality of the extracted DNA was checked by standard agarose gel electrophoresis. The DNA concentrations were measured by their absorbance at 260 nm.

PCR amplifications

To obtain the mixed community DNAs for DGGE analysis, the total DNA extracted from the activated sludge was used for PCR amplification (initial denaturation for 4 min at 94°C, 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 57°C and extension for 1.5 min at 72°C, with a final extension for 10 min at 72°C) of the 16S rRNA gene fragment region, at positions ~27 (27F-forward; 5'-AGAGTTTGATCMTGGCTCAG-3') and ~1492 (R1492-reverse; 5'-GGYTACCTTGTTACGACTT-3') (*Escherichia coli* numbering), according to the method of Lane (1991). The PCR products were purified, with a QIAquick PCR purification kit (Qiagen, Germany), and used for a DGGE-PCR template. For the DGGE analysis, PCR primers against the V2 region (357-518, *E. coli* numbering) were used for the amplification of the 16S rRNA gene. The PCR primers were, F357GC (5'-GCCCGCCG-CGCCCCGCGCCCG-GCCCGCCGCCCCCGCCCCCCT-ACGGGAGGCAG-CAG-3'), which contains a GC-rich clamp, which is specific for most Bacteria, and R518 (5'-ATTACCGCGGCT-GCTGG-3'), which is specific for most Bacteria, *Archaea* and *Eucarya* (Muyzer *et al.*, 1993; Van Hannen *et al.*, 1999). PCR amplification was performed with 5 μl of 10xPCR buffer (Promega, USA), 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 2 μM primer and 1 U of Taq DNA polymerase (Promega, USA); approximately 100 ng of template DNA and up to 50 μl of sterile Millipore water. The PCR cycling was performed with a Perkin-Elmer 9700 thermocycler. The temperature-cycling conditions were as follows: After a pre-incubation at 94°C for 4 min, a total of 25 cycles were performed at 94°C for 30 sec, T_A for 30 sec and 72°C for 30 sec. In the first 20 cycles, the T_A was decreased by 1°C, stepwise, every two cycles, from 65°C in the first cycle, to 56°C by the 20th. In the last five cycles, the T_A was 55°C. The cycling was followed by 8 min of incubation at 72°C. Three microliters of the amplified PCR products were examined on a 1% agarose gel, in TAE buffer (40 mM Tris, 20 mM acetate and 2 mM EDTA), and then purified with a QIAquick PCR purification kit (Qiagen, Germany). The Quantity of purified PCR products was examined using a UV spectrophotometer (UV-1601PC, Shimadzu, Japan). DGGE was performed using 1 mm-thick vertical gel, containing 8% acrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1), with a 40 to 60% denaturant gradient, where 100% denaturant was defined as 7 M urea plus 40% formamide. The same amount (approximately 2 μg) of PCR product was loaded in a final volume of 30 μl. The gels underwent electrophoresis at 60°C and 60 V for 30 min, and at 120 V for 13 h, using the D code mutation detection system (Bio-Rad, USA). The gels were stained for 20 min, with ethidium bromide, and washed twice for 10 min, with MilliQ H_2O, prior to UV transillumination. The DGGE gels were photographed using the Bio-Rad Geldoc Image Analyze system (Bio-Rad, UAS).

Sequencing and phylogenetic analysis of excised DGGE bands

Both prominent and unique DGGE bands were excised, and used for nucleotide sequence determination. For each band selected, only the middle portion was excised, with a sterile razor, and slices (approximately 30 mg [wet weight]) were placed in micro-centrifuge tubes, containing 100 μl of 1×TAE buffer. The tubes were then incubated overnight, at 4°C. Three μl of 1×TAE, containing DNA, was used as the template for the DGGE-PCR. The DNA was amplified by PCR, with the primers for DGGE described above, and run on a second DGGE gel. This procedure was repeated two or three times to obtain clean DNA products for the DNA sequencing (Nielsen *et al.*, 1999). The nucleotide sequences of the DGGE bands of interest were obtained by sequencing the excised fragment (purified fragment) ligated into a pGEM-T easy vector, which had 3'-T overhangs to facilitate cloning of the PCR products (Promega, USA). Ligation mixes were transformed into competent *Escherichia coli* DH5α-cells. White colonies on ampicillin (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium were picked, and the insert size determined by digestion with *Eco*RI (Takara, Japan). Clones containing an insert of the expected size were cultured in 3 ml of Luria broth, and then plasmids extracted with a plasmid extraction kit (Bioneer, Korea). The nucleotide sequences of the cloned products were determined from the plasmid DNA preparations, using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA) and ABI310 Sequencers (Applied Biosystems, USA) according to the manufacturer's instructions. Vector primer T7 was used for the sequencing reactions. Sequences obtained from 16S rDNA

Table 2. Summary of the operating results for the 5-stage BNR system process at various temperatures

Parameter	Influent (mg/l)	Effluent (mg/l)			
Temperature		11-15°C	15-20°C	20-25°C	25-28°C
TCODcr	274-348	40.6 (87%)*	35.2 (89%)	24.9 (91%)	22.4 (91%)
SCODcr	102-109	19.8 (81%)	20.2 (81%)	16.0 (84%)	15.6 (84%)
TN	25-31	6.2 (79%)	5.3 (82%)	3.9 (87%)	3.6 (86%)
NH_4+-N	25-31	3.4 (88%)	2.1(93%)	1.0 (97%)	0.7 (97%)
SP	3.8-4.5	0.62 (87%)	0.55 (86%)	0.49 (88%)	0.71 (81%)
SS	108-230	6.5 (90%)	14.0 (90%)	7.3 (93%)	9.4 (91%)

*removal efficiency (%). TCODcr; total chemical oxygen demand, as chromium, SCODcr; soluble chemical oxygen demand, as chromium, TN; total nitrogen, SP; soluble phosphate, SS; suspended solids.

libraries were checked for chimeras with the CHECK-CHIMERA software of the Ribosomal Database Project (Maidak *et al.*, 1997), and compared to those in the databases, using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1999), to identify the known sequences with high degrees of similarity. Partial sequences were manually compiled and aligned using Phydit software (Chun, 1995). Phylogenetic trees were generated using the neighbor-joining (Saitou and Nei, 1987), Fitch-Margoliash (Fitch and Margoliash, 1967), and maximum parsimony (Kluge and Farris, 1969) algorithms in the PHYLIP package. Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated, as described by Jukes and Cantor (1969). The robustness of the inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values >50% were shown on the trees.

Nucleotide sequence accession numbers

The 16S rDNA partial sequences obtained during this study have been submitted to the GenBank, EMBL and DDBJ nucleotide sequence databases, under accession no. from AY362830 to AY362845

Results and Discussion

Plant performance

Table 2 shows the average operating results from the 2 years of operation. The 5-stage BNR system efficiently removed NH_4^+-N (88~97%) and soluble phosphate (81~88%) from the municipal sewage. Despite the winter period (<15°C), the nitrogen and phosphate removal efficiencies were maintained at over 79 and 87%, respectively, which were much higher results than those previously reported (Ydstebo *et al.*, 2000). The effluent quality from this treatment process was consistently high, and the nitrogen and phosphate removal efficiencies were only slightly influenced by external temperature changes, and were stably maintained during the operation period.

Seasonal changes in microbial community composition

In order to investigate the microbial community structures according to the seasonal changes, the PCR-DGGE method was applied to the 5-stage BNR system. The results are shown in Fig. 2. The DGGE analysis indicated seasonal community changes were observed, however, the community profiles of the total bacteria of each reactor showed only minor sample to sample differences in the same season. In the case of the winter season, the community variation of each reactor was only low, although some bands appeared and others disappeared. Therefore, it was proposed that the bacterial population in the 5-stage BNR system was not influenced very much by the allochthonous bacteria from the wastewater during the same sea-

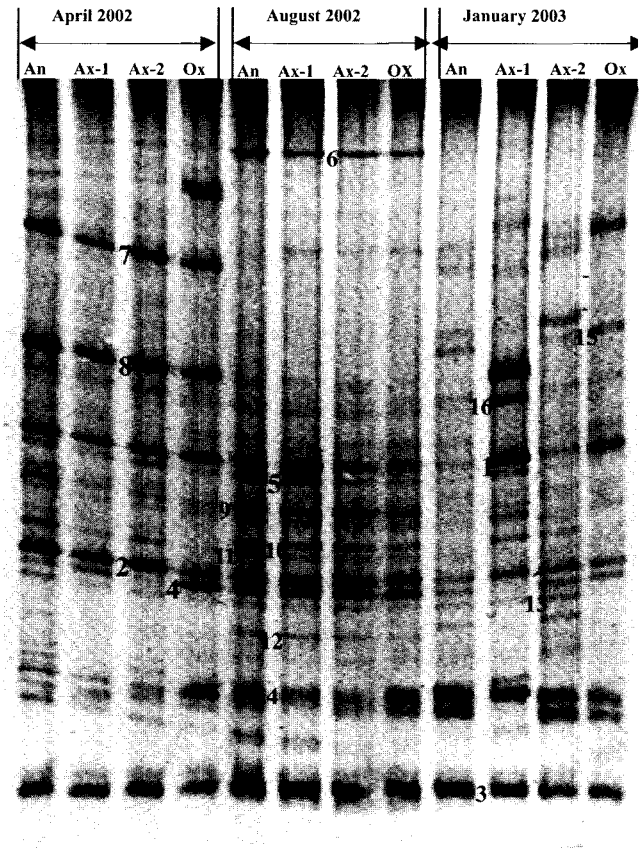

Fig. 2. DGGE of the PCR-amplified 16S rDNA fragments from the 5-stage BNR wastewater treatment process.

son, which suggests that this system can remove nitrogen and phosphorous in wastewater due to the stabilized bacterial population during the same season. This result was consistent with that of other researchers, who have shown analogous levels of bacterial community stabilities in a bench-scale system, as long as the bioreactor function also remained stable (Eichner *et al.*, 1999; Fernandez *et al.*, 1999).The population dynamics during the whole operational period (from spring to winter) were then analyzed (Fig. 2). Seasonal community changes were observed, and clearly expressed in the abundance of the distinct bands. Presumably, a stable reactor performance requires at least some degree of stability among the individual populations comprising the microbial community supported by such bioreactors, but flexibility is also needed to adapt the community structure in response to changing conditions. Our results also showed that the microbial community could adapt to changing environmental conditions (in this case, a change in temperature). Fernandez *et al.* (2000) showed that flexibility, with respect to bacterial community structure, leads to a more stable process performance. Fig. 2 showed that the number of major bands was higher in the summer sample, and were decreased during the winter period, indicating that the microbial community structure got simpler at low temperatures. Since the nitrogen and phosphate removal efficiencies were maintained at high levels throughout the winter operation period, the bacteria still remaining in the winter sample can be considered as important in the present 5-stage BNR sludge. The bacteria detected as dominant in the anoxic-1 reactor for the winter season may be the especially important denitrifying PAOs. Therefore, the focus of this study was to identify the predominant or unique bands that appear in the winter season. The major or unique bands on the DGGE gel (Fig. 2) were excised, and the DNAs recovered from these bands were sequenced and compared to publicly available databases. The DNAs recovered from No. 1 to 16 were successfully sequenced (Table 3).

Sequencing of DGGE bands

Bands 1, 2 and 3 appeared in all the reactors during the operating period. Band 8 appeared in the spring sample, but disappeared in the summer sample, and then reappeared in the anoxic-1 reactor of the winter sample, as a predominant group. The band intensities of bands 14 and 16 were increased as the temperature decreased. The band intensities of bands 4, 5, 9, 10, 11 and 12 decreased, or disappeared during the winter period. Bands 13 and 15 were newly appeared bands in the winter season, but only with slight intensities. Because there may be several biases, such as DNA extraction and PCR biases, it would have been dangerous to decide that bands observed with

Table 3. Nucleotide sequence similarity of the eluted DGGE bands.

Band[a]	Partial 16S rDNA sequence			Putative division
	Database similarity	number of nucleotides compared (bp)	Similarity (%)	
1	Uncultured bacterium ARFS-35(AJ277701)	171	97	Actinobacteria
2	*Microbacterium oxydans* (AJ576066)	176	100	Actinobacteria
3	Uncultured eubacterium clone F13.48 (AF495441)	168	98	-
4	*Janibacter terrae* (AF176948)	176	100	Actinobacteria
5	Bacterium Ellin325 (AF4987707)	178	90	Actinobacteria
6	Uncultured bacterium clone P4-29 (AF523328)	160	95	-
7	Uncultured bacterium clone 5g18 (AF522926)	176	99	-
8	Uncultured sludge bacterium A28b (AF234713)	158	100	-
9	*Actinobacterium* PI_CH2.1.C2 (AY162041)	171	100	Actinobacteria
10	Uncultured bacterium ASG6 (Aj514434)	193	98	-
11	Uncultured sludge bacterium H34 (AF234750)	167	98	α^b
12	Uncultured bacterium clone CCM12a (AY221043)	169	99	-
13	*Aquaspirillum psychrophilum* (AF078755)	193	98	β^c
14	Metal-contaminated soil clone K20-64 (AF145856)	158	94	-
15	Uncultured actinobacterium (AY193123)	167	99	Actinobacteria
16	Actinobacterium ANT9067 (AY 167331)	174	99	Actinobacteria

[a]: DEEG bands correspond to 1-16 bands on DGGE profile in Fig.2, [b]: Alpha subclass of proteobacteria, [C]: Beta subclass of proteobacteria, -: Unaffiliated group.

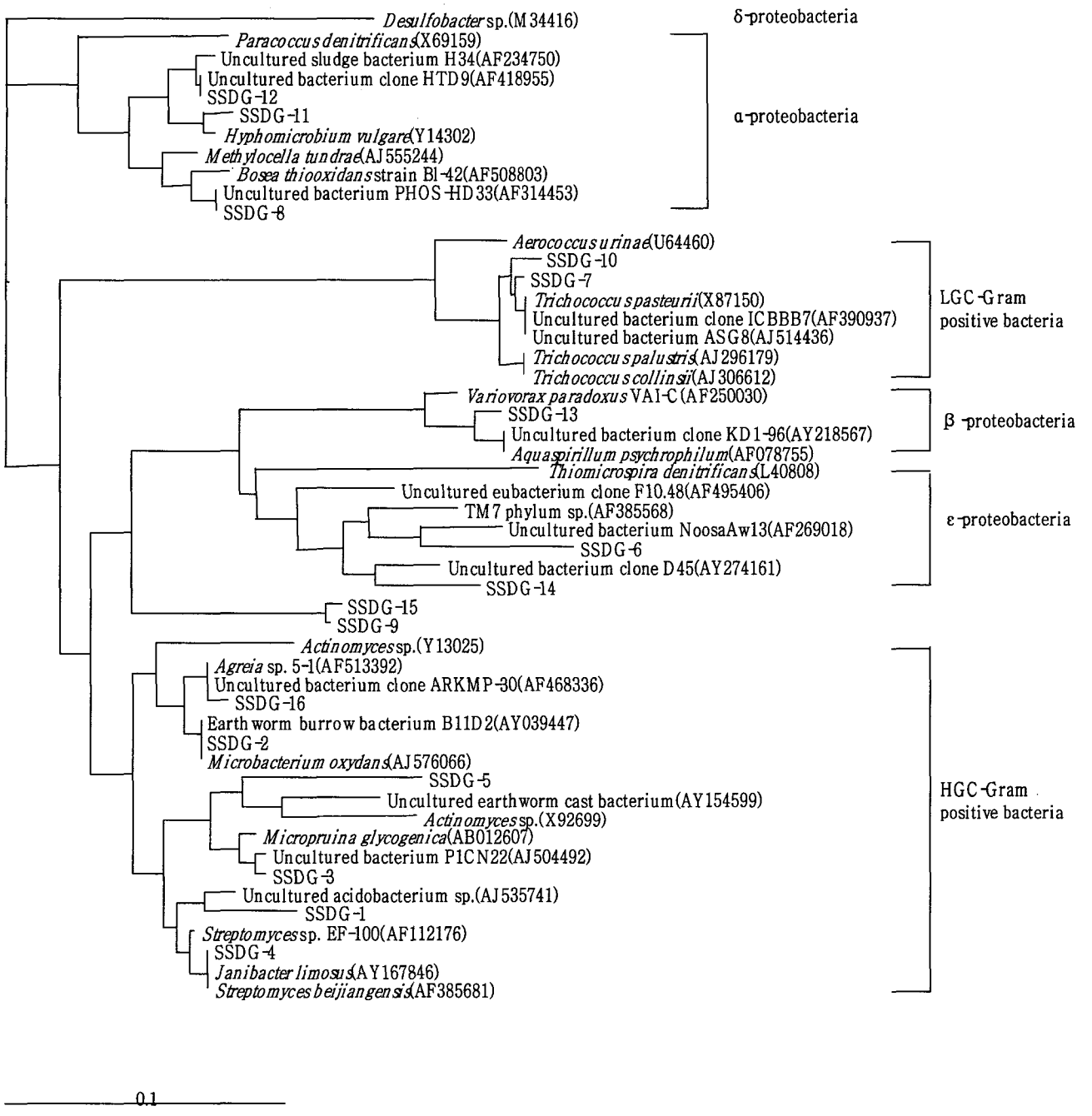

Fig. 3. Phylogenetic tree of the rDNA sequences of the DGGE bands. SSDG1~16; DGGE bands correspond to bands 1-16 on the DGGE profile in Fig. 2. The scale bar represents 0.1 substitution per nucleotide position.

strong intensities in the winter season represented the dominant species in this community. However, at least the appearance of a new band, or an increase of the same band intensities, with time, can be considered as an increase in the corresponding bacteria in the real community. Because bands 1, 2 and 3 were observed through all the seasons, bands 14 and 16 got thicker and thicker as the temperature decreased and bands 13 and 15 were new bands in the anoxic-2 or oxic tank of the winter period, respectively, even though their intensities were very weak, and band 8 was the most dominant group in the anoxic-1 reactor with the winter samples, these bands can be considered to represent bacteria that play key roles in the present 5-stage BNR process. Among these bands, bands 2, 13 and 16 were closely related to *Microbacterium oxydans* (97%), *Aquaspirillum psychrophilum* (98%) and *Actinobacterium* (99%), and the rest of the bands (bands 1, 3, 8, 14 and 15) were not closely related to previously characterized bacteria (Table 3). These data suggest the importance of culture-independent methods for the quality

control of wastewater treatment. In order to obtain more information for these uncultured bacterial groups, a phylogenetic distance tree for these bands was analyzed, and is shown in Fig. 3. It was found that the bacteria commonly contained in all the reactors, bands 1, 2 and 3, belonged to the high G+C Gram-positive bacterial group, on the basis of the analysis of the sequence excised from their DGGE bands and the determination of their phylogenetic affiliation. Band 16 also belonged to the high G+C Gram-positive bacterial group. In the case of band 15, this was closely related to uncultured actinobacterium, which belongs to the high G+C Gram-positive bacterial group, on the base of the 16S rDNA sequence analysis (Table 3), but it was positioned between the high G+C Gram-positive bacterial group, and members of the ε-Proteobacteria in Fig. 3. This may have been a consequence of the relatively short sequence used in the analysis. The dominant bacterial group in the anoxic-1 reactor, band 8, belonged to the α-Proteobacteria. In addition, bands 13 and 14 belonged to the β-Proteobacteria and ε-Proteobacteria, respectively. The phylogenetic analysis showed that there were four major bacterial groups in the sludge; Gram positive bacteria, with a high G+C content, the α-subclass of Proteobacteria, β-subclass of Proteobacteria and ε-subclass of Proteobacteria. Therefore, these bacterial groups are suspected to play an important role in the 5-stage BNR system. In this study, the seasonal bacterial population changes in the 5-stage BNR system were monitored using DGGE analysis. The number of major bands on the DGGE gel was higher in the summer sample, and decreased during the winter period, indicating that the microbial community structure got simpler at low temperatures. It is well known, and firmly established, that the rate of chemical and biochemical reactions slow down as the temperature decreases. Nevertheless, the reactor performance of the 5-stage BNR system did not decrease when the temperature decreased. In this study, the experiments were performed over temperatures ranging from <11 to 28°C The reason for the better system performance during the winter period was apparently related to the reduced competition for the substrate in the anoxic reactors, which resulted in an increased population of PAOs, and thus, it may be involved in maintaining the reactor performance. Erdal *et al.* (2003) also reported that PAOs were psychrophilic bacteria, and temperatures of 10°C, or less, gave them a growth advantage relative to the non-PAOs in activated sludge systems. They also postulated that cold temperatures, either partially or completely suppressed some of the metabolic pathways of the activated sludge microorganisms, without altering the dominant PAOs populations. Fig. 2 showed that the number of bands on the DGGE gel in the winter sample were less than those of the summer sample, and among them, several band's intensities were thicker in the anoxic-1 reactor, indicating that this could be considered as an increase in the corresponding bacteria in the real community. Therefore, the bacteria that remained in the winter sample can be considered as important in playing key roles in the present 5-stage BNR sludge. The bacteria that were detected as dominant in the anoxic-1 reactor may be the especially important denitrifying PAOs. Among them, some major DGGE bands were excised, reamplified and directly sequenced. It was revealed that most of the bacteria in anoxic-1 reactor were not closely related to previously characterized bacteria (Table 3). A phylogenetic analysis of the DGGE bands revealed they consisted of four different eubacterial phyla (Fig. 3). These results indicated that the PAOs in the activated sludges of the 5-stage BNR system, not only involved specific eubacterial phyla, but were distributing in various phyla. Also, the PAOs may not be composed of a few limited genospecies, but involve phylogenetically and taxonomically diverse groups of bacteria. Our data indicated, rather than there being a single dominant PAO, that several different bacterial groups may be important, but they were mainly to culture. Therefore, to define the microbial community structure of EBPR processes, there is a need to look more closely into the occurrence and behavior of each species of polyphosphate accumulating bacteria, in various EBPR processes, mainly by molecular methods, as many polyphosphate accumulating bacteria seem to be impossible to culture.

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