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Analysis of Microbial Communities Using Culture-dependent and Culture-independent Approaches in an Anaerobic/Aerobic SBR Reactor

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Comparative analysis of microbial communities in a sequencing batch reactor which performed enhanced biological phosphorus removal (EBPR) was carried out using a cultivation-based technique and 16S rRNA gene clone libraries. A standard PCR protocol and a modified PCR protocol with low PCR cycle was applied to the two clone libraries of the 16S rRNA gene sequences obtained from EBPR sludge, respectively, and the resulting 424 clones were analyzed using restriction fragment length polymorphisms (RFLPs) on 16S rRNA gene inserts. Comparison of two clone libraries showed that the modified PCR protocol decreased the incidence of distinct fragment patterns from about 63% (137 of 217) in the standard PCR method to about 34% (70 of 207) under the modified protocol, suggesting that just a low level of PCR cycling (5 cycles after 15 cycles) can significantly reduce the formation of chimeric DNA in the final PCR products. Phylogenetic analysis of 81 groups with distinct RFLP patterns that were obtained using the modified PCR method revealed that the clones were affiliated with at least 11 phyla or classes of the domain *Bacteria*. However, the analyses of 327 colonies, which were grouped into just 41 distinct types by RFLP analysis, showed that they could be classified into five major bacterial lineages: α, β, γ- *Proteobacteria*, *Actinobacteria*, and the phylum *Bacteroidetes*, which indicated that the microbial community yielded from the cultivation-based method was still much simpler than that yielded from the PCR-based molecular method. In this study, the discrepancy observed between the communities obtained from PCR-based and cultivation-based methods seems to result from low culturabilities of bacteria or PCR bias even though modified culture and PCR methods were used. Therefore, continuous development of PCR protocol and cultivation techniques is needed to reduce this discrepancy.

Keywords: microbial community, PCR, culturability

Prokaryotes are among the most important contributors to bioremediation and wastewater treatment processes (Briones and Raskin, 2003; Seviour *et al*., 2003; Lim *et al*., 2005). Therefore, investigation of the microbial structure and function of activated sludge is of great importance for gaining a better general understanding of wastewater treatment processes. Although efforts have been made to reveal the microbial communities in activated sludge on the basis of traditional cultivation methods, these methods cannot be directly applied to analyses of microbial diversity in entirety because it is now widely recognized that only small portions of the total cell counts in environmental samples can be cultured (Kämpfer *et al*., 1996). However, cultivation-based study still remains important, since the biotechnological role of prokaryotes in environmental processes can be estimated quite well when the prokaryotes are successfully cultivated and characterized. Recently, culture-independent molecular approaches using PCR-based methods have been used for studies of microbial ecology in activated sludge (Eschenhagen *et al*., 2003; Jeon *et al*., 2003b; Kim *et*

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al., 2004). However, although analysis of 16S rRNA gene sequences has been used to determine the microbial communities of sludge without the normal limitations of culturing methods, a severe issue with PCR-based methods is the potential generation of artifacts such as PCR error or the formation of chimeric molecules during the amplification of mixed microbial templates (Wang and Wang, 1997; Qiu *et al*., 2001; Thompson *et al*., 2002).

Activated sludge processes with cyclic changes of anaerobic and aerobic conditions have been used to remove phosphate from wastewater and are becoming more important for reducing the eutrophication from aquatic water (Mino *et al*., 1987; Jeon and Park, 2000; Jeon *et al*., 2001). Therefore, an understanding of the microbial community responsible for phosphorus removal is a prerequisite to understanding the EBPR mechanism and controlling the EBPR processes. Although PCR-based methods are generally used for the analysis of enhanced biological phosphorus removal (EBPR) sludge without the limitations of cultivation, cultivation-based approaches are certainly still necessary to reveal the function of EBPR bacteria. Therefore, many researchers have endeavored to increase the culturability of bacteria (Janssen *et al*., 2002; Davis *et al*., 2005). In this study, we performed comparative analyses of the microbial communities of EBPR sludge using PCR-based methods to analyze 16S rRNA gene sequences and also using a culture-based approach.

Materials and Methods

Operation of sequencing batch reactor

A cylindrical vessel with a 4-liter working volume was used for sequencing batch reactor (SBR) operation; it was operated in a fill-and-draw mode with a cycle of 8 hours. Microbial inoculum was obtained from an activated sludge treatment plant at Pohang University of Science and Technology. Each cycle consisted of 20 min of anaerobic filling, 1 h 40 min of anaerobic reaction, 4 h 20 min of aerobic reaction, 70 min of settling, and 30 min of decanting. Two liters of clarified supernatant were withdrawn at the end of the settling phase. Amounts of 770 mg/L sodium acetate, 40 mg/L NH_4^+-N, and 15 mg/L PO_4^{3-}-P were loaded into the SBR. The preparation of the synthetic wastewater and the operation of the SBR have been described elsewhere (Smolders *et al*., 1994; Jeon *et al*., 2003b). The soluble phosphate and nitrate in the solution were analyzed using an ICS-1000 ion chromatograph (Dionex Corp., USA). The mixed liquor suspended solid (MLSS) was analyzed as described by the American Public Health Association (APHA) (1995).

Cultivation and identification of isolates

The sludge sample was dispersed in PBS buffer (phosphate buffered saline, pH 7.2). The resultant suspension was serially diluted in 10-fold steps by the addition of 1 ml of the previous dilution to 9 ml PBS buffer, and 0.1 ml aliquots from each of the diluted suspensions were spread onto R2A agar plates and incubated under aerobic conditions at 20°C for up to 1 month in the dark. Colonies were randomly selected and crude lysates containing genomic DNA from respective colonies were prepared by boiling a small amount of cell material from colonies in 100 μl of 5% Chelex 100 solution (BioRad, USA) for 10 min. The preparations were then placed on ice for 10 min, followed by centrifugation at 16,000 × g for 10 min. The supernatant was used as the template in the PCR. For some isolates, cell material was added directly to the PCR without this initial boiling step ('colony PCR'). The PCR reactions of the 16S rRNA gene (~1,500 base-pair product) were performed using the eubacterial primers 27f and 1492r (Lane, 1991), and were carried out in a 50-μl thermal cycler (MJ Research, USA) with 1 μM of primers and 0.1 U of *Taq* polymerase (Solgent, Korea) using a cycling regime of 94°C for 3 min (1 cycle); 94°C for 1 min, 60°C for 45 sec, and 72°C for 1 min (32 cycles); and 72°C for 10 min (1 cycle). The amplicons were double-digested with *Hae*III and *Hha*I. Restriction fragment length polymorphism (RFLP) patterns were analyzed on 2.5% MetaPhore agarose (BioWhittaker, USA) gels with a 100-bp ladder (Bioneer, Korea), while colonies were grouped according to their RFLP patterns and representative PCR products containing distinct RFLP patterns were ligated into the pCR2.1 vector (Invitrogen, USA) according to the recommended protocol of the manufacturer. They were then sequenced as described previously (Jeon *et al*., 2003a).

Extraction of total genomic DNA

A modification of the DNA extraction methods of Zhou *et al*. (1996) was used to isolate the genomic DNA from the sludge. A sludge sample of 5 ml was collected from the SBR by centrifugation (10,000 × g, 3 min) at the end of the aerobic stage and was then resuspended in 0.75 ml of 50 mM Tris (pH 8.0) and 50 mM EDTA. The cell suspension was frozen at –20°C and thawed on ice for 45 min. Lysozyme was added at a concentration of 2 mg/mL, and the suspended solution was incubated for 15 min at 37°C. The samples were sonicated twice for 30 sec at power level 5 and 100% active cycles (Bandelin Electronic UW 3100, Germany). The effectiveness of the cell lysis procedure was confirmed by microscopic examination of the samples before and after the lysis

treatment. We added 75 μl of 20% sodium dodecyl sulfate [wt/v] to the sample solution, which was then stirred gently for 15 sec with a pipette. The genomic DNA was then purified by 700 μl of TE buffer-equilibrated phenol and phenol-chloroform-isoamyl alcohol (100:24:1 [v/v/v]) extraction. RNase treatment and ethanol precipitation were carried out according to the method of Zhou *et al*. (1996).

Library constructions of 16S rRNA genes and analysis
The PCR reactions of the 16S rRNA gene (~1,500 base-pair product) were performed by using the eubacterial primers, 27f and 1492r (Lane, 1991). The PCR amplification for the first (standard) library was carried out in a 50-μl thermal cycler (MJ Research, USA) with 1 μM of primers and 0.1 U of AccuPrime *Taq* polymerase (Invitrogen, USA) using a cycling regime of 94°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 45 sec, and 72°C for 1 min (32 cycles); and 72°C for 10 min (1 cycle) (Eschenhagen *et al*., 2003). The PCR amplification for the second (modified) library was performed to minimize the accumulation of the known artifacts (*Taq* errors, chimeras, and heteroduplex molecule) using the following conditions on three replicates for each sample: 94°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 45 sec, and 72°C for 2 min (15 cycles); and 72°C for 5 min (1 cycle) (Thompson *et al*., 2002). The three replicates of PCR amplifications were combined, purified using a PCR purification kit (Qiagen, USA), and resuspended in 40 μl of milliQ water. The resupended PCR products were amplified again by five additional PCR cycles. The standard and modified PCR products were then ligated into the pCR2.1 vector, respectively.

After blue-white screening of colonies, inserts were analyzed by RFLP analysis using vector-specific primers (5'-GTAACGGCCGCCAGTGTGCT and 5'-C AGTGTGATGGATATCTGCA) (Jeon *et al*., 2003a). Clones were grouped according to their RFLP patterns and representative clones containing distinct RFLP patterns were sequenced as described previously (Jeon *et al*., 2003a). The 16S rRNA gene sequences obtained were checked for their chimeric properties using the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) and were assigned into their taxonomical hierarchy using the Classifier of Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp). The 16S rRNA gene sequences were aligned together with those of representative members of selected genera from GenBank using the CLUSTAL W program (Thompson *et al*., 1994). The phylogenetic trees were constructed using the algorithm of the Kimura 2-parameter model for the neighbor-joining method in PHYLIP software, version 3.6 (Felsenstein, 2002).

Results and Discussion

SBR performance
The SBR was continuously operated with acetate as the sole carbon source for more than 4 months. Phosphate release during the anaerobic period and phosphate uptake during the aerobic period gradually increased over the operation time and complete EBPR and carbon removal were accomplished after approximately 30 days (data not shown). At this stage, MLSS (about 3,000 mg/L) and the concentration profiles of phosphate and carbon compounds were relatively constant.

Library constructions of 16S rRNA genes
More than 200 clones from each of the two PCR-derived 16S rRNA gene libraries constructed from the same SBR sludge were analyzed by comparing the RFLP fragment patterns of amplified 16S rRNA genes. The first library was constructed by employing standard amplification protocols. For the second library, a modified protocol suggested by Klepac-Ceraj *et al*. (2004) was used to minimize artifacts such as *Taq* polymerase errors and chimeric molecule formation. RFLP fragment comparison of the two libraries showed that the change to the amplification protocol alone decreased the incidence of distinct RFLP fragment patterns from about 63% (137 of 217) in the standard library to about 34% (70 of 207) in the modified library, which indicated that the low cycle PCR can quantitatively reduce the abundance of chimeric DNA in the final PCR product of complex ecological genomic DNA. Consequently, our experiments showed that standard amplification protocols can overestimate microbial communities if they contain a large fraction of chimeric DNA. Many researchers also have reported from quantitative analysis that avoidance of primer limitation, low cycle PCR, and longer extension time can decrease the formation of PCR bias (Thompson *et al*., 2002; Acinas *et al*., 2004; Klepac-Ceraj *et al*., 2004). However, because primers are usually added in sufficient amounts in standard PCR conditions, a low cycle PCR reaction and longer extension time can be critical factors to minimize PCR artifacts such as *Taq* polymerase errors and the generation of chimeric molecules to a significant extent.

Comparison of bacterial diversity between culture-based method and 16S rRNA gene clone library
Under aerobic conditions, the number of CFU (colony forming units) on R2A media continuously increased for 40 days (the viable count achieved after 40 days of incubation was about two times higher than that observed after two days of incubation), which

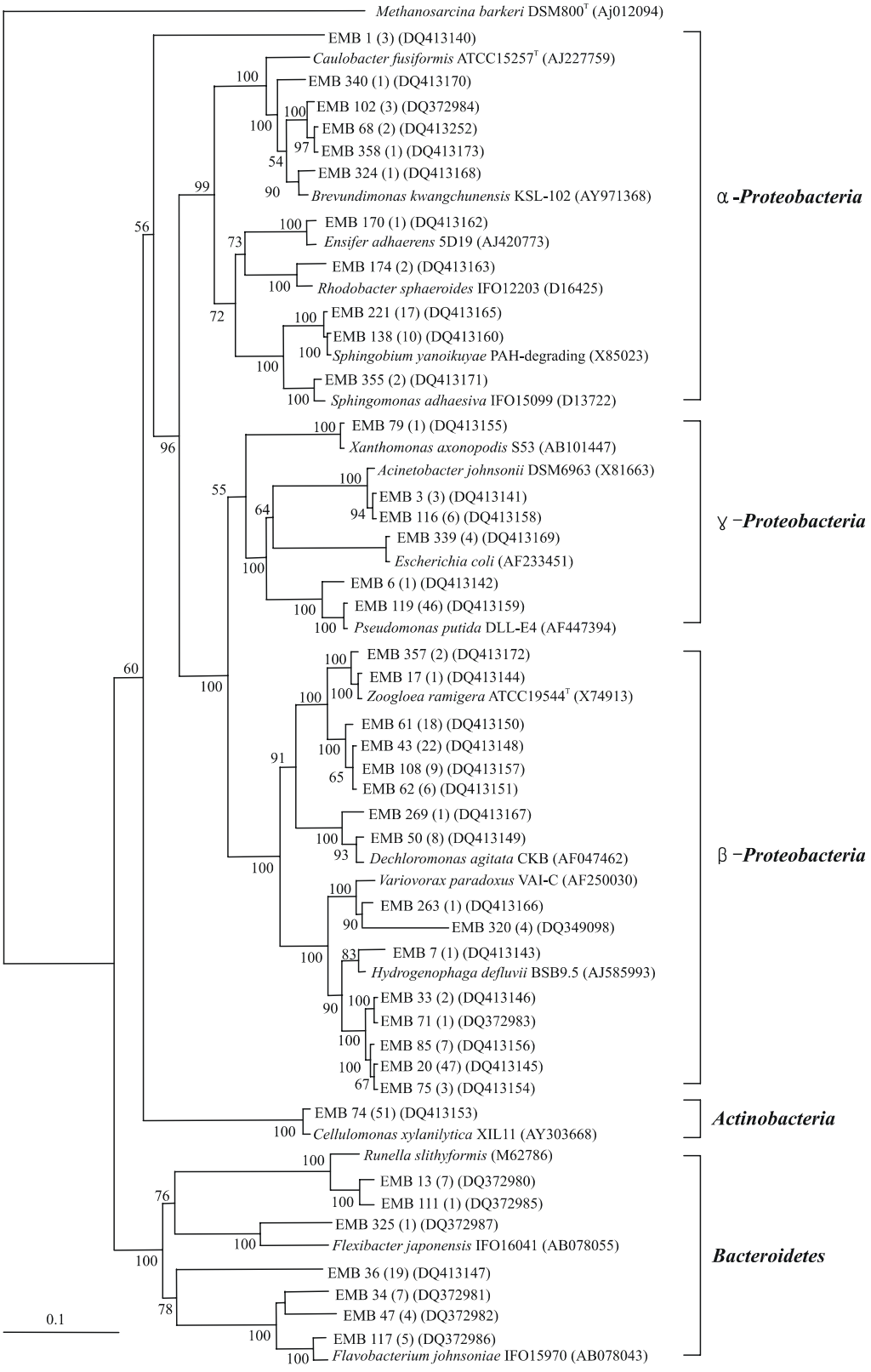

Fig. 1. Phylogenetic relationships of bacteria from SBR sludge grown on R2A media. Bootstrap values are shown in percentages of 1000 replicates, when higher than 50%. Numbers in parentheses indicate frequencies of colonies exhibiting the same restriction pattern. *Methanosarcina barkeri* DSM800^T was used as an outgroup. The scale bar is equal to 0.1 changes per nucleotide position.

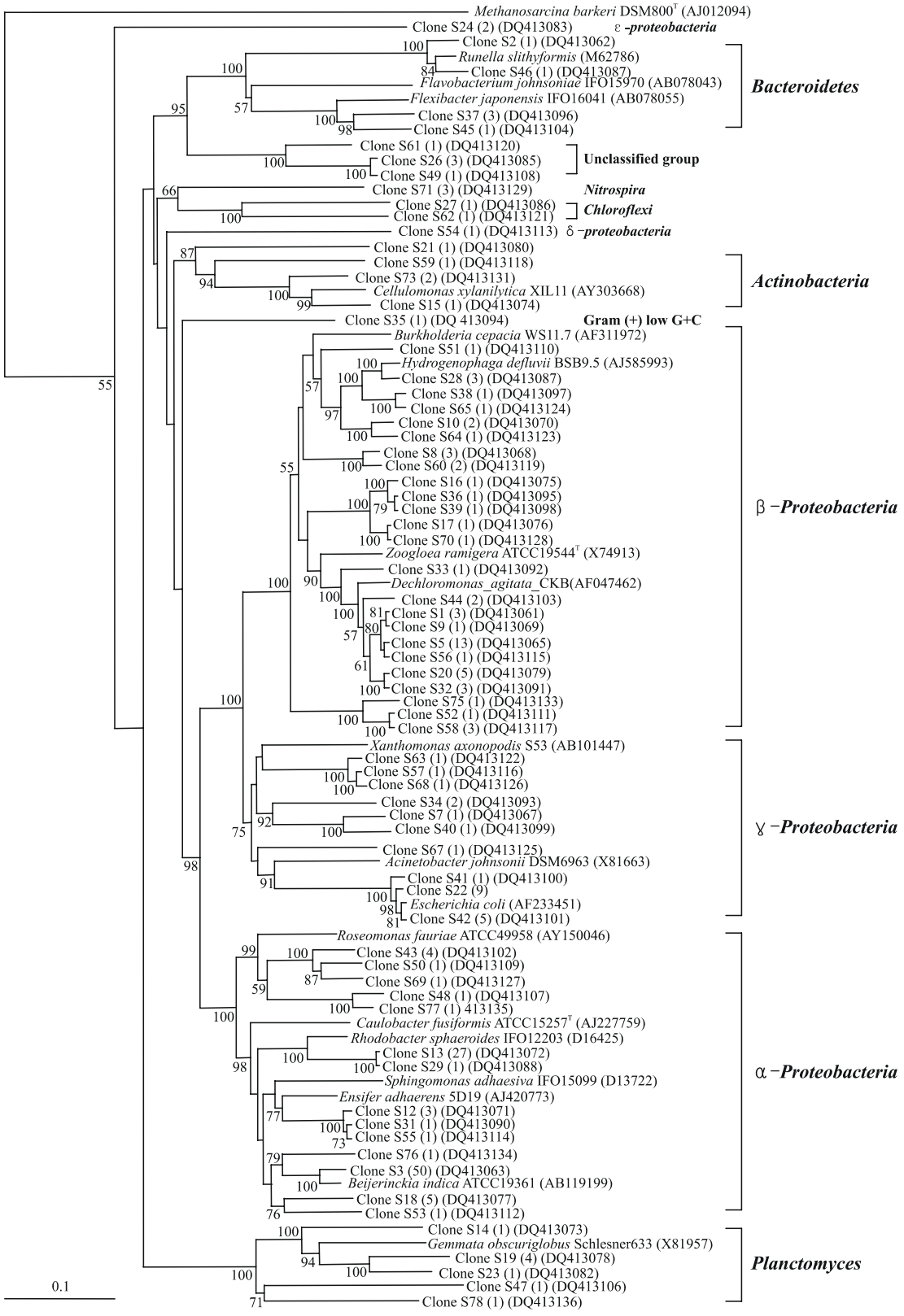

Fig. 2. Phylogenetic analysis of 207 cloned bacterial 16S rRNA gene sequences from the 16S rDNA library of SBR sludge. Bootstrap values are shown in percentages of 1000 replicates, when higher than 50%. Numbers in parentheses indicate frequencies of clones exhibiting the same restriction pattern. *Methanosarcina barkeri* DSM800^T was used as an outgroup. The scale bar is equal to 0.1 changes per nucleotide position.

indicates that the culturability of bacteria was increased strictly by the extension of culture time (Janssen *et al*., 2002). Identification of the microbes grown on R2A media was performed on the basis of 16S rRNA gene sequences after RFLP analysis. Three-hundred and twenty-seven colonies were selected randomly and their 16S rRNA genes were PCR-amplified. RFLP analysis of the 16S rRNA genes grouped the randomly picked colonies into 41 distinct types, and the analyses of 16S rRNA gene sequences with distinct fragment patterns showed that the colonies were affiliated with only five major bacterial lineages: α, β, γ- *Proteobacteria* (13.5, 39.1, and 18.7% of the total number of colonies selected from R2A agar medium, respectively), *Actinobacteria* (14.2%), and the phylum *Bacteroidetes* (13.5%) (Fig. 1). Previous researchers have reported that the *Rhodocyclus*-like group within the beta subclass of *Proteobacteria* is important for EBPR in the SBR process and potentially acts as a biological phosphorus remover, but this group has not been cultured (Jeon and Park, 2000; Kong *et al*., 2004). Our research also showed that bacteria belonging to the *Rhodocyclus*-related group were not retrieved by the cultivation method.

A total of 207 clones from the 16S rRNA gene library constructed by the modified PCR method to reduce PCR bias were analyzed based on RFLP fragment patterns and 16S rRNA gene sequences in order to estimate the bacterial diversity of EBPR sludge. Phylogenetic analysis of 81 distinct types with distinct fragment patterns revealed that the clones were classified into at least 11 phyla or classes of the domain *Bacteria*: α, β, γ-*Proteobacteria* (47.3, 25.6, and 11.1%, respectively), *Actinobacteria* (2.4%), the phylum *Bacteroidetes* (2.9%), *Planctomyces* (3.9%), *Nitrospira* (1.5%), *Chloroflexi* (1.0%), ε-*Proteobacteria* (1.0%), δ-*Proteobacteria* (0.5%), low G+C Gram-positive, and an unclassified group (2.4%) (Fig. 2). The 16S rRNA gene clone library showed that the largest groups of clones belonged to members of the *Proteobacteria* beta subclass, which have been reported likely to be responsible for the removal of biological phosphorus. Some groups belonging to *Planctomyces* (3.9%), *Nitrospira* (1.5%), *Chloroflexi* (1.0%), ε-*Proteobacteria* (1.0%), δ-*Proteobacteria* (0.5%), and the unclassified group (2.4%), all of which were observed in the 16S rRNA gene clone library. These findings indicate that the microbial community compositions of EBPR sludge determined by the cultivation method with a long incubation period and by the modified PCR method were still quite different, although the difference is smaller than that generally reported for activated sludge (perhaps up to 15% and often less than 5%) (Amann *et al*., 1995).

A significant discrepancy between microbial communities determined by culture-dependent and -independent analyses has been shown by many previous investigations (Amann *et al*., 1995; Ellis *et al*., 2003). Several explanations for these differences might be possible. First, a well-recognized primary reason for such differences is the PCR bias associated with the use of molecular methods. Second, many portions of the bacterial communities, including anaerobic bacteria, may be extremely difficult to cultivate in general. In this study, the discrepancy between the communities obtained from PCR-based and cultivation -based methods seems to have resulted from low culturabilities of bacteria or PCR bias. Therefore, continual development of PCR protocols and cultivation techniques are needed in order to reduce this discrepancy.

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