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Microbial Communities of Activated Sludge Performing Enhanced Biological Phosphorus Removal in a Sequencing Batch Reactor Supplied with Glucose

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Abstract Microbial communities were analyzed in an anaerobic/aerobic sequencing batch reactor (SBR) fed with glucose as a sole carbon source. Scanning electron microscopy (SEM) showed that tetrad or cuboidal packet bacteria dominated the microbial sludge. Quinone, slot hybridization, and 16S rRNA gene sequencing analyses showed that the Proteobacteria beta subclass and the Actinobacteria group were the main microbial species in the SBR sludge. However, according to transmission electron microscopy (TEM), the packet bacteria did not contain polyphosphate granules or glycogen inclusions, but only separate coccus-shaped bacteria contained these, suggesting that coccus-shaped bacteria accumulated polyphosphate directly and the packet bacteria played other role in the enhanced biological phosphorus removal (EBPR). Based on previous reports, the Actinobacteria group and the Proteobacteria beta subclass were very likely responsible for acid formation and polyphosphate accumulation, respectively, and their cooperation achieved the EBPR in the SBR operation which was supplied with glucose.

Key words: EBPR, sequencing batch reactor, phosphorus removal, wastewater

Activated sludge processes with cyclic changes of anaerobic and aerobic conditions have been used for phosphate removal from wastewater. Several biological models have been proposed to explain how this enhanced biological phosphorus removal (EBPR) is achieved [16, 28, 42]. However, knowledge of the biochemical reactions involved in the EBPR process has mostly been derived from indirect observations and theoretical consideration. Therefore, the identification of the phylogenetic and taxonomic groups of bacteria responsible for phosphorus removal remains to be done by environmental scientists in order to understand the EBPR mechanism and to control the EBPR processes.

Since Fuhs and Chen [13] first described *Acinetobacter* spp. as a key microorganism in EBPR, many studies have focused on this bacterial genus [3, 12, 24]. However, Jerkins and Tandoi [17] and van Loosdrecht *et al.* [39] showed that *Acinetobacter* spp. did not perform the key biochemical transformations observed in EBPR processes. The previous bias originated from the fact that a majority of the microorganisms in the activated sludge were non-culturable [18, 40]. In recent years, new attempts have been made to describe microbial communities without direct cultivation. Studies using immunofluorescence [10] and quinone profiling [14] have indicated that the numbers of *Acinetobacter* spp. are comparatively low in the EBPR sludge. Meanwhile, quinone analysis showed that members of the *Proteobacteria* beta subclasses and the *Actinobacteria* group were abundant in the EBPR system [14]. It has been shown in 16S rDNA clone library studies using the PCR (Polymerase Chain Reaction) approach and rRNA *in situ* hybridization that members of the beta subclass of *Proteobacteria* are also a major population in the EBPR system [5, 6, 11, 41]. However, conflicting results have been reported for *Actinobacteria*. According to *in situ* hybridization, a high proportion of the microbial population in the EBPR system was *Actinobacteria* [22, 41]. However, in other research, this phylogenetic group was not a major population [6, 35].

A high frequency of the *Actinobacteria* group has been mainly detected in the activated sludge when glucose has been supplied as a carbon source. The *Actinobacteria* of Gram-positive cocci with a morphology of clusters or tetrads and high G+C DNA content have also been found in activated sludge performing the EBPR, when acetate and glucose were supplied [25, 26, 27, 34]. However, these bacteria did not exhibit the characteristics consistent with the EBPR mechanism of activated sludge. On the other

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hand, Nakamura *et al.* [28] reported that *Microlunatus phosphovorus* NM1, Gram-positive cocci with high G+C DNA content, played a key role for the EBPR, when peptone and glucose were supplied. Nevertheless, the role of the *Actinobacteria* in the EBPR sludge still needs to be investigated. Glucose has been reported to be detrimental to EBPR, as it has to be first converted to short chain fatty acids by acidogenic bacteria to accomplish the EBPR [8, 23]. It can be inferred that cooperation of two kinds of bacteria, i.e. acidogenic bacteria and polyphosphate-accumulating bacteria, is necessary to accomplish the EBPR when glucose is supplied as a carbon source. Therefore, in this study, the microbial communities of the sludge were analyzed to investigate whether both of these bacteria were present at a high frequency, when the EBPR occurred in a sequencing batch reactor (SBR) supplied with glucose as a sole carbon source.

Materials and Methods

Operation of SBR

A cylindrical vessel with a 4-liter working volume was used for the SBR, and it was operated in a fill-and-draw mode with a cycle of 8 h. Microbial inoculum was obtained from an activated sludge treatment plant. Each cycle consisted of 15 min anaerobic fill, 2 h anaerobic reacting, 4 h 10 min aerobic reaction, 60 min settling, 30 min decant, and 5 min idle phase. Two liters of clarified supernatant were withdrawn at the end of the settling phase. Glucose as a sole carbon source was used at 562.5 mg per liter of synthetic wastewater. The preparation for the synthetic wastewater and SBR operation have been described elsewhere [16]. The amounts of 40 mg/l NH_4^+-N and 15 mg/l PO_4^{3-}-P were loaded into the SBR. Mean cell residence time (sludge age) was controlled to about 10 days by withdrawing sludge from the reactor at the end of the aerobic phase, and the temperature was controlled at 20°C.

Chemical Analyses

Soluble phosphate and nitrate in solution were analyzed using DX-120 ion chromatography (Dionex Co., U.S.A.), and total organic carbon (TOC) was measured with a TOC analyzer (TOC-5000A, Shimatzu Co., Japan). The mixed liquor suspended solid (MLSS) and total phosphate of the sludge were analyzed by the methods described by the American Public Health Association (APHA) [2]. The polyhydroxyalkanoic acids (PHA) and glycogen in the sludge were analyzed according to the method of Jeon and Park [16].

Electron Microscopy

Sludge samples for electron microscopic studies were collected from the reactor at the end of the aerobic stage. The sludge samples were fixed with 3% glutaraldehyde and 1% osmium tetroxide. For scanning electron microscopy (SEM), the fixed samples were dried by a critical point dryer using liquid carbon dioxide as the transition fluid. The dried samples were sputter-coated with gold under vacuum and examined with a scanning electron microscope (S-2460N, Hitachi Co., Japan). For transmission electron microscopy (TEM), the fixed samples were embedded in an epoxy resin and polymerized in an oven for 48 h at 60°C. The hardened samples were trimmed with a glass knife and sectioned to 100 nm with a diamond knife on an Ultramicrotome (MT-7000, RMC Inc., AZ, U.S.A.). The sectioned samples were stained with 0.2% uranyl acetate and lead citrate and examined with a transmission electron microscope (H-7000, Hitachi Co., Japan).

Quinone Analysis

Quinones were extracted from the sludge three times with a chloroform-methanol mixture (2:1 [v/v]), evaporated under vacuum, and re-extracted three times with n-hexane-water (1:1 [v/v]). The extracts were purified by Sep-Pak Plus Silica (Waters Co., MA, U.S.A.). Quinone components were separated and identified with an HPLC (Waters Co., MA, U.S.A.) equipped with a Waters 996 PAD (photodiode array detector), a 4.6 i.d.×250 mm ODS column (Alltech Co., IL, U.S.A.), and an IBM PC for data analysis using the Millenium program (Waters Co., MA, U.S.A.). Standard ubiquinones (Qs) and menaquinones (MKs), with respective UV spectra and extinction coefficients, were used for the identification and measurement of quinone homologues [15, 19]. Qs and MKs with n-isoprene units in their side chains are designated Q-n and MK-n, respectively. Partially hydrogenated MKs are designated MK-n (Hx), where x indicates the number of hydrogen atoms required for saturation in the side chain.

rRNA Extraction and Slot Hybridization

The total RNA from the sludge was recovered by a modified method of the Ultraspec RNA isolation kit (Biotecx Lab., TX, U.S.A.). Sludge samples were collected from the reactor by centrifugation (10,000 ×g, 3 min) at the end of the anaerobic and aerobic stages, and 10 ml of Ultraspec solution were added. The samples were resuspended and sonicated for 2 min (2 sec On; 1 sec Off; intensity 35) on ice with an ultrasonic generator (Hielscher Systems GmbH, Germany). Then, 2.4 ml of chloroform was added to the samples, and the mixtures were vigorously vortexed. The samples were placed on ice for 5 min and centrifuged at 15,000 rpm for 15 min at 4°C. Then, the crude rRNA extracts were purified by the method of Lin and Stahl [21] and treated with RNase free DNAse.

The rRNA-targeted oligonucleotides were designed with reference to several previous reports [1, 18, 32]. Sequences, target positions, and bacterial specificities of the signature

Table 1. Oligonuleotide probes, their sequences, target positions, and specificities.

Probe	Sequence (5'-3')	Target position	Specificity
EUB	GCTGCCTCCCGTAGGAGT	16S, 338-355	Eubacteria
ALF	CGTTCGYTCTGAGCCAG	16S, 19-35	α-Subclass proteobacteria, several δ-subclass proteobacteria, most spirochetes
BET	GCCTTCCCACTTCGTTT	23S, 1027-1043	β-Subclass proteobacteria
GAM	GCCTTCCCACATCGTTT	23S, 1027-1043	γ-Subclass proteobacteria
DEL	CGGCGTCGCTGCGTCAGG	16S, 385-402	Most members of δ-subclass of *Proteobacteria*, few Gram(+) bacteria
CF	TGGTCCGTGTCTCAGTAC	16S, 319-336	Cytophaga-flavobacterium cluster of most CFB-phylum bacteria
CTE	TTCCATCCCCCTCTGCCG	16S, 659-676	*Rhodocyclus purpureus, Comamonas testosteroni, Brachymonas denitrificans, Leptothrix discophora*
HGC	TATAGTTACCACCGCCGT	23S, 1901-1918	Gram (+) with high DNA G+C content
ACA	ATCCTCTCCCATACTCTA	16S, 652-669	*Acinetobacter* species
AER	CTACTTTCCCGCTGCCGC	16S, 66-83	All hitherto sequenced *Aeromonas* spp. except *A. schubertii* (1 mismatch); all other available sequences (16S and 23S) had at least 2 mismatches
PSE	GCTGGCCTAGCCTTC	23S,1432-1446	Most true *Pseudomonas* spp.
NIT3	CCTGTGCTCCATGCTCCG	16S, 1035-1048	*Nitrobacter* ssp.
Nso190	CGATCCCCTGCTTTTCTCC	16S, 190-208	Ammonia-oxidizing β-*Proteobacteria*

Y=C or T.

probes are listed in Table 1. The rRNA hybridization and dehybridization were conducted to quantify specific rRNA in the SBR sludge as described in previous papers [1, 18, 20, 32]. The total rRNA abundance was inferred by the use of a universal hybridization probe (EUB) complementary to all characterized 16S rRNA of eubacteria [36]. Hybridization signals were quantified with PhosphoImager and ImageQuant software (Molecular Dynamics, NJ, U.S.A.).

PCR Amplification and Sequencing of 16S rRNA Genes

Modification of the DNA extraction methods of Bond *et al.* [6] and Lin and Stahl [21] were used to isolate the genomic DNA from the sludge. Sludge samples were collected by centrifugation (10,000 ×g, 3 min) from the SBR at the end of the aerobic stage. Mechanical disruption of the sludge was achieved using the Mini-beadbeater (Biotecx Lab., TX, U.S.A.) as described in the above references. The effectiveness of the cell lysis procedure was confirmed by microscopic examination of samples taken before and after mechanical disruption.

Amplification of 16S rDNA was performed as described elsewhere [4]. PCR on 16S rDNA was performed using the eubacterial primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-AGAAAGGAGGTGATCCAGCC-3'). The PCR products of 16S rDNA were ligated into the pGEM-T Easy Vector System (Promega Co., WI, U.S.A.). The ligation product was transformed into XL1-Blue competent cells by electroporation using an *E. coli* pulser (Bio-RAD Lab., CA, U.S.A.). Nucleotide sequencing was performed with T7 and SP6 sequencing primers on an ABI model 377 instrument (Applied Biosystems, CA, U.S.A.).

Phylogenetic Analysis

The 35 partial (~400 bp) sequences obtained were compared with available 16S rRNA gene sequences from GenBank using the BLAST program to determine their approximate phylogenetic affiliations. Using SIMILARITY_MATRIX (version 1.1) in the Ribosomal Database Project (RDP), similarity values between 16S rRNA gene sequences were calculated [7]. The phylogenetic trees were constructed using the neighbor-joining method (Kimura 2-parameter) within the PHYLIP package from a distance matrix calculated by CLUSTAL W [33, 38]. The stability of the relationships was assessed based on a bootstrap analysis of 1,000 data sets using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE, which are all part of the PHYLIP package.

RESULTS

SBR Performance

The SBR was continuously operated for more than 18 months with glucose as a sole carbon source. Phosphate release during the anaerobic period and phosphate uptake during the aerobic period gradually increased with the operation time, and complete EBPR was accomplished after about 90 days. Profiles of other important compounds such as orthophosphate, nitrate, TOC, and PHA (the sum of 3-hydroxy butyric acid [3HB], 3-hydroxy valeric acid [3HV], 3-hydroxy-2-methyl butyric acid [3H 2MB], and 3-hydroxy-2-methyl valeric acid [3H 2MV]) are shown in Fig. 1. Glucose was not detected in the solution of SBR, however, TOC decreased slowly, suggesting that the

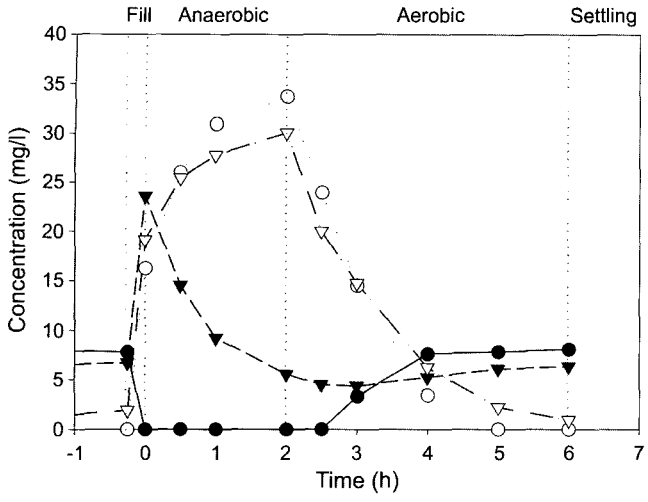

Fig. 1. Typical profiles of soluble orthophosphate P (○), nitrate N (●), TOC (▼), and PHA-C (▽) concentrations during anaerobic and aerobic reactor cycle stages.

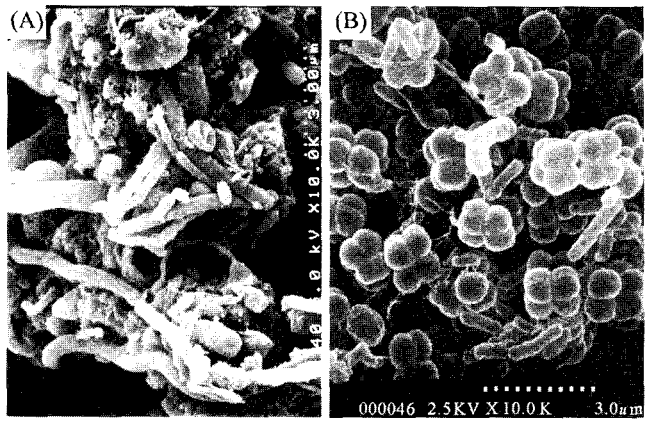

Fig. 2. Electron scanning photomicrographs (×10,000) of (A) inoculum, a mixture of coccus-, rod-, and filamentous-shaped bacteria and (B) SBR sludge, dominated by coccus-shaped microorganisms of tetrad or eight-packet cells.

glucose was converted to short chain fatty acids (SCFAs) immediately upon feeding. In the subsequent aerobic stage, PHA was nearly completely metabolized and orthophosphate decreased completely. The total phosphorus contents of the sludge at the end of the anaerobic and aerobic phases were about 3.3 and 4.9%, respectively. At the end of the aerobic phase, MLSS (about 3,600 mg/l) and the concentration profiles of phosphate and carbon compounds were relatively constant.

Electron Microscopic Analysis

The SEM images showed the presence of several morphologically distinct bacteria in the inoculum, including coccoid, rod, and long filamentous cells (Fig. 2A). However, the microbial communities were simplified after extended operation under the same conditions for more than 18 months, as shown in Fig. 2B. At this time, coccus-shaped microorganisms (0.7 μm in diameter) with mostly tetrad or cuboidal packet cells dominated the microbial sludge in the SBR. Other coccoid and rod bacteria were also present, but less frequently in the sludge. The relationship between morphological characteristics and polyphosphate accumulation was investigated by using TEM. It is a well-known fact that double staining using uranyl acetate and lead citrate highlights glycogen and PHA inclusions and makes polyphosphate granules appear black [9, 31]. The TEM images of the sludge showed that the dominating packet microorganisms did not contain polyphosphate granules or glycogen inclusions inside the cells and had a thick cell wall layer (Fig. 3A). The rod-shaped bacteria of about 0.3 μm in diameter did not contain polyphosphate granules and glycogen inclusions, either (Fig. 3B). Only the relatively large and separate coccus-shaped bacteria (about 1.2 μm diameter) contained small black polyphosphate granules and a large white inclusion (Fig. 3C). The white inclusions were presumed

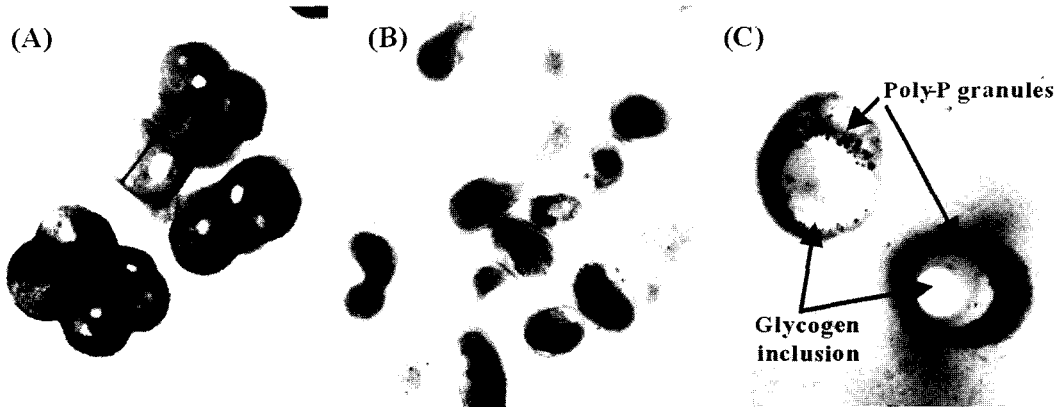

Fig. 3. Transmission electron microscopic observations of activated sludge from the SBR.
(A) Cuboidal cells (×20,000), (B) rod-shaped cells (×30,000), and (C) separate coccus-shaped cells with polyphosphate granules and a glycogen inclusion (×20,000).

to be glycogen granules, because the sludge sample for the TEM analysis, collected from the reactor at the end of the aerobic stage of the SBR cycle, had a low PHA content (Fig. 1).

Quinone Analysis

The quinone compositions of the sludge from the SBR reactor and the inoculum were determined by HPLC equipped with PAD after partial purification of the quinone extract with Sep-Pak Plus Silica, which enhanced the accuracy and reliability of the quinone analysis. The UV spectra of quinones and standard quinones were used for the identification and verification of the peaks. Figure 4 shows the HPLC elution profiles of the microbial quinones extracted from the inoculum and the SBR sludge. While the quinone extracts of the inoculum had complicated HPLC profiles, those of the SBR sludge showed relatively simple elution patterns. Among the ubiquinones of the inoculum, Q-9 (species belonging to the gamma subclass of *Proteobacteria*) and Q-10 (species belonging to the alpha subclass of *Proteobacteria*) appeared as two major peaks. However, Q-8 (species belonging to the beta subclass of *Proteobacteria* or some members of the gamma subclass) was not detected, shown in Fig. 4A. A peak was detected at a retention time similar to that of Q-8, as in Fig. 4A. However, co-injection with Q-8 standard and scanning by PAD both showed that this peak was not of quinone compounds. A few other peaks did not show typical absorption spectra of Qs or MKs by PAD, either, suggesting that they were not quinones. On the other hand, in the SBR sludge, Q-8 was the predominant type of quinone (about 68% of the total Qs on an area basis) and Q-10 the second most common component (about 32% of the total Qs).

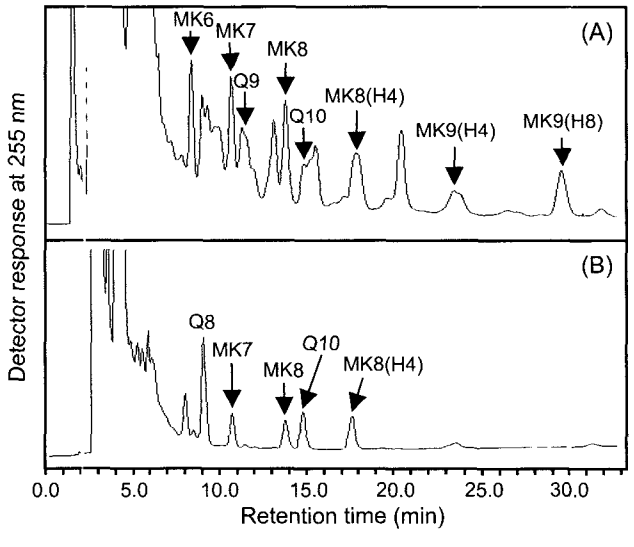

Fig. 4. HPLC elution profiles at 255 nm of microbial quinones from (A) inoculum and (B) SBR sludge.

However, Q-9 was not detected in the SBR sludge, suggesting that the gamma subclass of *Proteobacteria*, such as *Acinetobacter* ssp., and *Pseudomonas* ssp., was not the main population. The spectral analysis showed that all these Qs had an absorption maximum at 275 nm and, therefore, they were ascertained as being Qs.

Similarly, the MK patterns in the inoculum were complicated by MK-6, MK-7, MK-8, MK-8 (H4), MK-9 (H4), and MK-9 (H8). In contrast to the SBR sludge, MK-8 (H4), one of the long-chain partially saturated homologs, comprised about 41% of the total MKs as the main MK (Fig. 4B). The MK profiles showed that MK-7 and MK-8 (bacteria in the Cytophaga-*Flavobacterium* group and the *Plantomyces* group, respectively) comprised about 29% and 30% of the total MKs, respectively. All these MKs had two absorption maxima at 247 and 266 nm, and were therefore assigned as MKs.

Slot Hybridization

In our study, organism abundance was estimated from the fractional contribution of its specific rRNA molecules to the total ribosomal RNA. There were no significant differences in the abundance for the anaerobic and aerobic stages (Fig. 5). Ribosomal RNA molecules belonging to the beta subclass of the *Proteobacteria* (BET probe) were most dominant in rRNA extracted from the SBR sludge (Fig. 5). Next in abundance were the rRNAs matching HGC (complementary to a region of the 23S rRNA specific for Gram (+) bacteria with a high G+C content) and CTE probe (complementary to a region of the 16S rRNA specific for *Rhodocyclus purpureus*, *Comamonas testosteroni*, *Brachymonas denitrificans*, and *Leptothrix discophora*). However, *Acinetobacter* ssp. (ACE probe), *Aeromonas* ssp. (AER probe), and *Pseudomonas* ssp. (PSE probe), which historically have been considered to dominate phosphate-

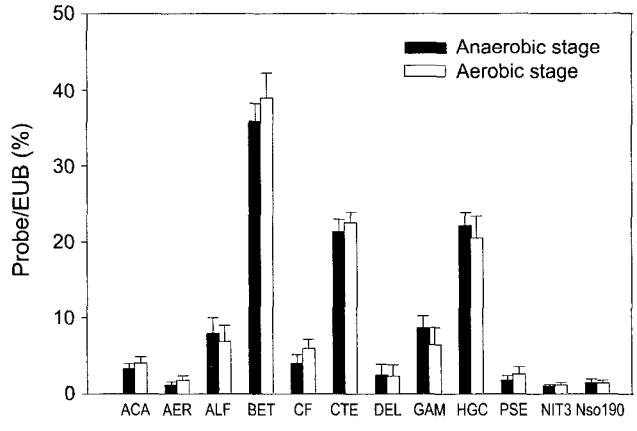

Fig. 5. Abundance of specific microbial rRNA inferred by nucleotide probe hybridization relative to total RNA extracted from sludge sampled from the SBR fed with glucose at the end of each anaerobic and aerobic period.
The bar graph represents the average of multiple hybridization experiments.

Table 2. Phylogenetic position and frequency of clones in the SBR clone library[a].

Phylum	Closest group	Identity (%)	No. of clones
Alpha subclass *Proteobacteria*	*Paracoccus* sp.	97	2
	Ochrobactrum intermedium	95	1
	Dechlorospirillum sp.	91	1
Beta subclass *Proteobacteria*	*Rhodocyclus tennus*	96	7
	Zooglea ramigera	96	2
	Alcaligenes defragrans	94	1
Actinobacteria	*Micropruina glycogenica* Lg2	97	9
	Kineococcus-like bacterium	96	1
	Terrebacter sp.	98	1
Verrucomicrobia	*Prosthecobacter* sp.	90	5
	V. spinosum	86	3
Cytophaga	*Runella* sp.	97	2

[a]A species was defined as a group of clones having >97% sequence similarity.

removing sludge populations, constituted less than 10% of the total rRNA.

16S rRNA Gene Sequencing and Phylogenetic Analysis

To obtain more detailed taxonomic information on the EBPR sludge, clone libraries of 16S rDNA were constructed from the EBPR sludge. Partial sequences of the 16S rRNA genes (about 400 nucleotides in length) were determined and compared for 35 SBR clones. Those clones that did not have >97% similarity with any other clones were considered as different species. The bacterial "species" compositions of the SBR clones are presented in Table 2. Most clones from the SBR sludge were affiliated with the beta subclass of the *Proteobacteria* and the *Actinobacteria* group. Within the beta subclass, major clones were closely assigned to *Rhodocyclus tenuis* (about 96% sequence identity). Most of the clones belonging to the *Actinobacteria* were assigned to *Micropruina glycogenica* Lg2 (about 97% sequence identity). The closest clones to the *Verrucomicrobium* group were also present with a low sequence similarity in considerable numbers. The clones of the alpha subclass of the *Proteobacteria* and Cytophaga group were also present. Representatives of these main clones were further sequenced up to more than 1,500 nucleotides in length and compared with the sequences available in GenBank. The resulting phylogenetic tree showed that the dominating clones of the *Proteobacteria* beta subclass and the *Actinobacteria* group were most closely related to *Propionivibrio limicola* GolChi1 and *Micropruina glycogenica* Lg2, respectively (Fig. 6).

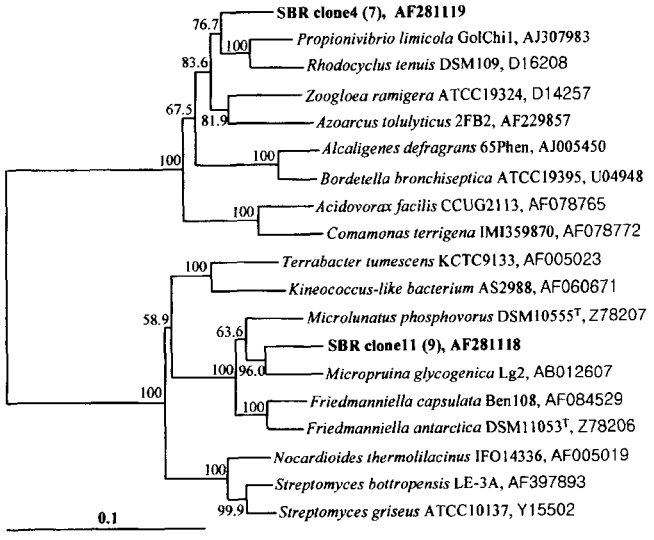

Fig. 6. Phylogenetic tree based on 16S rRNA gene sequences showing the position of the dominant clones of the beta subclass of *Proteobacteria* and the *Actinobacteria* group. Numbers in parenthesis indicate the number of clones having >97% sequence identity.

Scale bar represents 0.1 substitution per nucleotide position. Bootstrap probabilities are indicated at the branch points.

Discussion

It has been reported that members of the *Proteobacteria* were abundant and could be responsible for EBPR in the EBPR system [5, 6, 11, 14, 41]. On the other hand, conflicting results for *Actinobacteria* have been reported for the EBPR. Some researchers reported that *Actinobacteria* were the main microbial population in the EBPR system [14, 22, 41], whereas others claim the *Actinobacteria* group was not the major population [6, 35]. Some *Actinobacteria* of Gram-positive cocci with a morphology of clusters or tetrads and high in G+C content have been isolated from sludge supplied with glucose [25, 26, 27, 29, 34]. In this study, the microbial communities of the sludge were investigated by performing a complete EBPR in an SBR using a polyphasic approach incorporating electron microscopic analysis, quinone analysis, and slot hybridization and a sequencing approach to 16S rRNA genes. To clarify the conflicting results on *Actinobacteria*, glucose was supplied as a sole carbon source to the SBR.

The quinone profiles showed that Q-8 was predominant among quinones in the SBR sludge. This suggests that the beta subclass of *Proteobacteria* was the most abundant species, which is in good agreement with previous findings by other researchers [7, 11, 14, 15]. Slot hybridization and 16S rRNA gene sequencing studies also showed that the beta subclass of *Proteobacteria* contained the most dominant microorganisms. Electron microscopic analysis showed that the separate coccus-shaped microorganisms of about 1.2 μm in diameter contained small black polyphosphate granules, which implied that they are responsible for EBPR. Phylogenetic analysis revealed that the dominating clones of the *Proteobacteria* beta subclass were most closely

related to *Rhodocyclus tenuis*. Many researchers have reported that the beta subclass of *Proteobacteria*, especially the *Rhodocyclus* group, has an important role in enhanced biological phosphorus removal [6, 11, 14, 20, 40]. Therefore, it was inferred that the *Rhodocyclus* group of coccus shape within the *Proteobacteria* beta subclass was responsible for biological phosphorus removal in the SBR process supplied with glucose as a sole carbon source.

The SBR sludge also contained MK-8 (H4), one of the long-chain partially saturated homologs, as one of the main quinones, suggesting that *Actinobacteria* are one of the major phylogenetic groups. *Actinobacteria* were not found in the SBR sludge with acetate as a sole carbon source although the same inoculum was used (data not shown). Therefore, *Actinobacteria* may play some important role related to glucose metabolism for phosphorus removal. The bacteria bearing the rRNAs matching the HGC probe [complementary to a region of the 23S rRNA specific for Gram (+) bacteria with a high G+C content] were one of the dominant populations, and the *Actinobacteria* group was also detected as one of the major populations in the PCR cloning approach to 16S rDNA genes [30]. The phylogenetic analysis indicated that the dominating clones of the *Actinobacteria* group were most closely related to *Micropruina glycogenica* Lg2. Electron microscopic analysis showed that the cuboidal packet microorganisms dominated the SBR sludge, however, they did not contain polyphosphate granules or glycogen inclusions. They might be Gram-positive bacteria, because they have thick cell walls [3, 34] (Fig. 3A). Some of the *Actinobacteria* group, high G+C Gram-positive coccus-bacteria of tetrads or packet shape, have been detected in EBPR sludge [25, 26, 27, 34, 37]. However, these bacteria did not show the consistent characteristics of the EBPR mechanism of activated sludge. Therefore, it was presumed that the cuboidal packet microorganisms could be assigned to the *Actinobacteria* group.

It has been reported that glucose should first be converted to short chain fatty acids (SCFAs) by acidogenic bacteria (AB) for the EBPR by polyphosphate-accumulating organisms (PAO), because it is by itself detrimental to EBPR [8, 23]. Jeon and Park [16] also suggested that, when glucose was supplied as the sole carbon source, the EBPR was accomplished by the cooperation of two kinds of microorganisms; AB, especially lactate-producing organisms, and PAO. It can be inferred that two kinds of organisms, i.e. AB and PAO, are needed for the EBPR with glucose as a carbon source. Therefore, it can be claimed that the packet bacteria (*Actinobacteria*) and the separate coccus-shaped bacteria (the beta subclass of *Proteobacteria*) converted glucose to SCFAs and accumulated polyphosphate, respectively, and finally that the EBPR was achieved by their cooperation in the present study on the SBR operation with glucose.

For a complete understanding of the EBPR mechanisms, more definitive identification remains to be established as well as identification of the physiological characteristics of the beta subclass of *Proteobacteria* and *Actinobacteria*. Only pure cultures of the microorganisms in the EBPR process would provide a basis for the understanding of their characteristics and the EBPR mechanisms.

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