Characterization of the High-Density Bacteria in Biological Phosphorus Removal Process by using Buoyant Density Separation

TAKASHI KONDO¹, YOSHITAKA EBIE², SATOSHI TSUNEDA¹, and YUHEI INAMORI²

¹ Department of Chemical Engineering, Waseda University
³ 4–3–4–1 Ohkubo, Shinjuku, Tokyo 169–8555, Japan
² Research Center for Material Cycles and Waste Management, National Institute for Environmental Studies/16–2 Onogawa, Tsukuba, 305–8506, Japan

Abstract

To evaluate the effectiveness of the separation of polyphosphate-accumulating organisms (PAOs) and glycogen accumulating organism (GAOs)/G-bacteria from the phylogenetically high-diverse activated sludge using the buoyant density separation, activated sludge was collected from the acetate-fed SBR and the microbial community was determined. The activated sludge sample collected at the end of the aerobic phase was subjected to the buoyant density separation. The microbial communities of separated sludge were fingerprinted by terminal restriction fragment length polymorphism (T-RFLP) analysis. The microbial community analysis exhibited that Candidatus ‘Accumulibacter phosphatis’ known as PAOs, Defluvicoccus-relative G-bacteria and the group GB known as GAOs and some organisms playing an important role for nitrogen removal existed in the SBR. The T-RFLP analysis for each fraction of the buoyant density separation revealed that Cand. ‘Accumulibacter phosphatis’ was selectively concentrated in the high-density fraction. Although some unidentified peaks were also the prominent, Defluvicoccus vanus-relative G-bacteria were failed to concentrate. The unique T-RF derived from organisms including the group GB was the prominent in the high-density fraction. The Acidobacteria, the Bacteroidetes and the Nitrospira were disappeared in the high-density fraction. Even though some bacteria related to phosphorus removal were eliminated, most of bacterial strains which do not play an important role for phosphorus removal could be eliminated by the buoyant density separation.

Keywords: 16S rRNA gene; buoyant density separation; enhanced biological phosphorus removal (EBPR); polyphosphate accumulating organisms (PAOs); terminal restriction fragment length polymorphism (T-RFLP)

INTRODUCTION

Enhanced biological phosphorus removal (EBPR) processes such as anaerobic-anoxic-oxic (A2O) are widely used to remove inorganic phosphate (Pi) from wastewater. EBPR process essentially contains the first anaerobic and the subsequent aerobic period. In initial anaerobic period, polyphosphate-accumulating organisms (PAOs) assimilate volatile fatty acids (VFAs) such as acetate with releasing Pi, and transform VFAs into polyhydroxy-alkanoates (PHA)¹,². In the subsequent aerobic period, PAOs accumulate large amount of Pi as polyphosphate (PolyP) in excess of released Pi. PolyP is broken down for serving as an energy source¹,².

Recent studies using culture-independent molecular techniques demonstrated that members of the phylogenetically defined
Rhodocyclus-related PAO (Candidatus ‘Accumulibacter phosphatis’) and Actinobacterial PAO (APAO) play an important role for phosphorus removal in EBPR processes. On the other hand, some bacteria called glycogen-accumulating organisms (GAOs) and/or G-bacteria, which have a distinctive morphology of cocci in tetrads, sheets or clusters, had become dominant in the deteriorated EBPR. Culture-independent molecular techniques defined that GAOs belong to the members of the group GB in the Gammaproteobacteria and G-bacteria belong to the members of Defluvicoccus vanus in Alphaproteobacteria.

The characterizations of PAOs and GAOs/G-bacteria are still necessary because the water profile and/or the staining for intercellular compounds indicated the existence of these bacteria. One promising approach to effectively separate and to enrich PAOs and GAOs/G-bacteria is to use buoyant density separation based on the biochemical model of PAOs, which accumulates high-density materials such as PolyP, PHA and glycogen.

In this study, effectiveness of the separation of PAOs and GAOs/G-bacteria from the phylogenetically high-diverse activated sludge using the buoyant density separation was evaluated with cloning and terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene.

MATERIALS AND METHODS

Sequencing batch reactor design and operation The sequencing batch (SBR) has 4.7-l working volume and was operated with a cycle of 8 h, consisting of a filling period (15 min), an anaerobic period (90 min), an aerobic period (300 min), a settling period (60 min), and withdrawing period (15 min). In the withdrawing period, 2,350 ml of the supernatant was withdrawn and 2,350 ml fresh synthetic wastewater was added to each reactor in the next filling period. The hydraulic retention time (HRT) was adjusted to 16 h. Synthetic wastewater contained CH₃COONa as a sole organic carbon source, 384 mg l⁻¹; KH₂PO₄, 65.9 mg l⁻¹; (NH₄)₂SO₄, 142 mg l⁻¹; MgSO₄·7H₂O, 101 mg l⁻¹; CaCl₂·2H₂O, 14.7 mg l⁻¹ and mineral salt solution, 1.5 ml l⁻¹. The sludge retention time (SRT) was adjusted to 20 days by withdrawing 235 ml of mixed liquor at the last 10 min in the aerobic stage once per day. MLSS was maintained to 2,600 mg l⁻¹ and temperature was adjusted at 20±1°C. The reactor was seeded with an activated sludge obtained from an EBPR plant operating on municipal wastewater at Tokyo (Japan).

Water quality analysis. Water quality analysis was performed according to standard methods. Dissolved ammonium, orthophosphate, nitrite and nitrate were analyzed by Traacs 2000 (Bran+Luebbe K. K., Japan) after filtration with 0.2 μm filters (Millipore, United States). Soluble TOC was measured by TOC–5000A (SHIMADZU, Japan). MLSS and the phosphorus content in biomass at the end of the aerobic period were also determined using standard methods.

Diversity and phylogenetic analysis. A sludge sample was collected from the SBR at the end of the aerobic phase. Total DNA was extracted from the sample using Isoplant (Nippon Gene, Japan) according to the manufacturer’s instructions. The 16S rRNA genes were amplified by PCR amplification by using universal primers, 341f and 907r. The PCR mixture contained 0.5 mM of each primer, 200 mM of dNTP, 1.0 mM MgCl₂, 1.25 U of Takara Ex Taq DNA polymerase (TAKARA BIO, Japan), 5 μl of 10× PCR Ex Taq buffer, and sterile water added to a final volume of 50 μl. The PCR amplifications were conducted in a model 9700 thermal cycler (Applied Biosystems, USA) using the following protocol: 1 min at 94°C, 25 cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C), 1 min at 72°C. The presence of PCR product was confirmed by 2% agarose gel electrophoresis and the subsequent staining of the gels with ethidium bromide. PCR products purification, cloning, plasmid DNA preparation and sequencing with an ABI PRISM 3100-Avant DNA Sequencing system (Applied Biosystems, Japan) were performed as described previously. A database search was conducted using BLAST from the DDBJ (http://www.ddbj.nig.ac.jp/search/blast-e. html). The sequences determined in this
study and those retrieved from the database were aligned using Clustal W. Phylogenetic trees were constructed using Clustal W and Tree View by the neighbor-joining method.

**Buoyant density separation.** Sludge sample was collected from the SBR at the end of the aerobic phase. Sixty-four milliliter of each activated sludge sample was concentrated to 16 ml by centrifuge (5,000 × g, 10 min) (MX–300; TOMY SEIKO, Japan). Concentrated sludge samples were dispersed by ultrasonic disrupter (UR–20P; TOMY SEIKO, Japan) with the intensity 5 for 10 min and divided into four 15 ml tubes (4 ml for each tube). Six ml of Percoll (Amersham Bioscience Co., USA) was added the tubes, and then the tubes were centrifuged (5,000 × g, 60 min). After centrifugation, 4 ml of the sample was collected from the bottom of the tubes and referred as the high-density fraction (HD). The residual of the tubes was also collected and referred as the low-density fraction (LD). These fractions were washed 3 times with sterile saline solution (NaCl, 8.5 g l⁻¹).

**T-RLP analysis.** After the buoyant density separation, total DNA of HD, LD and the original sludge (OS) was extracted and microbial communities of each sample were fingerprinted by T-RLP analysis. T-RLP analysis was performed as described previously. In brief, PCR amplification was performed using FAM-labeled primer 341f and non-labeled primer 907r using the same procedure described above. Fluorescently labeled PCR products were purified and aliquots of purified PCR products were cleaved for 4 h in a water bath at 37°C with two different restriction endonucleases in digestions with a single tetrameric enzyme each for bacterial 16S rRNA genes, MspI [C‘CGG] (where the prime shows the site of cleavage) and CfoI (an isoschizomer of HhaI) [GCG‘C] (Promega, USA). Aliquots of the digest were purified by ethanol precipitation. The precipitate was mixed with 0.5 μl of GeneScan–500 size standard (Applied Biosystems) and 15 μl deionized formamide. After denaturing the DNA at 94°C for 5 min and immediate chilling on ice, the fluorescently labeled terminal fragments were analyzed by electrophoresis on an ABI 3100 Avant Genetic Analyzer automated sequence analyzer (Applied Biosystems) in GeneScan mode as described previously. Signals with a peak area contribution below 1% were regarded as background noise and excluded from analysis. The relative abundance of terminal fragments was determined by calculating the ratio between the peak height of each peak and the total peak height of all peaks within one sample. Lengths of predominant bacterial 16S rRNA gene terminal fragments were theoretically compared with the clone library generated in this study using GENETYX-MAC software (Genetyx, Japan).

**RESULTS**

**Performance of the sequencing batch reactor.** The SBR was operated for more than 2 years after inoculation of activated sludge originating from an EBPR plant. The profile of water quality in one cycle showed a typical EBPR profile, with a rapid carbon consumption and phosphorus release by PAOs during an anaerobic stage and luxury phosphorus uptake during the subsequent aerobic stage (Fig.1). Sufficient nitrogen removal was also achieved by nitrifying and denitrifying bacteria. Phosphorus content of sludge was kept from 6 to 8 % (mg-P mg⁻¹–MLSS) for two years.

![Fig. 1 The profile of soluble carbon, nitrogen and phosphorus concentrations in SBR](image-url)
**16S rRNA gene diversity.** To investigate the bacterial community structure in the SBR, 16S rRNA gene was amplified with a universal primer set, and then cloned and sequenced. A total of 60 clones were obtained and their 16S rRNA gene sequences were analyzed. Most clone sequences were affiliated with the Betaproteobacteria (35 clones), followed by the Bacteroidetes (11 clones), and the Gammaproteobacteria (6 clones) (Table 1).

The affiliations of these molecular isolates are depicted in the phylogenetic trees as shown in Fig. 2 a–c. Most of the clones belonging to the Betaproteobacteria were affiliated with the family Rhodocyclaceae; twenty-six clones were affiliated with Cand. ‘Accumulibacter phosphatis’ (Rhodocyclus sp., AJ224937), indicating the prominence of Cand. ‘Accumulibacter phosphatis’ in the SBR. The other clones belonged to the genus Zoogloea, the genus Dechloromonas and the genus Thauera, which have been reported as the representatives of acetate-utilizing denitrifying bacteria (Fig. 2a). One clone was affiliated with the genus Nitrosomonas, representative of ammonia-oxidizing bacteria. The genus Malitia, which was recently isolated from activated sludge as a polyP and PHA accumulating bacteria, was also detected in the SBR (SBR-79). The PAOs competitors, the group GB belonging to the Gammaproteobacteria (AF361090-3) and the Defluvitococcus-relative G-bacteria belonging to the Alphaproteobacteria (DQ146465 and DQ146467), were detected in the SBR (Fig. 2b).

Eleven clones belonged to the phylum Bacteroidetes (Fig. 2c). All of these belonged to the order Sphingobacterales. None of these sequences were closely related to the type strain, however, these sequences were related to the sequences obtained from the acetate-fed SBRs by Dabert et al. (AF314419, AF314421, AF314424 and AF314433) and McMahon et al. (AF502207 and AF502208). Other clones belonged to the following phylogenetic lineage: phylum Nitrospira, three clones; phylum Acidobacteria, one clone (Fig. 2c).

**Table 1** Number of the clones obtained in this study.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaproteobacteria</td>
<td></td>
</tr>
<tr>
<td>Rhodospirillaceae</td>
<td>3</td>
</tr>
<tr>
<td>unclassified Alphaproteobacteria</td>
<td>1</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td></td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>1</td>
</tr>
<tr>
<td>Nitrosonomadaceae</td>
<td>1</td>
</tr>
<tr>
<td>Rhodocyclusae</td>
<td>33</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td></td>
</tr>
<tr>
<td>unclassified Gammaproteobacteria</td>
<td>6</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td></td>
</tr>
<tr>
<td>Sphingobacterales</td>
<td>10</td>
</tr>
<tr>
<td>unclassified Bacteroidetes</td>
<td>1</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>1</td>
</tr>
<tr>
<td>Nitrospira</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
</tr>
</tbody>
</table>

**Buoyant density separation and T-RFLP analysis.** After the buoyant density separation, the sludge was clearly separated into the two parts; the high-density fraction (HD) concentrated at the bottom of the tubes and the low-density fraction (LD) floated on the surface. Total DNA of HD, LD and the original sludge (OS) was extracted and microbial communities of each sample were fingerprinted by T-RFLP analysis.

No difference in the number of unique T-RFs between OS and LD was confirmed (Fig.3), and the microbial communities in OS and LD were almost the same. On the other hand, the number of unique T-RFs was significantly decreased in HD (Fig.3). It exhibited that some bacterial strains were selectively concentrated in HD. In the case of MspI digestion, eight predominant peaks were confirmed in OS and LD: 72-bp, 103-bp, 112-bp, 138-bp, 158-bp, 163-bp, 209-bp and 274-bp T-RFs whereas only three T-RFs, 72-bp, 163-bp and 274-bp, were confirmed in the HD. For the CfoI digestion, 31-bp, 40-bp, 74-bp, 122-bp, 212-bp, 241-bp, 560-bp and 580-bp T-RFs were predominant in OS and LD. The peaks of 560-bp and 580-bp T-RFs were derived from undigested fragments. In HD, most of these undigested fragments were disappeared and only two T-RFs, 40-bp and 241-bp T-RFs, were the prominent.
Fig. 2 Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the Betaproteobacteria (a), Alpha- and Gammaproteobacteria (b), Bacteroidetes and other bacteria (c). The trees were constructed using the neighbor-joining method. Genetic distances were calculated using Kimura's two-parameter method. The number on the nodes indicates the number of times the species (shown on the right) grouped together in 1000 bootstrap samples. Bootstrap values below 800 are not shown. The root of the tree was determined using the 16S rRNA gene of Methanosarcina mazei (AB065295) as an outgroup. The theoretical length of terminal restriction fragments in \textit{in silico} analysis is shown on the gray box. Scale bar indicates the 10% estimated difference in nucleotide sequence position. Clones obtained in this study are in boldface. The number of clones obtained is shown in parentheses.
Identification of the organisms concentrated in the high-density fraction. To identify the organisms concentrated in the high-density fraction (HD), sequences of 16S rRNA genes obtained were analyzed in silico with respect to MspI and CfoI restriction sites. Rich et al. reported that individual T-RRFs differed in size by an average of 1.5-3 bp over a range of 0.3-5.1 bp. In this study, a gap of less than 5 bp was found acceptable for the identification of the predominant T-RRFs.

The results of the identification of the T-RRFs in HD are shown in Table 2. In the case of MspI digestion, the 163-bp T-RRFs represented the clones affiliated with the relatives of the genus Nitrosococcus, the group GB belonging to the Gammaproteobacteria, Malikia sp., Zoogloea sp. and Cand. ‘Accumulibacter phosphatis’ belonging to the Betaproteobacteria. The 274-bp T-RRF was generated by only Nitrospira sp. Unfortunately, the 72-bp T-RRF, one of the predominant peaks, was not identified by the estimated T-RRFs with in silico analysis. On the other hand, the disappeared peaks were identified as follows. The peaks of 209-bp and 158-bp T-RRFs, represented the Bacteroidetes. The Alphaproteobacteria (136- or 138-bp T-RRF) including the Defluvicoccus-relative G-bacteria, The Acidobacteria (139-bp T-RRF), and SBR-02 (243-bp T-RRF) belonging to the Gammaproteobacteria, Dechloromonas sp. (103-bp T-RRF) and Thauera sp. (103-bp T-RRF) were also not concentrated in HD.

As for CfoI digestion, 241-bp T-RRF, one of the predominant peaks in HD, represented the clones SBR-33 and 37 affiliated with Dechloromonas sp. and SBR-09, 33 and 37 affiliated with Cand. ‘Accumulibacter phosphatis’. Dechloromonas sp. was judged to be not concentrated in HD according to the results of MspI digestion (Table 2). Therefore, this T-RRF represented only Cand. ‘Accumulibacter phosphatis’. The relative abundance of this T-RRF was increased from 18% to 50%. All the clones, which generate 163-bp T-RRF with MspI digestion except for the clones SBR-33 and 37, generate 40-bp T-RRF with CfoI digestion and the further distinction of these bacterial strains was impossible. On the other hand, the 31-bp T-RRF derived from Nitrospira sp. was not confirmed in HD.

Fig. 3 Bacterial community structure in each fraction as determined by T-RFLP analysis using the restriction enzymes MspI(a) and CfoI(b).
although the 274-bp T-RF with MspI digestion was confirmed in HD. These results indicated that *Nitrospira* sp. was not concentrated in the HD, and the 274-bp T-RF with MspI digestion must represent the other unidentified bacterial strain. The T-RF derived from the *Alphaproteobacteria* (the 212-bp T-RF), the *Acidobacteria* (the 31-bp T-RF) and the *Bacteroidetes* (the 79-bp, 125-bp and undigested T-RFs) was not confirmed in the HD as same as the results of MspI digestion.

### DISCUSSION

For the selective concentration of PAOs and GAOs/G-bacteria, we focused on the buoyant density separation. The EBPR sludge sample for the evaluation of the effectiveness of the buoyant density separation was collected from the acetate-fed SBR with efficient phosphorus removal (Fig.1). The microbial community analysis by cloning exhibited that *Cand.* ‘Accumulibacter phosphatis’ was the predominant in this EBPR process. The competitor for carbon uptake in the anaerobic phase, the group GB and the
Defluvicoccus vanus-relative G-bacteria also existed in the SBR. The uncultured bacteria affiliated with the Bacteroidetes, which were detected in this reactor, were closely related to the sequences obtained in the acetated-fed SBR by Dabert et al. and McMahon et al. Nitrospira sp., Nitrosomonas sp., Zoogloea sp., Thauera sp. and Dechloromonas sp. which play an important role for nitrogen removal were observed. These results revealed that the microbial community in this reactor consisted of the bacterial members which generally exist in the EBPR processes.

The sludge sample, collected at the aerobic phase, was subjected to the buoyant density separation, and then the microbial communities in each fraction were fingerprinted by T-RFLP analysis (Fig.3). The number of peaks in the HD was lower than that in the OS and LD, indicating the selective concentration of some bacterial strains. To identify the organisms concentrated in the HD, sequences obtained were analyzed in silico with respect toMspI and CfoI restriction sites. In this study, most of the sequences affiliated with the Betaproteobacteria and the Gammaproteobacteria generated the same T-RFs: 103-bp and 163-bp T-RFs with MspI digestion; 40-bp and 241-bp with CfoI digestion. Therefore, the low-resolution of T-RFLP analysis was obtained for the Betaproteobacteria and the Gammaproteobacteria in this study.

The relatives of the genus Nitrospira, the group GB belonging to the Gammaproteobacteria and Malhia sp., Zoogloea sp. and Cand. ‘Accumulibacter phosphatis’ belonging to the Betaproteobacteria were judged to be concentrated in the HD. However, these organisms except for a part of Cand. ‘Accumulibacter phosphatis’ generated the same T-RFs with both MspI and CfoI digestion. Therefore, it was not clear which bacterial strain was concentrated in the HD. SBR-09, SBR-33 and SBR-37 affiliated with Cand. ‘Accumulibacter phosphatis’ generated 241-bp T-RF with CfoI digestion. It revealed that Cand. ‘Accumulibacter phosphatis’ was the prominent in the HD. The relative abundance of this T-RF was increased to 50 % in the HD of the sludge sample collected at the end of the aerobic phase. The 40-bp T-RF with the CfoI digestion, generated by the other sequences affiliated with Cand. ‘Accumulibacter phosphatis’ (SBR-05, SBR-21, SBR-27, SBR-40 and SBR-43), was also prominent in the HD, then the relative abundance of Cand. ‘Accumulibacter phos-phatis’ should be higher than 50%. The 72-bp and the 274-bp T-RFs with the MspI digestion were not affiliated by the in silico analysis. This is due to some unavoidable bias associated with cloning in Escherichia coli and/or the underestimation of the limited number of rRNA gene clones sequenced. Anyway, these results suggested that Cand. ‘Accumulibacter phosphatis’ and some unidentified bacterial strains were selectively concentrated by the buoyant density separation in this study.

On the other hand, the Defluvicoccus vanus-relative G-bacteria were not concentrated in the HD whereas Meyer et al. reported that this organism became the prominent in the propionate-fed SBR with no phosphorus removal and accumulated PHA, which is the high-density compound. Defluvicoccus-relative G-bacteria have been seen in the sludge appearing cocci in package of tetrad and sometimes called tetrad forming organisms (TFOs). This morphology, different from cocci- and rod-shape PAOs and GAOs, might affect the buoyant density separation. Using a pure culture strain, Nishino et al. reported that the effectiveness of the buoyant density was changed with the physiological states of the bacteria. In this reactor, efficient phosphorus removal maintained more than 2 years without unidentified deterioration described elsewhere. Therefore, PAOs in this study must have maintained the high growth activity and it resulted in the prominence in this reactor. On the other hand, the low detection frequency for the clone library suggested that GAOs/G-bacteria maintained a little population. This difference in activity among each bacterial strain should also affect the effectiveness of the buoyant density separation but it is impossible to make the adequate explanation for this phenomenon.

The T-RFs derived from the clones affiliated with the Acidobacteria, the Bacteroidetes and the Nitrospira, which are not related to
PAOs nor GAOs/G-bacteria candidates, were disappeared in the HD. Therefore, bacteria which play unimportant role for phosphorus removal could be eliminated by the buoyant density separation, however, some important bacteria, such as the Defluvicoccus vanus-relative G-bacteria, were also eliminated in this study.

The buoyant density separation for the concentration of PAOs has been performed in several studies16–22). Hung et al.19) reported PAOs as determined by polyP staining were enriched from 14% to 43–48% in the high-density fraction. The effectiveness of concentration of PAOs is the same as this study when all the polyP-positive cells in Hung et al.19) were considered as Cand. ‘Accumulibacter phosphatis’. Their further molecular analysis by cloning of the high-density fraction revealed that only 6 clones of total 70 clones were affiliated with Cand. ‘Accumulibacter phosphatis’22) but further information of the other clones are not available because their sequences have not been submitted in database. Therefore, the comparison of other clones in high-density fraction could not be made.

The buoyant density separation condition in their study19) was different from that in this study. According to their protocol, sludge samples collected from full-scale EBPR plants were added to centrifuge tubes containing a 20% Percoll and centrifuged for 60 min at 32,000 × g19). On the other hand, the concentration of Percoll was higher (60% Percoll, final concentration) and the centrifugation force was quite lower (5,000 × g) in this study. These differences in the separation condition were considered to affect the results. The buoyant density separation condition in this study was almost the same as reported by Schuler et al. 21). According to their procedures, activated sludge samples collected from full-scale EBPR plants and acetate-fed SBR were added in 45% Percoll solution, then centrifuged for 4 min at 4,000 rpm. Subsequently, small pellet was resuspended in 60% Percoll and centrifuged. The operational condition for separation was almost the same while the centrifugation time was different.

However, the results of microbial community analysis were different. They analyzed the microbial community in the high-density fraction by PCR-DGGE analysis21). The DGGE-bands with greater intensity in the high density fraction than the low-density fraction or the original sludge sample were considered as PAOs candidates and were sequenced. As a result, 5 of 12 PAOs candidates were affiliated with the Gammaproteobacteria and 2 of them were related to the sequences identified in the deteriorated EBPR by Nielsen et al.17). The sequences identified by Nielsen et al.17) are grouped into the group GB. Only one PAOs candidate was affiliated with the Betaproteobacteria, however, it was uncertain whether this candidate was related to Cand. ‘Accumulibacter phosphatis’ or not because of the absence of the sequence in database. The simple explanation for this difference in the results of microbial community analysis in the high-density fraction could not be made, but it might be caused by the differences in the microbial community and the activity in each bacteria derived from the differences in the reactor operational conditions.

In this study, Cand. ‘Accumulibacter phosphatis’ known as PAOs, the group GB and Defluvicoccus-relative G-bacteria known as GAOs/G-bacteria were identified in the SBR. Some bacterial strains were separated in the HD by the buoyant density separation. Judging from the relative abundance of the 239-bp T-RF with CfoI digestion, Cand. ‘Accumulibacter phosphatis’ was concentrated from 18% to 50% by the buoyant density separation. Some unidentified bacterial strains were also concentrated in the HD. On the other hand, Defluvicoccus vanus-relative G-bacteria was failed to concentrate by unknown reasons. The Acidobacteria, the Bacteroidetes and the Nitrospira, which are not related to PAOs nor GAOs/G-bacteria candidates, were disappeared in the HD. Even though some bacteria related to phosphorus removal were eliminated, most of bacterial strains which do not play an important role for phosphorus removal could be eliminated by the buoyant density separation.

The buoyant density separation requires only the centrifuge which was equipped in
most laboratory and Percoll. Moreover, after separation, each fraction can be re-cultured, and then it could be subjected to the further analysis such as isolation, ecophysiology analysis and so on36, 37). However, the concentration effectiveness by the buoyant density separation is still not clear and further studies for estimation is necessary.

REFERENCES


16) Meyer RL, Saunders AM, and Blackall LL: Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing. Microbiology-SGM 152,


34) McMahon KD, Dojka MA, Pace NR, Jenkins D, and Keasling JD: Polyphosphate Kinase from Activated Sludge Performing


   (Submitted 2006. 10. 6)
   (Accepted 2006. 12. 5)