Chapter 4

Characterization of the high-density bacteria in biological phosphorus removal process by using buoyant density separation

SUMMARY

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, which are not related to PAOs nor GAOs/G-bacteria candidates, were disappeared in 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.

4.1 INTRODUCTION

Enhanced biological phosphorus removal (EBPR) processes such as anaerobic-anoxic-oxic (A_2O) 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 polyhydroxyalkanoates (PHA) (Mino *et al*., 1998; Seviour *et al*., 2003). 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 source (Mino *et al*., 1998; Seviour *et al*., 2003).

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 (Crocetti *et al*., 2000; Hesselmann *et al*., 1999; Kong *et al*., 2004, 2005; Liu *et al*., 2001). On the other hand, some bacteria called glycogen-accumulating organisms (GAOs)/G-bacteria had become dominant in the deteriorated EBPR (Cech and Hartman, 1993; Mino *et al*., 1998; Seviour *et al*., 2000, 2003). 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* (Beer *et al*., 2004; Crocetti *et al*., 2002; Kong *et al*., 2001, 2002a, 2002b, 2006; Meyer *et al*., 2006; Nielsen *et al*., 1999; Wong *et al*., 2004).

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 (Hung *et al*., 2002; Shuler *et al*., 2000,

2002; Zilles *et al*., 2002).

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.

4.2 MATERIALS AND METHODS

4.2.1 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 CH_3COONa as a sole organic carbon source, 384 g/m^3; KH_2PO_4, 65.9 g/m^3; $(NH_4)_2SO_4$, 142 g/m^3; $MgSO_4 \cdot 7H_2O$, 101 g/m^3; $CaCl_2 \cdot 2H_2O$, 14.7 g/m^3 and mineral salt solution (Smloders *et al*., 1994), 1.5 mL/L. 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).

4.2.2 Water quality analysis

Water quality analysis was performed according to standard methods (APHA, 1992). 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 (APHA, 1992).

4.2.3 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 (Muyzer *et al*., 1993). The PCR mixture contained 0.5 mM of each primer, 200 mM of dNTP, 1.0 mM $MgCl_2$, 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 94ºC, 25 cycles (60 s at 94ºC, 60 s at 55ºC, 60 s at 72ºC), 1 min at 72ºC. 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 sequeincing with an ABI PRISM 3100-Avant DNA Sequencing system (Applied Biosystems, Japan) were performed as described previously (Osaka *et al*., 2006). A database search was conducted using BLAST from the DDBJ. The sequences determined in this study and those retrieved from the database were aligned using Clustal W (Thompson *et al*. 1994). Phylogenetic trees were constructed using Clustal W and Tree View (Page 1996) by the neighbour-joining method (Saitou and Nei 1987).

4.2.4 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 (MX-300; TOMY SEIKO, Japan). Concentrated sludge samples were dispersed by ultrasonic disrupter (UR-20P; TOMY SEIKO) 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 (8.5 g L^{-1} NaCl).

4.2.5 T-RLP analysis

T-RFLP analysis was performed as described previously (Hoshino *et al*., 2005). 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 37ºC with two different restriction endonucleases in digestions with a single tetrameric enzyme each for bacterial 16S rRNA genes, *Msp*I [C'CGG] (where the prime shows the site of cleavage) and *Cfo*I (an isoschizomer of *Hha*I) [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 94ºC 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 (Lukow *et al*., 2000). 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).

4.3 RESULTS

4.3.1 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-MLSS) for two years.

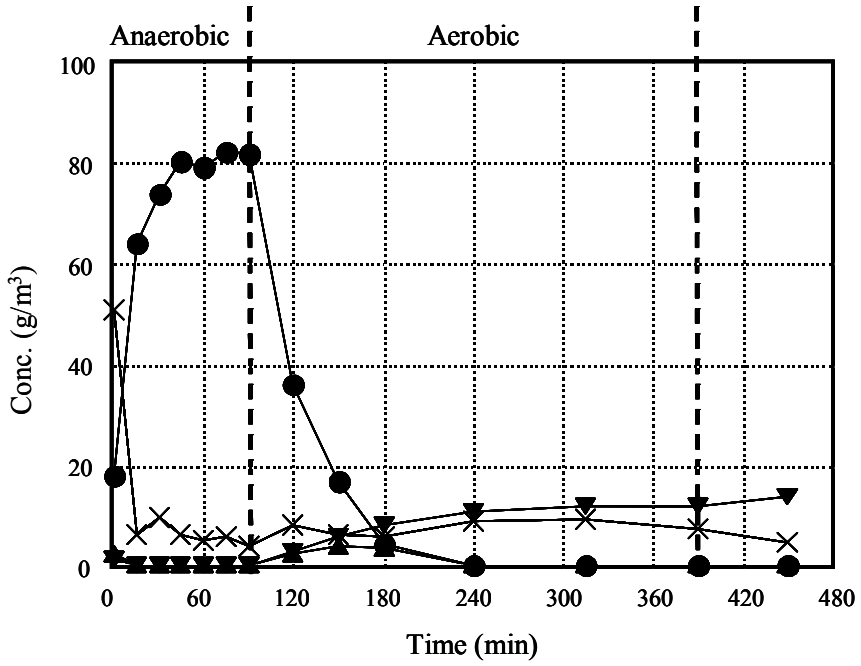

Fig. 1 The profile of soluble carbon, nitrogen and phosphorus concentrations in SBR.(× S-TOC, ▼ NO_3-N, ▲ NO_2-N and ● PO_4-P).

4.3.2 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) (Table1).

Table 1 Number of the clones obtained in this study

Group	No. of clones
Alphaproteobacteria	
Rhodospirillaceae	3
unclassified *Alphaproteobacteria*	1
Betaproteobacteria	
Comamonadaceae	1
Nitrosomonadaceae	1
Rhodocyclaceae	33
Gammaproteobacteria	
unclassified *Gammaproteobacteria*	6
Bacteroidetes	
Sphingobacteriales	10
unclassified *Bacteroidetes*	1
Acidobacteria	1
Nitrospira	3
Total	60

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 (Osaka *et al*., 2006) (Fig.2 a). One clone was affiliated with the genus *Nitrosomonas*, representative of ammonia-oxidizing bacteria. The genus *Malikia*, which was recently isolated from activated sludge as a polyP and PHA accumulating bacteria, was also detected in the SBR (SBR-79) (Spring *et al*., 2005). The PAOs competitors, the group

GB belonging to the *Gammaproteobacteria* (AF361090-3) and the *Defluvicoccus*-relative G-bacteria belonging to the *Alphaproteobacteria* (DQ146465, DQ146467), were detected in the SBR (Fig.2b).

Eleven clones belonged to the phylum *Bacteroidetes* (Fig.2c). All of these belonged to the order *Sphingobacteriales*. 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) (Dabert *et al*., 2001; McMahon *et al*., 2002). Other clones belonged to the following phylogenetic lineage: phylum *Nitrospira*, three clones; phylum *Acidobacteria*, one clone (Fig. 2c).

(a)

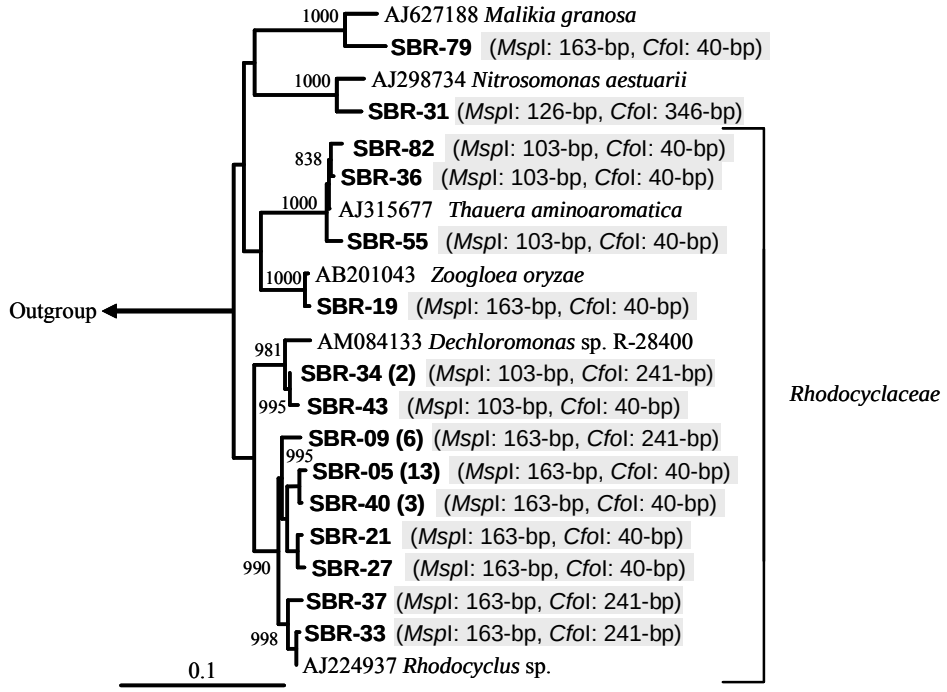

Fig. 2 Phylogenetic dendrogram based on 16S rRNA gene sequences in relation to members of the *Betaproteobacteria* (a), *Alpha-* and *Gammaproteobacteria* (b), Bacteroidetes and other bacteria (c). The trees were constructed using the neighbor-joining method. Genetic distances were calculated using Kimura's 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 *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.

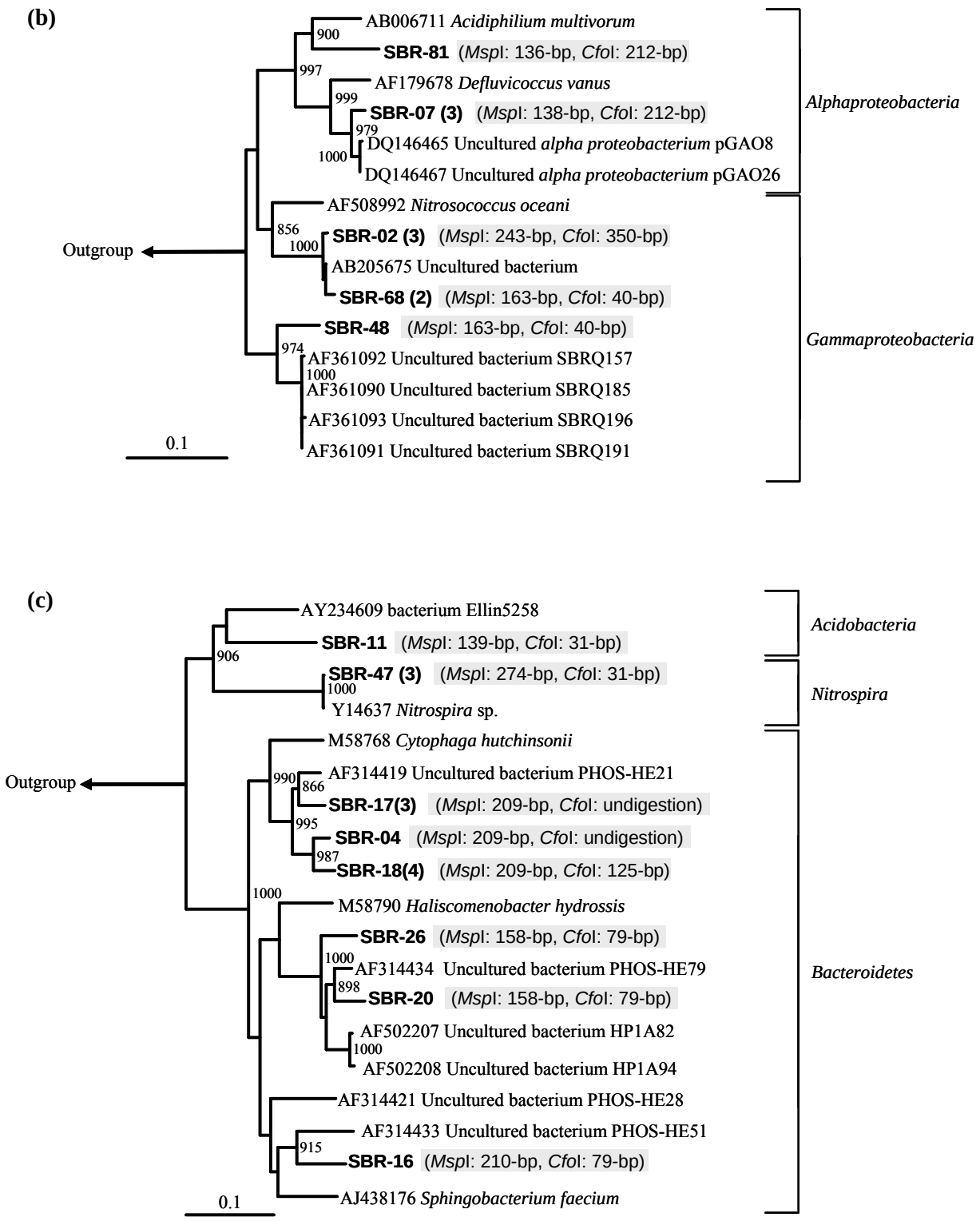

Fig. 2 continued.

4.3.3 Buoyant density separation and T-RFLP analysis

Sludge sample was collected from the SBR at the end of the aerobic phase, and subjected to the buoyant density separation procedure. 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.

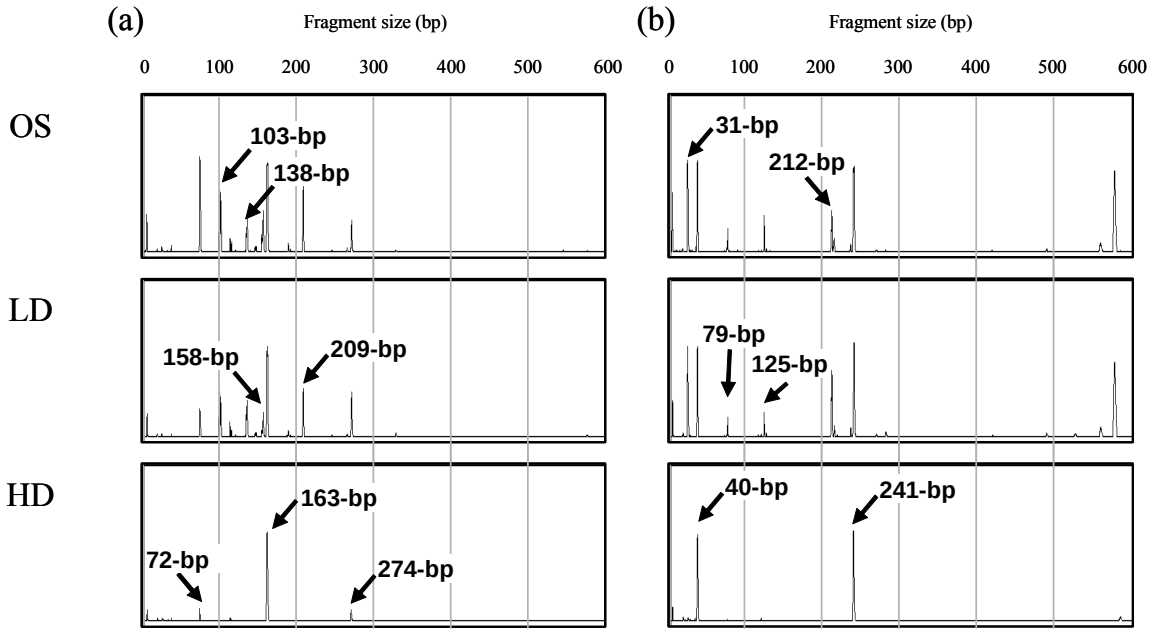

Fig. 3 Bacterial community structure in each fraction as determined by T-RFLP analysis using the restriction enzymes *Msp*I(a) and *Cfo*I(b).

In the case of *Msp*I 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 *Cfo*I 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.

4.3.4 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 *Msp*I and *Cfo*I restriction sites. Rich *et al.* reported that individual T-RFs differed in size by an average of 1.5-bp over a range of 0.3-5.1-bp (Rich et al., 2003). In this study, a gap of less than 5-bp was found acceptable for the identification of the predominant T-RFs.

The results of the identification of the T-RFs in HD are shown in Table 2. In the case of *Msp*I digestion, the 163-bp T-RFs 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-RF was generated by only *Nitrospira* sp. Unfortunately, the 72-bp T-RF, one of the predominant peaks, was not identified by the estimated T-RFs with *in silico* analysis. On the other hand, the disappeared peaks were identified as follows. The peaks of 209-bp and 158-bp T-RFs represented the *Bacteroidetes*. The *Alphaproteobacteria* (136- or 138-bp T-RF) including the *Defluvicoccus*-relative G-bacteria, The *Acidobacteria* (139-bp T-RF), and SBR-02 (243-bp T-RF) belonging to the *Gammaproteobacteria*, *Dechloromonas* sp. (103-bp T-RF) and *Thauera* sp. (103-bp T-RF) were also not concentrated in HD.

Table 2 Relative abundance of the T-RFs in the HD and comparison of the 16S rRNA genes from the clone libraries with the T-RFs obtained by digestion with *Msp*I (a)/*Cfo*I (b) and with sequences in the DDBJ. The clones, which were identified with both *Msp*I and *Cfo*I digestion, are shown on the gray box.

Fragment size (T-RFs)	Relative aboundance (%) OS	LD	HD	Clone	Most similar sequence
(a)					
72-bp	13	4.7	11	n.d.	
163-bp	13	21	74	SBR-05	AJ224937 *Rhodocyclus* sp.
				SBR-09	AJ224937 *Rhodocyclus* sp.
				SBR-19	AB201043 *Zoogloea oryzae*
				SBR-21	AJ224937 *Rhodocyclus* sp.
				SBR-27	AJ224937 *Rhodocyclus* sp.
				SBR-33	AJ224937 *Rhodocyclus* sp.
				SBR-37	AJ224937 *Rhodocyclus* sp.
				SBR-40	AJ224937 *Rhodocyclus* sp.
				SBR-48	AF361091 Uncultured bacterium SBRQ191
				SBR-68	AB205675 Uncultured bacterium
				SBR-79	AJ627188 *Malikia granosa*
274-bp	4	10	9.7	SBR-47	Y14637 *Nitrospira* sp.
(b)					
40-bp	22	17	45	SBR-05	AJ224937 *Rhodocyclus* sp.
				SBR-19	AB201043 *Zoogloea oryzae*
				SBR-21	AJ224937 *Rhodocyclus* sp.
				SBR-27	AJ224937 *Rhodocyclus* sp.
				SBR-36	AJ315677 *Thauera aminoaromatica*
				SBR-40	AJ224937 *Rhodocyclus* sp.
				SBR-43	AM084133 *Dechloromonas* sp. R-28400
				SBR-48	AF361091 Uncultured bacterium SBRQ191
				SBR-55	AJ315677 *Thauera aminoaromatica*
				SBR-68	AB205675 Uncultured bacterium
				SBR-79	AJ627188 *Malikia granosa*
				SBR-82	AJ315677 *Thauera aminoaromatica*
241-bp	13	18	50	SBR-09	AJ224937 *Rhodocyclus* sp.
				SBR-33	AJ224937 *Rhodocyclus* sp.
				SBR-34	AM084133 *Dechloromonas* sp. R-28400
				SBR-37	AJ224937 *Rhodocyclus* sp.

As for *Cfo*I digestion, 241-bp T-RF, one of the predominant peaks in HD, represented the clones SBR-34 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 *Msp*I digestion (Table2). Therefore, this T-RF represented only *Cand.* 'Accumulibacter phosphatis'. The relative abundance of this T-RF was increased from 18% to 50%. All the clones, which generate 163-bp T-RF with *Msp*I digestion except for the clones SBR-33 and 37, generate 40-bp T-RF with *Cfo*I digestion and the further distinction of these

bacterial strains was impossible. On the other hand, the 31-bp T-RF derived from *Nitrospira* sp. was not confirmed in HD although the 274-bp T-RF with *Msp*I digestion was confirmed in HD. These results indicated that *Nitrospira* sp. was not concentrated in the HD, and the 274-bp T-RF with *Msp*I 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 *Msp*I digestion.

4.4 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 nutrient 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.* (2001) and McMahon *et al.* (2002). *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 to *Msp*I and *Cfo*I 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 *Msp*I digestion; 40-bp and 241-bp with *Cfo*I digestion. Therefore, the resolution of T-RFLP analysis in this study ranged from the genus-level to the order-level, and the low-resolution was obtained for the *Betaproteobacteria* and the *Gammproteobacteria*.

The relatives of the genus *Nitrosococcus*, the group GB belonging to the *Gammaproteobacteria* and *Malikia* sp., *Zoogloea* sp. and *Cand.* 'Accumulibacter phosphatis'

belonging to the *Betaproteobacteria* were judged to be concentrated in HD. However, these organisms except for a part of *Cand.* 'Accumulibacter phosphatis' generated same T-RFs with both *Msp*I and *Cfo*I 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 *Cfo*I 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 phosphatis' should be higher than 50%. The 72-bp and the 274-bp T-RFs with the *Msp*I 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, *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 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 (Meyer et al., 2006). *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.* (2003) reported that the effectiveness of the buoyant density was changed with the physiological states of the bacteria. In this reactor, efficient nutrient removal maintained more than 2 years without unidentified deterioration described elsewhere (Seviour *et al*., 2003). Therefore, PAOs in this study must have maintained the high growth activity and it resulted in the prominence in this reactor whereas GAOs and 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 HD. Therefore, bacteria which play unimportant role for phosphorus removal could be eliminated by the buoyant density separation; however, some important bacteria were also eliminated in this study.

The buoyant density separation for the concentration of PAOs has been performed in several studies (Hung *et al*., 2002; Shuler *et al*., 2000, 2002; Zilles *et al*., 2002). Hung *et al.* (2002) 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 their study 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' but further information of the other clones is 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 study 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 × g (Hung *et al*., 2002). 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.* (2002). 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 analysis (Shuler *et al*., 2002). 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*. (1999). The sequences identified by Nielsen *et al.* (1999) 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 know as GAOs/G-bacteria were identified in the SBR. Some bacterial strains were separated in HD by the buoyant density separation. Judging from the relative abundance of the 239-bp T-RF with *Cfo*I digestion, *Cand.* 'Accumulibacter phosphatis' was concentrated from 18% to 50% by the buoyant density separation. Some unidentified bacterial strains were also concentrated in 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 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 has some advantages. This separation method 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 on (Nishino *et al*., 2003; Jetten *et al*., 2005). However, the concentration effectiveness by the buoyant density separation is still not clear and further studies for estimation is necessary.

4.5 CONCLUSIONS

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. Main conclusions are as follows.

(1) *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.

(2) *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.

(3) The Acidobacteria, the Bacteroidetes and the Nitrospira, which are not related to PAOs nor GAOs/G-bacteria candidates, were disappeared in 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.

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