Comparing results of cultured and uncultured biological methods used in biological phosphorus removal

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Abstract

Increasing attention has been paid to phosphate-accumulating organisms (PAOs) for their important role in biological phosphorus removal. In this study, microbial communities of PAOs cultivated under different carbon sources (sewage, glucose, and sodium acetate) were investigated and compared through culture-dependent and culture-independent methods, respectively. The results obtained using denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction-amplified 16S rDNA fragments revealed that the diversity of bacteria in a sewage-fed reactor (1#) was much higher than in a glucose-fed one (2#) and a sodium acetate-fed one (3#); there were common PAOs in three reactors fed by different carbon sources. Five strains were separated from three systems by using a phosphate-rich medium; they were from common bacteria isolated and three isolates could not be found in DGGE profile at all. Two isolates had good phosphorus removal ability. When the microbial diversity was studied, the molecular biological method was better than the culture-dependent one. When phosphorus removal characteristics were investigated, culture-dependent approach was more effective. Thus a combination of two methods is necessary to have a comprehensive view of PAOs.

Key words: phosphate-accumulating organisms (PAOs); denaturing gradient gel electrophoresis (DGGE); biological phosphorus removal; culture-dependent approach; culture-independent approach

Introduction

There have been many investigations to identify phosphate-accumulating organisms (PAOs) (Fuhs and Chen, 1975; Wagner *et al*., 1994; Sudiana *et al*., 1998; Wang and Park, 1998; Philip *et al*., 1998; Daniel *et al*., 1998; Gregory *et al*., 2002; Julie *et al*., 2002; Che *et al*., 2003; Johwan *et al*., 2002), *Acinetobacter* was first separated and was thought to be important members of PAOs (Fuhs and Chen, 1975; Wentzel *et al*., 1986). However, some evidence showed that *Acinetobacter* might not be the dominant PAOs (Wagner *et al*., 1994; Sudiana *et al*., 1998; Wang and Park, 1998; Philip *et al*., 1998; Daniel *et al*., 1998; Gregory *et al*., 2002), for it is easily isolated and identified based on culture-dependent identification methods, such as dilution or plating on various nutrient agar plates. Only less than 1% bacteria on earth can be cultured (Amann *et al*., 1995) and conventional pure-culture methods can identify only a few microbes (Norman, 1997). Recently, the culture-independent methods had been used in preference to traditional isolation techniques for analyzing microbial community analysis (Richard *et al*., 2003).

By culture-independent methods, *Rhodocyclus* genus of β-protebacteria has gained increasing attention as the most possible candidate for PAOs (Philip *et al*., 1998; Daniel *et al*., 1998; Gregory *et al*., 2002; Julie *et al*., 2002; Johwan *et al*., 2002; Che *et al*., 2003). Moreover, there are some other possible PAOs, like *Tetrasphaera* spp. and members of *Mcrolunatus* spp. (Eschenhagen *et al*., 2003), members of *Accumulibacter phosphatis*, the novel γ-*Porteobacterial* group, filamentous group resembling *Candidatus Nostocoidia* limicola (Liu *et al*., 2001) and *Cytophagales* (Dabert *et al*., 2001). The difference between cultured and uncultured results in biological phosphorus removal systems fed with different carbon sources was not analyzed in previous works. Therefore, the purpose of this study was to compare the microbial information obtained from direct amplification of rRNA genes of the sludge samples in biological phosphorus removal systems fed by different carbon sources with PAOs isolated from the same sludge samples, and to evaluate the biological methods that describe PAOs.

1 Materials and methods

1.1 Sequencing batch reactor operation

Three laboratory-scale sequencing batch reactors (SBRs), each with an 18 L working volume, were operated

and had gained good enhanced biological phosphorus removal performance after about 90 d. All of them were seeded from a full-scale SBR, which was used to treat wastewater from a hotel and had achieved biological phosphorus removal when it was used as seed in this experiment. They were operated under sequencing anaerobic-aerobic conditions with an 8-h cycle, which consisted of 2.5 h anaerobic, 3 h aerobic, and 2.5 h settling fill/draw and idle stages. During the anaerobic phase, nitrogen gas was purged into reactors for 15 min to get anaerobic conditions.

Phosphorus removal efficiencies under different pH, temperature, and sludge retention time (SRT) conditions were investigated in batch experiments (data are not shown here). Results showed that different systems reached optimal phosphorus removal at pH 7.2. It was also found that temperature had no obvious effect on phosphorus removal in the interval of 10 to 25°C. When SRT was 7 and 20 d, all systems achieved better phosphorus removal. Thus, the operation temperature was fixed at 22°C; pH values were controlled around 7.0 ± 0.2 by adding 0.5 mol/L HCl or 0.5 mol/L NaOH; extra sludge was withdrawn from the system at the end of each cycle to maintain the SRT around 7 d and mixed liquid suspended solid (MLSS) around 3000 mg/L.

1.2 Wastewater

Domestic sewage and two kinds of synthetic domestic wastewater were used in this study. The synthetic water was prepared by mixing tap water with glucose and acetate, respectively. To avoid fermentation, the above components were added to the water tank before the start of each cycle. The compositions of synthetic wastewater for the three reactors are shown in Table 1. Domestic sewage, glucose, and sodium acetate were used in three reactors as carbons, respectively.

The biological treatment units were run for over 3 months under controlled conditions to reach steady phosphorus removal performance. Then the tests were carried out.

1.3 Chemical analysis

Chemical oxygen demand (COD), PO_4^{3-}, NH_4-N, NO_3-N, MLSS, and MLVSS were analyzed according to Standard Methods (SEPAC, 1989). Total organic carbon

Table 1 Composition of synthetic wastewater for three reactors

Compound (mg/L)	1# Reactor	2# Reactor	3# Reactor
Sewage (1#)/glucose (2#)/ sodium acetate (3#)		589.5	741.4
P (KH_2PO_4)	4.0–7.2	15.0	15.0
NH_3-N (NH_4Cl)	35.1	40.0	40.0
TOC	104.7	202.4	347.4
COD	285.4–326.8	600.0	600.0
Mg ($MgCl_2$)	/	10.0	10.0
Cu ($CuSO_4$)	/	0.1	0.1
Ca ($CaCl_2$)	/	5.0	5.0
Mn ($MnSO_4$)	/	0.1	0.1
Zn ($ZnCl_2$)	/	0.1	0.1
Peptone	/	10.0	10.0

"/" no data.

(TOC) was measured by a TOC analyzer (TOC-V$_{CPN}$, SHIMADZU). Dissolved oxygen (DO) was measured by a DO meter (JPB-607). pH was measured by an acidometer (PHB3-pH).

1.4 PCR-DGGE (denaturing gradient gel electrophoresis) analysis

1.4.1 Sludge sample preparation and DNA extraction

Sludge samples were collected from each reactor at the end of aerobic phases. Part of the sample was used for isolation experiments, and the remainder was treated as described by Johwan *et al.* (2002). Total DNA was extracted from sludge samples by a rapid freeze-and-thaw method from Tsai and Olson (1992).

1.4.2 Primers and PCR amplification

16S rDNA fragments were amplified with the same primers as described by Johwan *et al.* (2002). To minimize nonspecific annealing of the primers to target DNA, a "hot start" technique was carried out (Muyzer, 1993). Then touchdown PCR was performed (Muyzer *et al.*, 1998). 3 µl of the PCR product was electrophoresed on 1% (w/v) agarose gel in 0.5×TAE for 30 min at 100 V, and then checked with ethidium bromide (EB) staining before being used for DGGE analysis.

1.4.3 DGGE analysis

Twenty-five micrometers PCR product was loaded onto a 6% (w/v) polyacrylamide gel (acrlamide/bis-acrylamde ratio, 37.5:1) in 0.5×TAE buffer (20 mmol/L Tris base, 10 mmol/L glacial acetic acid, 0.5 mmol/L EDTA, pH 8.0) for 24 h at 100 V and 60°C. The polyacrylamide gel contained a linear gradient ranging from 30% to 70% denaturant. After electrophoresis, the gel was stained for 10 min with EB, and then scanned with UV transilluminator (BIO RAD Gel Doc 1000) combined with a digital camera.

1.4.4 Sequencing of DGGE bands

Bands in the DGGE gel of 16S rDNA fragments were excised with a razor blade. The DNA was retrieved from the acrylamide block with Centriluter (Amicon) at 150 V for 3 h. The DNA was reamplified using GC-clamp-EUB341f and UNIV 907r. The clones were PCR-amplified again with the same primers mentioned earlier and were analyzed by DGGE. The clones that produced a DGGE band at the same position as the excised band were selected for subsequent sequence analysis. The sequence analysis of selected clones was performed on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, USA) according to the manufacture's guidelines.

1.5 Isolation experiments

1.5.1 Culture medium

The two kinds of culture medium used in phosphate uptake and release experiments were phosphate and non-phosphate mediums. Phosphate medium contained 5.0 g sodium acetate, 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4·7H_2O$, 0.25 g KH_2PO_4, 0.2 g $CaCl_2·2H_2O$, and 0.6 g acid hydrolysis casein in 1000 ml tap water; the final pH

was 7.0. Nonphosphate medium contained 5.0 g sodium acetate, 2.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $CaCl_2 \cdot 2H_2O$ in 1000 ml tap water; the final pH was 7.0. When phosphate uptake characteristics were being studied, air was pumped into the phosphate medium to increase the dissolved oxygen concentration to more than 2 mg/L, whereas for phosphate release study, nonphosphate medium was used and the system was kept anaerobic by purging nitrogen for 15 min at the start of the experiment.

Different types of phosphate-accumulating microorganisms can be discriminated by their colony morphology on beef grease peptone media. The enrichment culture was modified by adding 20 mg/L KH_2PO_4. The bacterial cells that have good phosphorus-accumulating capability become dominant and can therefore be easily separated. Bacteria morphology and physiology experiments were carried out according to Dong and Cai (2001).

1.5.2 Identification of isolated bacterium by molecular biological methods

DNA from the pure culture was extracted and PCR was carried out by the method adopted by Hiraishi (1992). To reduce the possible bias caused by PCR amplification, rDNA was amplified in triplicate tubes and these were combined for the next cloning step. The rDNA fragments were cloned into plamids using the TA cloning kit.

Clonal rDNA were prepared from randomly selected recombinants and used as template for sequencing. Then sequencing was conducted the same way as described in Section 1.4.4.

2 Results and discussion

2.1 SBR operation

After more than a three-month operation, three SBRs obtained good EBPR (enhanced biological phosphorus removal) performance. Phosphorus removal performance of 1# reactor supplied with sewage was the best. Its phosphorus removal efficiency was nearly 100% after a 70-d operation, which may be because of the fluctuant phosphorus in the influent. Most of the time in this experiment, phosphate concentration in sewage was below 5 mg/L. Reactor 3# supplied with sodium acetate did not achieve the best phosphorus removal as described by literature (Wang, 2001) (phosphorus removal efficiency was 90.94% after a 98-d operation), whereas 2# supplied with glucose gained better result (phosphorus removal efficiency was 96.9% after a 94-d operation). The reason might be that the phosphorus-removing ratio between PAOs and extracellular exopolymers was different because of the different carbon sources (Liu *et al*., 2004).

2.2 Fingerprinting of reactor communities by DGGE

Sludge was sampled at the end of anaerobic phase and was treated before being used for gene extracting. Fig.1 shows the gene DNA from sludge was about 23 kb. Then the DNA was amplified with "touch down" PCR process. The gene fragments of predicted length of about 500 bp was obtained (Fig.2) and used for the following DGGE
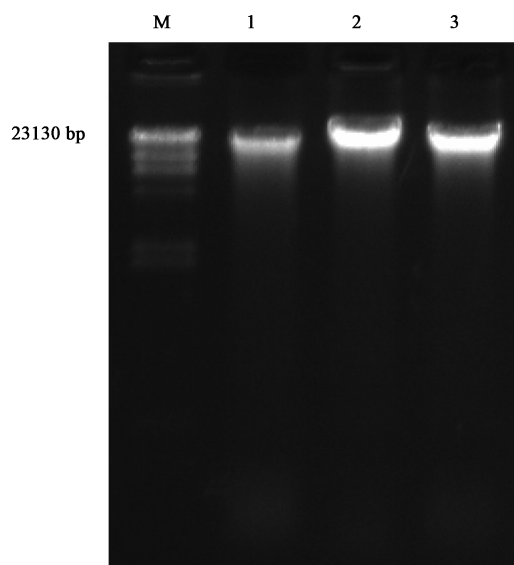

Fig. 1 Agarose gel electrophoresis of genomic DNA of sludge samples from three reactors. M: λDNA/Hind III.

analysis.

Of the PCR-amplified 16S rDNA, bacterial community structures and phylogenetic affiliation of three SBRs were screened using DGGE (Fig.3). The existing visible bands demonstrated the complexity and diversity of microbial ecology in sludge samples. From the DGGE profiles, it could be found that the sludge sample from 1# reactor that was supplied with sewage as carbon source presented a larger number of bands. Bands 1, 2, 3, 4, and 5 existed in all three reactors, bands 8, 9, 12, and 13 existed only in 1# reactor, bands 6 and 7 existed only in 2# reactor, and band 11 existed only in 3# reactor, whereas band 10 exited in both 1# and 3# reactors.

Bands 1–13 were excised from DGGE polyacrylamide gel and their sequences were analyzed. Comparison of 16S rDNA sequences of bands from DGGE with sequences available in GenBank database revealed high similarity
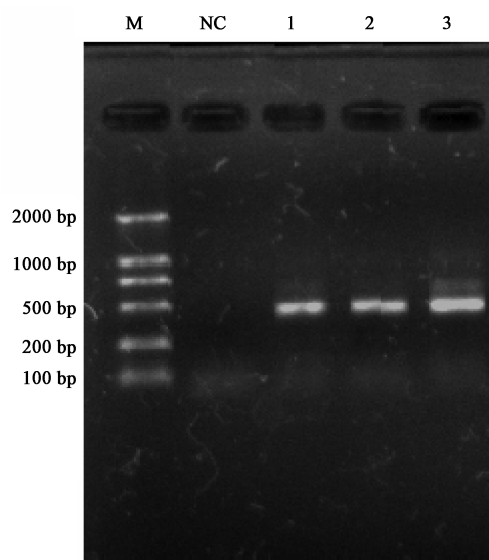

Fig. 2 Agarose gel electrophoresis of PCR amplified 16S rDNA gene from three reactors sludge samples. M: DL2000; NC: negative control.
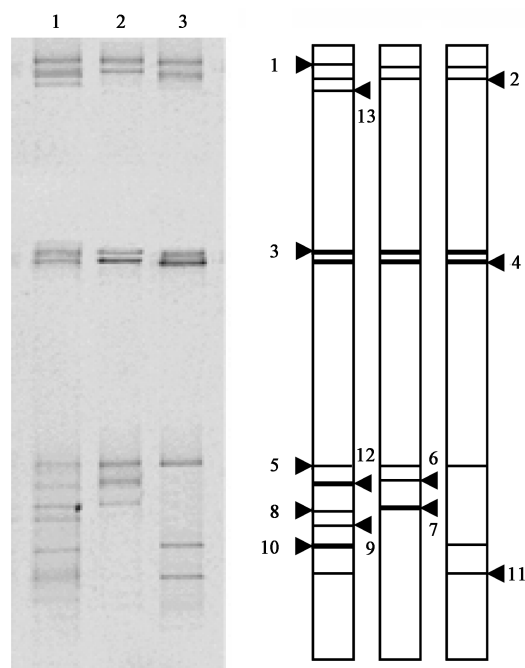
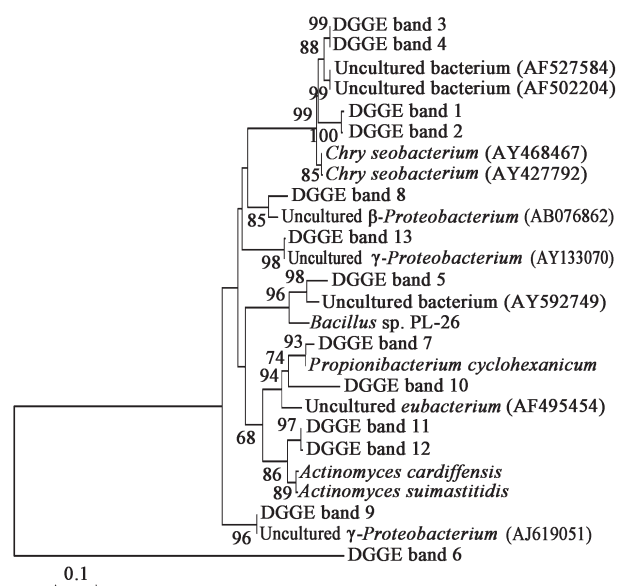Fig. 3 DGGE analysis of 16S rDNA fragments obtained from three reactors.

Fig. 4 Phylogenetic tree of 16S rDNA excised from DGGE profiles. Evolutionary distance and parsimonious analyses were carried out in MEGA 3.0. The scale indicates 0.1 nucleotide substitution per nucleotide position. The tree was rooted on midpoint.

values (Table 2). Sequence analysis revealed that DGGE bands 1, 2, 3, and 4 are closely related to genus *Flavobacterium* (Fig.4). Furthermore, bands 3 and 4 are uncultured bacterium and are highly related to the bacterium that were submitted by the Department of Chemical Engineering, University of California at Berkeley in 2002 from a laboratory scale sequencing batch reactor carrying out enhanced biological phosphorus removal. DGGE bands 5 and 6 have high similarity to genus *Bacillales*. Bands 7, 10, 11, and 12 are related to the genus *Actinobacteridae*. Band 8 is closely related to genus β-*Protebacteria*, whereas bands 9 and 13 are related to genus γ-*Protebacteria*. The uncultured bacteria (bands 3 and 4: AF527584 and AF502204) that were proposed to be PAOs could be detected in all three reactors as also *Bacillales* (bands 5). However, genus of β-*Protebacteria* and γ-*Protebacteria* (corresponding to bands 8, 9, and 13) only existed in sewage-fed reactor. There were several uncultured bacteria found in this study: for bands 3, 4, 5, 8, 9, 10, and 13, the locus codes are AF527584, AF502204, AY592749, AB076862,

AJ619051, AF495454, and AY133070, respectively.

2.3 Characteristics of cultured bacteria

At the end, there were five strains separated by phosphate-rich medium from sludge in three reactors. They were named PAO1-1, PAO1-2, PAO2-1, PAO2-2, and PAO3-1, respectively. Many other isolates (14) from the three reactors were clarified to have the same physiochemical and morphological characteristics with the above five ones. Transmission electron micrographs of five strains are shown in Fig.5. Five obtained nucleotide sequences were filed in GenBank under the accession numbers AY994313, AY994315, AY994312, AY994314, and AY994311, respectively. Comparison of 16S rDNA sequences of the five strains with sequences available in GenBank database revealed a high similarity value of 99% related to *Alcaligenes* sp. YcX-20, 99% related to *Brucellaceae bacterium*, 98% related to *Providencia alcalifaciens*, 99% related to *Kurthia gibsonii*, and 99% related to *Alcaligenes* sp. IS-170, respectively.

The phosphorus removal characteristics of the five strains were studied (Table 3). The reactor operated under

Table 2 Identification of bands originating from three reactors and their similarity values from GenBank

| Reactor	Band	Organism	Similarity (%)
1#, 2#, 3#	1	*Chryseobacterium* sp.	97
1#, 2#, 3#	2	*Chryseobacterium* sp.	97
1#, 2#, 3#	3	Uncultured bacterium clone LPB28 16S rRNA gene	97
1#, 2#, 3#	4	Uncultured bacterium clone HP1B06 16S rRNA gene	96
1#, 2#, 3#	5	Uncultured bacterium clone Napoli-4B-10 16S rRNA gene	94
2#	6	*Bacillus* sp.	99
2#	7	*Propionibacterium cyclohexanicum* gene for 16S rRNA	98
1#	8	Uncultured β-*Proteobacterium* gene for 16S rRNA clone: OS1L-4.	96
1#	9	Uncultured γ-*Proteobacterium* partial 16S rRNA gene, clone MTAD19.	99
1#,3#	10	Uncultured *eubacterium* clone T19 16S rRNA gene	97
3#	11	*Actinomyces* sp.	97
1#	12	*Actinomyces suimastitidis* partial 16S rRNA gene, strain CCUG 39276.	96
1#	13	Uncultured γ-*Proteobacterium* clone ccs2170	99
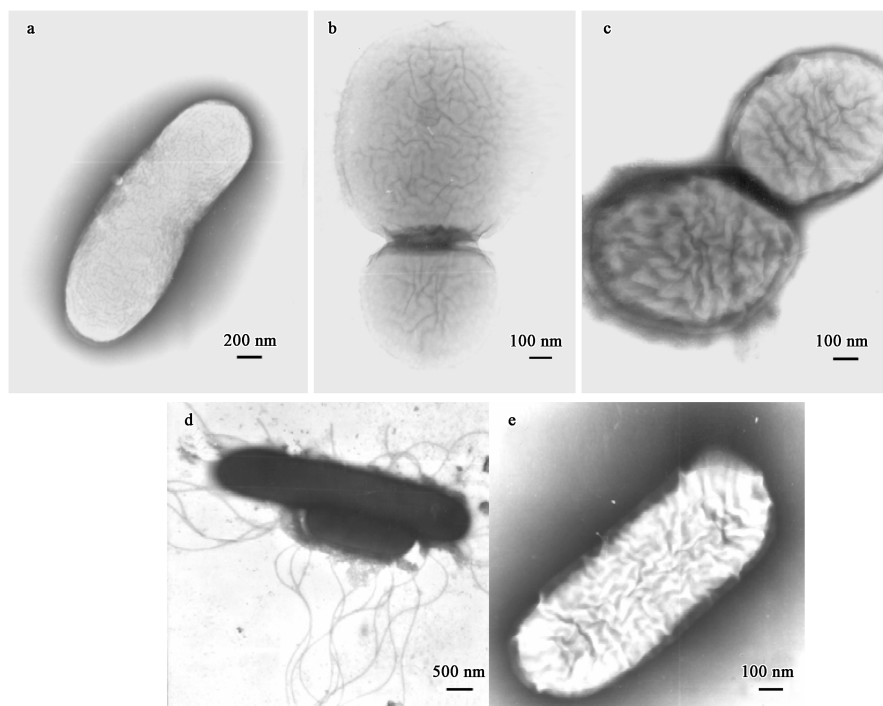
Fig. 5 Transmission electron micrographs of five strains. (a) PAO1-1; (b) PAO1-2; (c) PAO2-1; (d) PAO2-2; (e) PAO3-1.

conditions without inoculation of isolated strains reached steady phosphorus removal efficiency of 44.8% after about a 20-d operation. However, the addition of PAO 1-1 into reactor obtained obviously better phosphorus removal result. After a 10-d operation, the phosphorus removal efficiency was 90.7%. In the following days, the removal ratio reached was more than 91%. Addition of PAO3-1 pure culture also resulted in fine phosphorus removal efficiency. After a 15-d operation, the phosphorus removal efficiency was 83.5%, and then reached more than 82% in the following days. The other three strains of PAO1-2, PAO2-1, and PAO2-2 did not give good enhancing phosphorus removal effect.

Increasing attention has been paid to microbiological characteristics of enhanced biological phosphorus removal systems (Wang and Park, 1998; Gregory *et al.*, 2002; Che *et al.*, 2003). Carbon sources can affect microbial populations in biological biological phosphorus removal systems. The molecular biological method DGGE has been widely used to study microbial diversity in environmental samples (Muyzer *et al.*, 1993; Hanaki *et al.*, 2001; Ahn *et al.*, 2002; Díez *et al.*, 2001). The phylogenetic analysis of bands 1–4 of DGGE were sequenced and was seen to have high similarity with *Cytophaga-Plexibacter-Bacterodies* (CFB) group division (Fig.4), but CFB group bacteria were not

obtained in culture-dependent experiment results. It was found that CFB group bacteria were present at significant levels in both EBPR and standard processes (Akira *et al.*, 1998). PAO1-2 (AY994315) isolated from No. 1 reactor fed with real sewage had a 99% similarity with *Alcaligenes* sp. YcX-20; this kind of bacterium was not found in DGGE result. The sequence of PAO2-1 (AY994312) and PAO2-2 (AY994314) have 98% similarities with *Providencia alcalifaciens* and 99% with *Kurthia gibsonii* respectively, but these two kinds of bacterium separated from glucose-fed reactor were either not detected by DGGE method, or were they among the "uncultured bacteria clone" in DGGE result? The other two bacteria PAO1-1 and PAO3-1 (PAO313, PAO311) might be the ones detected by DGGE although the sequences were not identical to the group they belong to. Thus, the five isolated bacteria were not just among the 13 bands in DGGE profile. It is safe to say that the results of cultured and uncultured biological methods were obviously different when used to describe the different effects of different carbon sources on biological phosphorus removal.

DGGE patterns of DNA extracted from the sludge of the three reactors showed that the diversities of the bacterial communities in phosphorus removal systems fed on different carbon sources were different. The diversity

Table 3 Comparison of phosphorus removal efficiency between bioaugmented and un-bioaugmented systems

Reactor	Strain	Phosphorus removal ratio (%)						
		5 d	10 d	15 d	20 d	25 d	30 d	35 d
SBR1	PAO1-1	58.6	90.7	91.2	93.3	93.6	93.5	93.4
SBR2	PAO1-2	21.7	22.6	35.4	45.7	42.5	46.7	43.0
SBR3	PAO2-1	27.5	46.3	62.4	62.0	61.5	63.2	62.8
SBR4	PAO2-2	19.0	30.5	42.7	53.5	53.9	52.2	53.0
SBR5	PAO3-1	45.1	67.2	83.5	82.6	83.9	84.7	82.3
SBR6	–	20.3	20.5	33.4	44.8	50.2	49.7	46.3

of bacteria in sewage-fed reactor (1#) was much higher than in glucose-fed one (2#) and sodium acetate-fed one (3#). Five common bands (bands 1–5) were screened in three reactors; it meant that there were common bacteria in three reactors fed by different carbon sources. Furthermore, bands 3 and 4 had high similarity with registered uncultured PAOs (AF527584, AF502204). Thus, there are PAOs independent of carbon source. From the culture-dependent experiment results, little microbial diversity could be found, but the isolates with good phosphorus removal ability could be studied in detail (Table 3). PAOs isolated in this study were totally different from PAOs reported in literature (Fuhs *et al.*, 1975; Lacko *et al.*, 2003; Maszenan *et al.*, 2000; Nakamura *et al.*, 1995; Shoda *et al.*, 1980). Molecular biological method is indeed the better method to describe the effect of different carbon sources on biological phosphorus removal than culture-dependent approach. But when the phosphorus removal characteristics of bacteria were analyzed, the culture-dependent method played its own important role.

3 Conclusions

Thirteen bacteria were screened by PCR-DGGE and five bacteria were isolated by pure culture method from three biological phosphorus removal systems fed with different carbon sources, respectively. The results of cultured and uncultured biological methods were obviously different when used to describe the different effects of different carbon sources on biological phosphorus removal. When the microbial diversity is studied, molecular biological method is better than the culture-dependent approach, whereas, the culture-dependent method can be used to study phosphorus removal characteristics of bacteria in detail. Consequently, the complete portrait view of PAOs should be obtained by using both the culture-independent and culture-dependent biological methods. In the other words, different biological detecting methods are necessary to describe the biological characteristics of enhanced biological phosphorus removal systems.

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