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Analysis of bacterial community in bulking sludge using culture-dependent and -independent approaches

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Abstract

The bacterial community of a bulking sludge from a municipal wastewater treatment plant with anoxic-anaerobic-oxic process was investigated by combination of cultivation and 16S rRNA gene clone library analysis for understanding the causes of bulking. A total of 28 species were obtained from 63 isolates collected from six culture media. The most cultivable species belonged to γ-Proteobacteria including *Klebsiella* sp., *Pseudomonas* sp., *Aeromonas* sp. and *Acinetobacter* sp. Further analysis of these strains by repetitive sequence based on polymerase chain reaction (rep-PCR) technology showed that rep-PCR yielded discriminatory banding patterns within the same genus using REP and BOX primer sets. While the culture-independent assessment revealed that β-Proteobacteria was the dominant group in the bulking sample. Sequence analysis revealed that the highest proportion (14.7%) of operational taxonomic units was 98% similar to *Candidatus* Accumulibacter phosphatis, which is used to remove phosphorous from wastewater. Our results indicated that combining different approaches can produce complementary information, thus generate a more accurate view of microbial community in bulking sludge.

Key words: bulking sludge; cultivable bacteria; rep-PCR; 16S rRNA gene clone library

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Introduction

The anoxic-anaerobic-oxic (A2/O) process is widely used in wastewater treatment plant for phosphorus and nitrogen removal, however, sludge bulking and foaming are the most frequent operational problems in plants under this process. This is caused either due to the presence of non-degradable surfactants or the overgrowth of filamentous bacteria such as *Microthrix parvicella* (Rossetti et al., 2005), *Sphaerotilus natans* (Suzuki et al., 2002), Eikelboom type 021N, and *Thiothrix* spp. (Vaiopoulou et al., 2007). When the sludge bulking appears, it causes the poor settleability of sludge that results in a poor effluent quality, the loss of active biomass, increased costs, and poses a number of environmental and health hazards (Naidoo, 2005).

In spite of considerable efforts have been introduced, these results are still unsatisfied to suppress sludge bulking. Understanding the microbial ecology of activated sludge is the basis of pursuing suitable remediation to resolve this problem. In the past years, many researchers focused on the enumerating the bacterial community present in activated sludge by using molecular approaches, including denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone library analysis (Eschenhagen et al., 2003; Blackall et al., 1998; Choi et al., 2007; Boon et al., 2002). These culture-independent technologies can reveal more abundance than culture-dependent approaches in the terms of bacterial composition. However, there are several inherent biases regarding with PCR-based molecular methods, including primer preference; the efficiency of cell lyses; DNA extraction and purification (Snaidr et al., 1997). Therefore, isolation and characterization of bacteria from environmental samples is still essential, especially in bulking sludge. Firstly, it can provide useful and complement information about the structure of microbial communities; secondly, some common filamentous bacteria that caused sludge bulking and foaming have not been isolated for their hardly cultivable; thirdly, many works still rely on obtaining pure cultures, such as, some genera strains have great diversity at the species level which differ in their capabilities in the same ecosystem,

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so obtaining the pure cultures that will facilitate the research on their physiological-biochemical characteristics. It is also necessary to isolate more pure cultures from activated sludge for getting a better comprehension on the biodiversity associated with sludge bulking based on genomic fingerprinting technology. For instance, rep-PCR can be used to discriminate bacteria and fungi at the strain level. Previously, rep-PCR has been employed successfully to classify and differentiate among environmental strains (Sikora and Redžepović, 2003; Mohapatra and Mazumder, 2008), but the analysis of bacteria dwelling in bulking sludge by this method is seldom reported. Moreover, little research has focused on the analysis of microbial diversity of A2/O activated sludge using a combination of culture-dependent and -independent methods during bulking and foaming.

The objectives of this study are to evaluate the combination of 16S rRNA gene clone library and the cultivation for assessing of bacterial diversity of the microflora in bulking sludge, and to estimate biodiversity within the dominance of bacterial isolates using rep-PCR technology (REP-PCR and BOX-PCR). The present study could provide a more precise view of bacteria community composition in activated sludge in bulking stage.

1 Materials and methods

1.1 Sample collection and microscopic examination

Activated sludge was collected from aerobic tank of a wastewater treatment with the A2/O process located in a northern suburb of Beijing in winter 2009. The process presents a sequence of anoxic tank followed by a sequence of anaerobic tank to promote denitrification, and finally aerobic tank. When sampling where bulking had occurred, the temperature was approximately 4–8°C outside. The collected sample was immediately taken to laboratory and refrigerated at 4°C. Within a week, activated sludge sample was counterstained with 4',6-diamidino-2-phenylindole (DAPI). Staining was detected using fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany) equipped with appropriate filters. Besides, Gram staining was also performed under bright field illumination using the same microscope. Images were captured with the Canon PowerShot A640.

1.2 Isolation, purification and cultivation of activated sludge bacteria

Besides four media (CGYA, R2A, C and SCY) which were often used for cultivation of filamentous bacteria (Kämpfer, 1997), the other two most common growth media for microorganisms, Luria broth agar (Shanghai Sangon, China) and actinomycete isolation agar (Difco), were also employed in this study. All the media components were prepared in double, glass-distilled water and autoclaved at 121°C for 15 min except for stock solutions of vitamins and soluble carbohydrates were sterilized by membrane filtration (0.22 μm; Fisher Scientific Co., USA) and transferred aseptically as required. Aseptic techniques were used for all subsequent procedures involving sample collected. After pretreatment by sonication, a certain volume of activated sludge (10^{-3} dilution with sterile water) was spread into each culture medium containing agar. Cultivation was carried out at 22°C for 3–7 days. Individual colonies were picked randomly and further purified by restreaking at least three times onto plates with corresponding media until they were brought into pure cultures.

1.3 PCR amplification of 16S rRNA gene of cultivable bacteria

Bacterial DNA was extracted from the enrichment sample using E.Z.N.A.TM Bacteria DNA Kit (OMEGA, USA). 16S rRNA gene was then amplified by PCR from the extracted DNA. PCR mixture consists of 5 μL of 10× PCR buffer (Mg^{2+} plus), 1 μL of dNTP (10 mmol/L), 1 μL of each primer: forward primer (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′, 5 μmol/L) and reverse primer (1492r: 5′-GGTTACCTTGTTACGACTT-3′, 5 μmol/L), 0.5 μL of *Taq* polymerase (5 U/μL) and 2 μL of DNA template (approximately 100 ng). Double-distilled water was added until its final volume reached 50 μL, negative-DNA control was made in the same volume including identical components except the template DNA, which was replaced by 2 μL of sterilized double distilled water. The PCR conditions were an initial denaturation step at 95°C for 6 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and a final step at 72°C for 10 min. The PCR products were purified by E.Z.N.A.TM Gel Extraction Kit (OMEGA, USA).

1.4 Genomic DNA extraction and purification

Total community genomic DNA was extracted from 5 g of sludge sample according to method described by Zhou et al. (1996) with some modifications. The protocol encompassed grinding, three cycles of freezing and thawing, chemical lyses in a high-salt extraction buffer (1.5 mol/L NaCl) by heating suspension in the presence of sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB), and a proteinase K step. Finally, the genomic DNA was purified (according to the method described above) and quantified by spectrophotometry at 260 nm using the ND-1000 spectrophotometer (NanoDrop Technologies, Inc., DE, USA). The concentration was modified to 100 ng/μL. The obtained DNA was stored at –20°C and used as the template for further PCR amplification.

1.5 16S rRNA gene clone library construction

The purified PCR products were cloned into pGEM-T vectors, which were used to transform *Escherichia coli* DH5α competent cells. After blue-white screening, 316 white colonies were randomly selected and re-amplified by PCR. The positive reamplified products were digested by the restriction endonuclease *Rsa*I and *Msp*I (MBI Fermentas) at 37°C overnight. The system for the reaction of *Rsa*I and *Msp*I digestion consists of 1 μL buffer, 0.25 μL of each

enzyme, and 6 μL of purified clone PCR products. Double-distilled water was added until its final volume reached 10 μL. The digestion products were analyzed on a 3% agarose gel prestained with ethidium bromide by electrophoresis (3 hr, 80 V). Representatives of each group were selected for sequencing.

1.6 DNA sequencing and phylogenetic analysis

Cultivable isolates and representative clones were sequenced (Completed by Beijing Tsingke Biotech. Co., Ltd., China) and these sequences were compared with sequences in NCBI database using BLAST software (www.ncbi.nih.nlm.gov). The closest 16S rRNA gene sequences were aligned with Clustal X 1.83. Phylogenetic tree was constructed by the neighbor joining method, using MEGA 3.1.

1.7 Rarefaction analysis

Richness of bacterial community in bulking activated sludge was analyzed by rarefaction analysis using Analytic Rarefaction 1.3 software.

1.8 Rep-PCR analysis of dominant strains based on culture-dependent method

REP- and BOX-PCR analysis of the isolates were conducted to obtain their genomic fingerprints. The primers used were: for REP-PCR, REP 1R: 5′-IIIICGICGICATCIGGC-3′, REP 2-I: 5′-ICGICTATCIGGCTAC-3′, and for BOX-PCR, BOXA1R: 5′-CTACGGCAAGGCGACGCTGACG-3′. Rep-PCR was carried out in 50 μL of reaction mixture consisting of 50 pmol each of the primers for REP-PCR and BOX-PCR, 2.5 μL of 10× PCR buffer, 400 μmol/L dNTPs, 5.0 mmol $MgCl_2$, 2 U of *Taq* DNA polymerase (Fermentas, USA), and 50 ng of template DNA. PCR products were then examined through horizontal electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) at 80 V for 3 hr in TBE buffer. Gel images were captured using the GeneSnap system (Syngene, Cambridge, UK). All the amplifications were performed in triplicates three times to test the reproducibility of this method.

1.9 Nucleotide sequence accession numbers

The representative 16S rRNA gene clone sequences from phylogroups of clones have been deposited in a GenBank database under accession numbers HQ538624 to HQ538656. The representative isolates from 28 phylogroups of 63 strains are to be found under accession numbers HQ538657 to HQ538684.

2 Results and discussion

2.1 Microscope analysis

The activated sludge sample was analyzed by DAPI staining for microscopic examination. The image is shown in Fig. 1a. It demonstrated that sludge bulking in this plant was caused by the overgrowth of filamentous bacteria, and the morphological characteristics of dominant filamentous bacteria were closest to *Microthrix parvicella* include Gram-positive, no sheath, coiled and no branching occurred (arrow in Fig. 1b) (Jenkins et al., 1993). However, filaments exhibit false branching were also seen in slide (arrow in Fig. 1c), which indicated there were at least two types of filamentous bacteria coexist in bulking sludge.

2.2 Cultivable bacterial strains

Previous studies had focused on the isolation of bacterial strains from activated sludge. In the early years, Horan et al. (1988) had attempted to isolate both floc-forming and filamentous bacteria from activated sludge flocs. In the 1990s, Käpfer et al. (1996) collected samples from aerobic and anaerobic zones of a wastewater treatment plant showing enhanced phosphorous removal and characterization of 255 isolates from tryptone-soy agar and R2A agar. Until recent years, a few bacterial strains especially filamentous bacteria were cultivated by the method of dilution plate (Ramothokang et al., 2003) or micromanipulation (Blackall et al., 1996). However, it should be noted that a majority of microorganisms present in any environment have not been cultivated through general media (Ellis et al., 2003); thus, obtaining more unexplored microbes is an important prerequisite for better understanding what functions these strains have in this complex environment. In this study, we used six different media to enrich bacteria present in

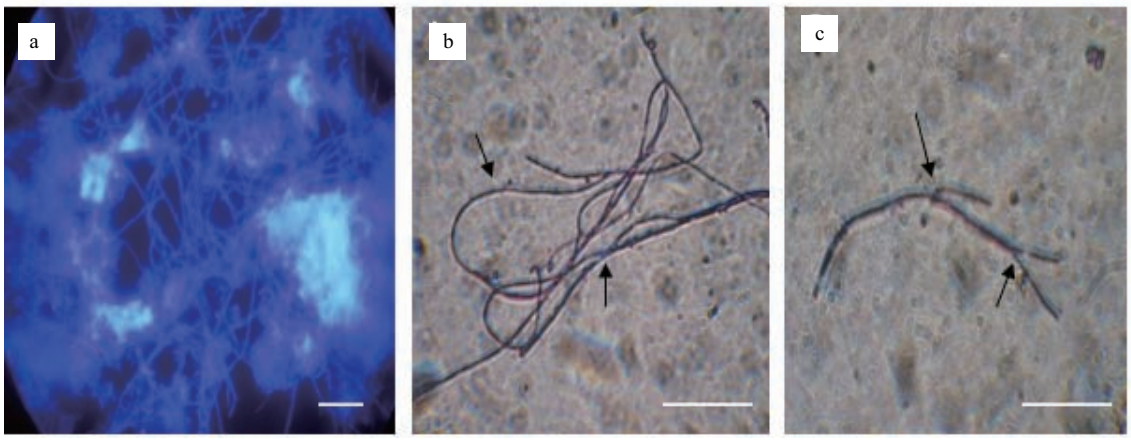

Fig. 1 Microscope image of DAPI-stained and Gram-stained activated sludge. The bar represents 5 μm.

the samples, and the isolates were selected based on morphology, size or color. There were 8–12 different strains isolated from each of the six media, resulting in a total number of 63 strains (named bk_1–bk_63, respectively). These strains were phylogenetically clustered into 6 major groups and 28 distinct lineages or species (the similarities of 16S rRNA gene sequences < 97%): 34 strains of γ-Proteobacteria (53.9%), 7 strains of β-Proteobacteria (11.1%), one strain of α-Proteobacteria (1.6%), 10 strains of Firmicutes (15.9%), 4 strains of Actinobacteria (6.4%) and 7 strains of Bacteriodetes (11.1%) (Table 1). This result is similar with the results reported by Kämpfer et al. (1996), which showed that members of γ-Proteobacteria were the most dominant group in collections, in contrast to the observation by Lu et al. (2006) which showed that β-Proteobacteria was the most dominant group in sludge samples. In addition, at the genus level, strains of the genera *Klebsiella*, *Pseudomonas*, *Bacillus, Aeromonas, Flavobacterium* and *Acinetobacter* (12.7%, 12.7%, 12.7%, 11.1%, 7.9% and 7.9%, respectively) were the most predominant microorganisms in the collection.

On the other hand, our results also coincided with the previous reports in which the Gram-negative (GN) bacteria constituted the most genera strains isolated from activated sludge (Pick, 1995), and the results of this study also strongly support this view. Among 63 isolates, 49 (78%) strains of bacteria were GN. *Klebsiella* and *Pseudomonas* were the most abundant GN genera, followed by *Aeromonas* and *Acinetobacter*. Merely 14 (22%) isolates belonged to Gram-positive (GP) bacteria. Genus *Bacillus* was the dominant GP bacteria, followed by the genus *Microbacterium*. Most of these strains may be closely related with the performance of activated sludge. *Klebsiella* spp. can degrade many organic compounds with widespread use such as chlorpyrifos (Ghanem et al., 2007) and 2,4,6-trinitrotoluene (Kim et al., 2002), which had been isolated from activated sludge. Both members of the genera *Aeromonas* and *Acinetobacter* were frequently isolated from wastewater treatment plants (Käpfer et al., 1996), their widespread can infer they play a significant role in this system. Most of *Baclilus* spp. can produce endospores that confer them survive in extreme environments; this genus is ubiquitous in nature with ability to degrade a wide range of substrates (Claus and Berkeley, 1986). *Pseudomonas* and *Flavobacterium* are widely distributed in a variety of habitats and are able to metabolize organic contaminants in the environment (Poornima et al., 2010; Ashiuchi et al., 1999).

Overall, these phylogenetical results of cultivable bacteria resemble that reported in previous studies in many ways. Unfortunately, no filamentous bacteria were observed on all these above media. It seems that optimizing medium components and developing a suitable method for sample pretreatment are still important for cultivating filamentous bacteria successfully if dilution plating method was adopted, or the filamentous bacteria present in this sample were not cultivable in these methods.

2.3 Rep-PCR fingerprinting

Rep-PCR has attracted a lot of attention in recent years, for its highly discriminatory, accurate, fast and low-cost advantages. To date, it has been applied for analyses of biodiversity among bacterial strains in water (Mohapatra and Mazumder, 2008), soil (Rejili et al., 2009), plants (Sikora and Redžepović, 2003), etc. However, there were no reports on the analysis of isolated bacteria diversity in activated sludge using this method. Meanwhile, it should be noted that different strains belonging to the same genus may play different roles in activated sludge. For this reason, it is urgent to obtain more detailed evaluation on biodiversity among the same genus; rep-PCR can be a suitable candidate reference method for this purpose.

In the present study, in order to determine the genetic diversity among our isolates, the dominant strains of pure cultures (number ⩾5) were selected for further analysis by rep-PCR technology.

Table 1 Identification of isolates based on 16S rRNA gene sequences analysis

Affiliation	Identification (the nearest genus)	Similarity (%)	Number of isolates	Strain No. (bk_)	OTU*
γ-Proteobacteria	*Aeromonas*	99–100	7	1, 3, 4, 5, 26, 52, 53	1
	Acinetobacter	98–99	5	2, 7, 13, 34, 42	3
	Pseudomonas	99–100	8	15, 22, 23, 30, 43, 44, 45, 49	2
	Shewanella	99	3	8, 16, 21	1
	Klebsiella	99–100	8	20, 24, 27, 29, 31, 47, 59, 63	1
	Kluyvera	99	1	32	1
	Serratia	99–100	2	6, 46	1
β-Proteobacteria	*Neisseria*	99	2	12, 37	1
	Janthinobacterium	99	2	51, 56	1
	Acidovorax	99	1	57	1
	Diaphorobacter	99	1	62	1
	Comamonas	99	1	18	1
α-Proteobacteria	*Brevundimonas*	98	1	28	1
Firmicutes	*Staphylococcus*	100	2	9, 11	1
	Bacillus	100	8	10, 14, 33, 35, 39, 40, 50, 60	4
Actinobacteria	*Kocuria*	100	1	17	1
	Microbacterium	99–100	3	36, 41, 55	1
Bacteriodetes	*Chryseobacterium*	98	2	19, 48	2
	Flavobacterium	97–99	5	25, 38, 54, 58, 61	3

* OTUs generated using a 16S rRNA percentage identity value of ⩾ 97%.

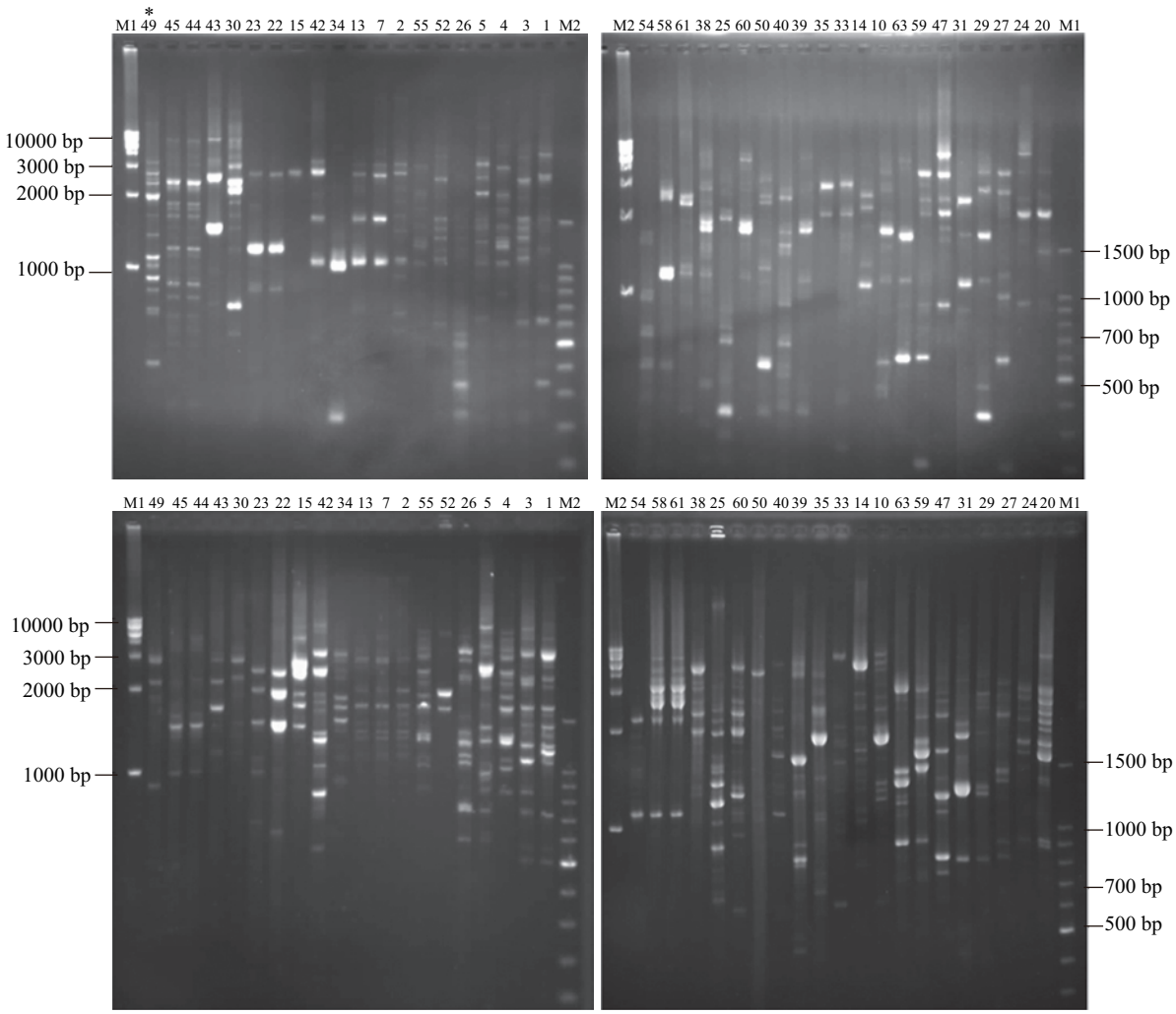

Fig. 2 Rep-PCR genomic fingerprints of 41 dominant strains generated with REP (top) and BOXAIR (bottom) primer set. Lane M1: 100 bp marker, lane M2: 1 kb marker. * The data represent strain number (bk_).

As shown in Fig. 2, it could be easily seen that REP- and BOX-PCR produced similar discrimination within each genus; both generated highly specific and reproducible patterns that enabled accurate strain differentiation. All isolates showed an abundance of repetitive sequences. The size of the bands was between 200 and 10,000 bp and their numbers ranged from 1 to 15 bands per profile. Most bands showed a molecular weight of between 500 and 3000 bp. In addition, the results have also proved to be very distinct and unique for each strain except for bk_58 and bk_61; bk_44 and bk_45; bk_2, bk_7 and bk_13. In contrast with the 16S rRNA gene analysis pattern of strains of the genera *Klebsiella*, *Pseudomonas, Bacillus, Aeromonas, Flavobacterium* and *Acinetobacter*, the rep-PCR profiles of cultivable dominant strains show the significant different patterns. Among *Klebsiella* and *Aeromonas* genera, all strains are different types from each other while only one type of species can be derived from 16S rRNA gene sequences. For *Pseudomonas*, total eight strains are allocated into seven types of species in contrast only two types defined by 16S rRNA gene sequences. Besides, it is interesting to find that no change occurred in the genus of *Acinetobacter*, which might be due to the high degree of similarity among three strains of *Acinetobacter*. Anyway, rep-PCR can generate relatively more complex results.

2.4 16S rRNA gene clone library

As many reports revealed that cultivable bacteria represent only a small proportion of natural microbial communities. Therefore, we chose 16S rRNA gene clone library as a culture-independent approach to survey bacterial community composition in bulking activated sludge. More than 200 positive clones were selected randomly for reamplification and 33 OTUs were obtained based on PCR-RFLP analysis. Rarefaction curves (plots of the cumulative numbers of OTUs as a function of clone

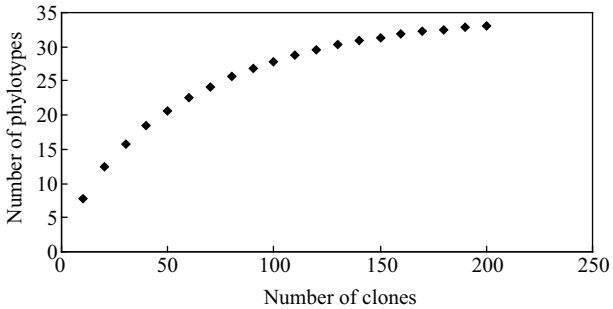

Fig. 3 Rarefaction curve for bacterial 16S rRNA gene clone library derived from bulking sludge.

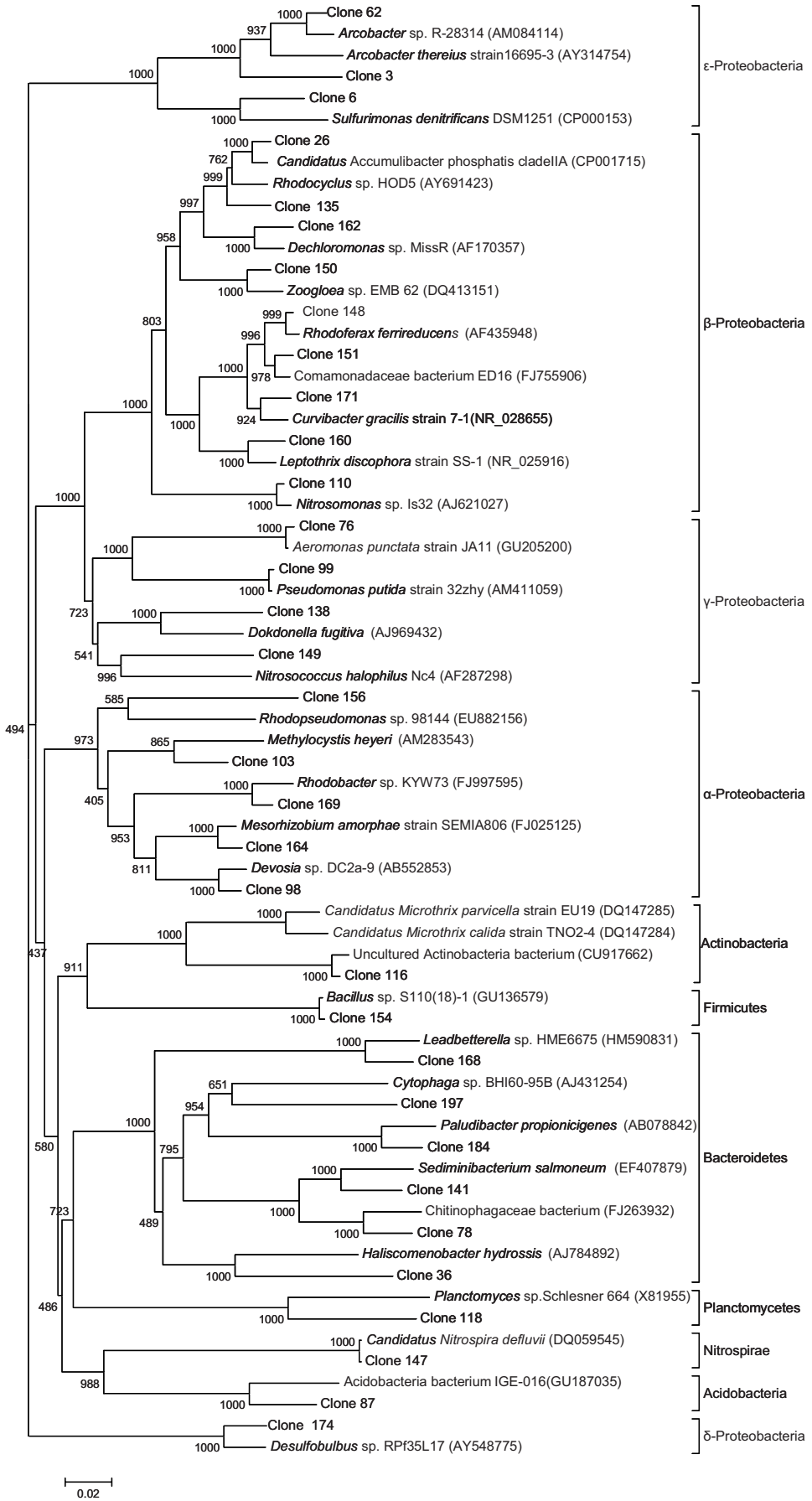

Fig. 4 Neighor-Joining tree deduced from partial sequences of 16S rRNA gene clones from bulking sludge sample. Bootrap confidence values obtained with 1000 resampling are given at the branch point.

number) indicated that the majority of the OTUs in the sample were detected (Fig. 3). Further sequencing and comparative analysis were conducted to elucidate the bacterial diversity. Sequence analysis revealed that 33.8% of clones with low similarity (< 97%) to those bacteria deposited in GenBank, suggesting a great abundance of novel strains exist in the activated sludge. In addition, results of phylogenetic analysis showed the sequenced clones fell into 7 groups. Among them, members of Proteobacteria were the dominant group, accounting for 69.61%, including α-Proteobacteria (8.82%), β-Proteobacteria (43.63%), γ-Proteobacteria (4.41%), δ-Proteobacteria (0.98%) and ε-Proteobacteria (11.76%), followed by Actinobacteria (13.73%), Bacteroidetes (9.80%), Firmicutes (2.94%), Planctomycetes (1.96%), Acidobacteria (1.47%) and Nitrospirae (0.49%), respectively. At the level of class, β-Proteobacteria (43.6%) was the dominant group in the bulking sludge, which was in accordance with previous studies (Rani et al., 2008). A phylogenetic tree was constructed together with their closest relatives derived from Genbank (Fig. 4).

Diversity index serves as a valuable tool to quantify diversity of a community and describe its numerical structure. As shown in Table 2, the high values of diversity indices and evenness indicated high microbial diversity in our sample using both culture dependent and independent methods; however, compared with results obtained by cultivation, only *Bacillus*, *Aeromonas*, and *Pseudomonas* species were identified in the clone library. It was worth noting that *Acinetobacter* spp., which was proposed as polyphosphate-accumulating bacteria, was not found in clone library, but isolated successfully in this study. Moreover, the clone library was dominated by β-Proteobacteria, which were not evident in the collection of isolates, due to microbes belonging to β-Proteobacteria often be underestimated by culture-dependent techniques (Vainio et al., 1997). These finding indicated that the analysis of microbial community composition of activated sludge by the method of cultivation and 16S rRNA gene clone library was still quite different; the same phenomenon was also observed by Lu et al. (2006). It is indicated that the most abundant bacteria might be not cultivable in the activated sludge.

In aerobic reactors, the polyphosphate accumulating organisms (PAOs) are considered to be as a group of microorganisms capable of accumulating phosphate by taking up soluble phosphates in the bulk solution (You et al., 2003). The 16S rRNA gene clone library constructed in the study also include genus *Pseudomona* (OTU16) (Ren et al., 2007) and *Rhodocyclus* group (OTU11) (Wang et al., 2009), which have been identified as having phosphorus removing ability. Moreover, OTU6 shows 98% identity to *Candidatus* Accumulibacter phosphatis clade IIA strain. UW-1, which is used to remove phosphorous from wastewater, were the most dominant OTU in clone library, representing 14.7% of the total clones. These findings indicated that the PAO still occupied the largest proportion even when bulking had occurred. In addition, although *Microthrix parvicella*, a filamentous bacterium causing bulking and foaming at lower temperatures were not detected, an OTU identified as belonging to uncultured Actinobacteria, which represents 13.7% of total clones also show 88% similarity with *Candidatus Microthrix calida* strain TNO2-4 and 87% with *Candidatus* Microthrix parvicella strain EU19. This finding may indicate the possibility of a new strain of filamentous microorganism associated with sludge bulking in activated sludge. The further work is in progress to confirm this speculation.

Table 2 Comparison of phylotype richness (S), diversity (H) and evenness (E) values of the bacterial population using culture dependent and independent methods

Index*	Cultivable isolates	16S rRNA gene clone libraries
S	28	33
H	3.06	3.01
E	0.92	0.86

* The Shannon-Weiner diversity index was calculated according to the formula described by Rani et al. (2008).

3 Conclusions

In this study, the bacterial community structure and composition in activated sludge obtained from aerobic zone of a wastewater treatment plant with A2/O process was investigated by cultivation techniques combined with 16S rRNA gene clone library in process of bulking. Phylogenetic analysis of the partial 16S rRNA gene sequences of 63 isolates showed that γ-Proteobacteria were the dominant microorganisms among the collection, followed by Firmicutes, Bacteriodetes, β-Proteobacteria and α-Proteobacteria. However, 11 classes were found in 16S rRNA gene clone library. While β-Proteobacteria were the most abundance phylogenetic group in these clones. Moreover, only *Bacillus*, *Aeromonas*, and *Pseudomonas* species isolated by cultivation method were identified in the clone library. These results reinforced the conclusion that different approaches should be performed to improve the understanding of microbial diversity in bulking sludge. In addition, rep-PCR technology was used to evaluate biodiversity within the dominance of bacterial isolates, and the results showed the highly discriminatory banding patterns using REP and BOX primer sets. As a sensitive and accurate method, rep-PCR could provide a more comprehensive evaluation of bacterial diversity in bulking sludge.

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