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The microbial community in a high-temperature enhanced biological phosphorus removal (EBPR) process



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ABSTRACT

An enhanced biological phosphorus removal (EBPR) process operated at a relatively high temperature, 28 °C, removed 85% carbon and 99% phosphorus from wastewater over a period of two years. This study investigated its microbial community through fluorescent *in situ* hybridization (FISH) and clone library generation. Through FISH, considerably more *Candidatus* "Accumulibacter phosphatis" (Accumulibacter)-polyphosphate accumulating organisms (PAOs) than *Candidatus* 'Competibacter phosphatis' (Competibacter)-glycogen accumulating organisms were detected in the reactor, at 36 and 7% of total bacterial population, respectively. A low ratio of Glycogen/Volatile Fatty Acid of 0.69 further indicated the dominance of PAOs in the reactor. From clone library generated, 26 operational taxonomy units were retrieved from the sludge and a diverse population was shown, comprising Proteobacteria (69.6%), Actinobacteria (13.7%), Bacteroidetes (9.8%), Firmicutes (2.94%), Planctomycetes (1.96%), and Acidobacteria (1.47%). Accumulibacter are the only recognized PAOs revealed by the clone library. Both the clone library and FISH results strongly suggest that Accumulibacter are the major PAOs responsible for the phosphorus removal in this long-term EBPR at relatively high temperature.

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1. Introduction

Enhanced biological phosphorus removal (EBPR) process is an activated sludge process tailored for phosphorus removal. This process is based on the enrichment of polyphosphate accumulating organisms (PAOs) in the activated sludge community [1]. Till date, *Candidatus* "Accumulibacter phosphatis", hereafter abbreviated as Accumulibacter, are the best known PAOs present in EBPR process. These organisms are able to store phosphate as intracellular polyphosphate via alternating anaerobic-aerobic conditions. Carbon sources, particularly volatile fatty acids (VFAs) are taken up by PAOs anaerobically and stored as polyhydroxyalkanoates (PHAs) using energy generated mostly from hydrolysis of polyphosphate and partly from degradation of glycogen. In the subsequent aerobic condition, a greater amount of phosphorus is taken up to replenish

polyphosphate reserve and accompanied by intracellular organic matter degradation for biomass growth. Phosphorus removal is achieved via removal of PAO biomass from the waste activated sludge.

As in the case of many biological wastewater treatment processes, microbial activity in EBPR process is affected by climates, particularly temperature. Temperature appears to be one of the key factors influencing the PAOs-GAOs (glycogen accumulating organisms) competition in the EBPR process [2–4]. Like PAOs, GAOs take up VFAs anaerobically without performing anaerobic phosphorus release or aerobic phosphorus uptake. When GAOs are present in significant numbers, they will compete with PAOs for carbon sources, which in turn limit the potential of PAOs for aerobic phosphorus uptake [4]. A group of GAOs named *Candidatus* 'Competibacter phosphatis', hereafter named Competibacter, has been commonly found in laboratory- and full-scale EBPR processes [5].

Successful EBPR operation has been observed at very low temperatures, as low as 5 °C [6], though a higher sludge age was necessary due to the decrease in process kinetics at low temperatures. Low temperatures in the range of 10–20 °C, have been found

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to favor the growth of PAOs and thus improve EBPR performance [2,7]. Lopez-Vazquez [8] claimed that at 10 °C, the anaerobic metabolism of GAOs, in particular the anaerobic glycogen hydrolysis, was inhibited and limiting the substrate uptake rate, thus, the growth of GAOs. At higher temperatures (> 20 °C), caused by seasonal variations [9,10] or geographical location [11] deterioration of EBPR capacity was observed. It is hypothesized that PAOs are less competitive than GAOs at higher temperatures. At the laboratory scale, researchers have studied the temperature effects on EBPR and the PAOs-GAOs competition [2,3,7,8,12]. In general, these reports agree that at temperatures higher than 20 °C, the EBPR activity tends to deteriorate and GAOs become dominant. These experimental evidences indicate that the operation of EBPR process in tropical climates could be challenging.

Nevertheless, there have been successful cases of EBPR process operated at 30 °C by Freitas et al. [13] with short sequencing batch reactor (SBR) cycle, and Winkler et al. [14] through selective sludge removal in a segregated aerobic granular biomass system. In our previous study on the long term performance evaluation of a lab scale EBPR process at 28 °C, stable phosphorus removal efficiency of 99% was observed over a period of two years [15]. From these studies, doubts are casted over the predominance of GAOs in high-temperature EBPR systems. Curiosity of the microbial population present that contributes to EBPR is also raised. Although much knowledge of EBPR microbiology has been accumulated over the years, microbial study of high-temperature EBPR system still lacking. Following the success in operating a lab-scale EBPR reactor at 28 °C [15], we further examine the microbial community involved. Firstly, the most recognized *Accumulibacter*-PAOs and *Competibacter*-GAOs were examined through fluorescent *in situ* hybridization (FISH). Secondly, a clone library was generated to assess the microbial community structure of the aforementioned 28 °C EBPR reactor. This work aimed to shed some lights on the bacterial groups that drive the high-temperature EBPR.

2. Materials and methods

2.1. Long term EBPR reactor operation at 28 °C

The EBPR process was operated over a two-year period in a SBR at 28 °C using synthetic wastewater, with solids retention time (SRT) and hydraulic retention time (HRT) set at 10 d and 10 h respectively, as detailed in Ong et al. [15]. The key features were that acetate, yeast extract and peptone were the main carbon sources, constitute to 50 mg C L⁻¹ in the feed. The carbon to phosphorus ratio (C:P) was 3:1.

Under steady state conditions, the process removed over 85% of total organic carbon and 99% phosphate, with phosphorus concentration in the effluent below 1.0 mg L⁻¹. Fig. 1 illustrates the biochemical transformations of a SBR cycle at 28 °C during the two-year operation. Sludge sample was collected from the reactor during steady state for the subsequent chemical and microbial analysis.

2.2. Microbial characterisation with fluorescent *in situ* hybridization (FISH)

Sludge samples were collected periodically and fixed in 4% paraformaldehyde. FISH was performed according to [16] to study the relative abundance of PAOs and GAOs in the microbial community. FISH samples were observed using a fluorescence microscope (Model DM 2500, Leica, German) and images were captured with a cooled charged-coupled device camera (Model DFC 310 FX, Leica, German). The oligonucleotide probes used in this study included 5' FITC labeled EUBmix (i.e., EUB338, EUB338-II, EUB338-III) that targets most of the bacteria, 5' Cy3 labeled PAOmix (i.e.,

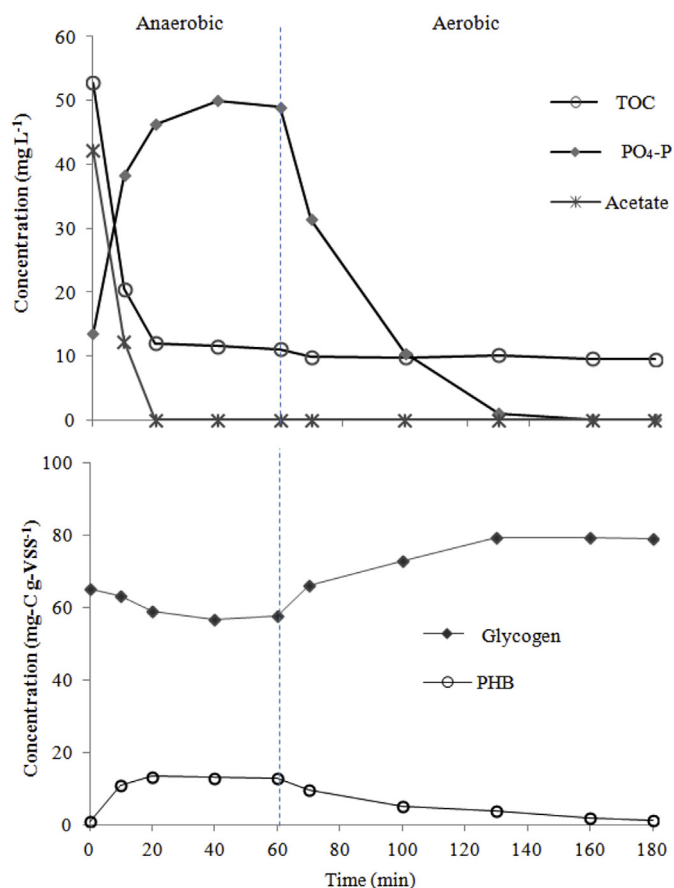


Fig. 1. Typical cyclic concentration profiles of phosphorus, TOC and acetate; and intracellular carbohydrate and PHB in a SBR cycle of day 700 during steady state of reactor operation.

PAO462, PAO651, PAO846) that targets most of the PAOs members in *Accumulibacter* [17] and 5' Cy3 labeled GB probe that targets most of the GAO members in *Competibacter* [17,18]. A minimum of 20 microscopic fields were captured randomly for each sample. FISH quantification of the PAOs and GAOs was done by image analysis software VideoTest- Morphology 5.1. The relative abundance of PAOs or GAOs was determined respectively as the ratio of the mean image areas with a positive signal for PAOmix or GB to the area with a positive signal for EUBmix.

2.3. Microbial characterization through DNA extraction and polymerase chain reaction (PCR) amplification

The sludge was extracted for total genomic DNA using ZR Soil Microbe DNA Micro Preps (Zymo Research, USA) according to the manufacturer's instructions. The DNA template was subjected to PCR by using bacterium specific 11f (5'-GTTTGATCCTGGCTCAG-3') and 1512r (5'-GGYTACCTTGTTACGACTT-3') primers. The PCR mixture contained 4 µL of DNA template, 3 µL of each primer (10 µM), 37.5 µL GoTaq[®] Green Master Mix 2X (Promega, USA), and 27.5 µL of sterile ultra pure water. PCR amplification was carried out in a thermocycler (MyCycler, Biorad, USA) with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min, followed by a final extension at 72 °C for 15 min. The PCR products were then purified by Wizard[®] SV Gel and PCR Clean-Up System (Promega, USA).

2.4. Microbial characterization through 16S rRNA gene clone library construction

Cloning of the purified PCR product was conducted using pGEM-T easy vector system (Promega, USA) and JM 109 Competent Cells (Promega, USA). After blue white screening, a total of 80 colonies were retrieved and sequenced.

2.5. Microbial characterization through DNA sequencing and phylogenetic analysis

Sequencing of the 16S rRNA genes of the clones was carried out by Protech Technology Enterprise Co. (Taiwan) using ABI 3730DNA analyzer (USA). The 16S rRNA sequences found in the sludge were compared with sequences in GenBank database using the BLAST software (www.ncbi.nlm.nih.gov). The closest sequences were aligned with and a phylogenetic tree was constructed by the neighbor-joining method using MEGA 5 [19].

3. Results and discussion

3.1. The abundance of Accumulibacter-PAOs and Competibacter-GAOs

Based on the FISH results, the number of Accumulibacter-PAOs contributed to $32 \pm 4\%$ of the total bacterial population while Competibacter-GAOs contributed $7 \pm 5\%$, as shown in Fig. 2. Throughout the two-year reactor operation, the ratio of Accumulibacter-PAOs population to Competibacter-GAOs population was relatively constant at around five.

We cross-checked the results of the FISH technique by noting that a main metabolic difference between PAOs and GAOs is in their primary energy source for anaerobic carbon uptake. While PAOs obtain energy from the hydrolysis of polyphosphate, GAOs use glycogen as their sole energy source. Thus, in a GAOs-enriched system, larger amount of glycogen is consumed anaerobically. By calculating the ratio of glycogen degradation to VFA uptake during the anaerobic phase (hereafter abbreviated as Gly/VFA), the relative activity of PAOs and GAOs can be estimated. In this study, the Gly/VFA ratio was 0.69, close to the Gly/VFA ratio reported in a few efficient EBPR systems (Table 1). This Gly/VFA ratio was also much lower than 1.12 reported in a GAOs-enriched system [24]. Thus, in addition to the higher abundance of Accumulibacter-PAOs shown by FISH, the low Gly/VFA ratio further supports that our EBPR reactor is a PAOs enriched system.

The success of maintaining a stable population of Accumulibacter-PAOs in a relatively high-temperature EBPR process for a long-term indicates the feasibility of the biological

phosphorus removal technology in warm temperature regions. Freitas et al. [13] and Winkler et al. [14] also shed some light on high temperature EBPR. Freitas et al. [13] found that through short 36-min SBR cycle, with 20 min of anaerobic phase and 10 min of aerobic phase, the sludge became more robust and ready to cope with typical disturbances, such as shock load, and PAOs-GAOs competition. As for Winkler et al. [14], selective removal of GAOs dominated at the top of the sludge bed had proven to be influential to the PAOs-GAOs competition in forming a desired microbial population for EBPR. Their approaches offer possibility to engineer the competition between PAOs and GAOs. The encouraging EBPR performance obtained by us and others [13,14] strongly suggests that the enrichment of PAOs at high temperature is possible with certain operating strategy and conditions. On the other hand, Cao [12] reported a poorly performed EBPR process operated at 30 °C, with 22% of GAOs and Accumulibacter-population fluctuated between 7 and 30% in the total bacterial population and the phosphorus concentration at the end of aerobic condition was in the range of 3.0–6.5 mg L⁻¹. Although Accumulibacter-PAOs were present in Cao's [12] system, they did not seem to contribute much to phosphorus removal. A few other studies also reported that EBPR performance deteriorates at temperatures above 20 °C, hypothesized to result from a shift in the community from PAOs to GAOs [2,3,7]. The reasons for the different EBPR performance in the past studies are not clear yet. It could be due to the seed sludge used which maybe geographically specific, or differences in wastewater composition, operational conditions and so forth. Thus more intensive effort is needed to identify a suitable operation strategy for EBPR at high temperatures.

3.2. The EBPR microbial population from 16S rRNA gene retrieval and phylogenetic analysis

In order to have more detailed taxonomic information on the microbial community involved in our high temperature EBPR process, a clone library of the EBPR sludge was constructed. A total of 80 clones were selected. The partial 16S rRNA sequence of 600–800 bps was identified for each clone. These partial 16S rRNA sequences were grouped into 26 OTUs (operational taxonomy units). The phylogenetic analysis categorized the sequenced clones into 7 groups. The numerically largest bacterial group is the Proteobacteria, accounting for 69.6%, including α -proteobacteria (13.8%), β -proteobacteria (43.6%), γ -proteobacteria (12.3%), followed by Actinobacteria (13.7%), Bacteroidetes (9.8%), Firmicutes (2.94%), Planctomycetes (1.96%), Acidobacteria (1.47%). At the level of class, β -proteobacteria was the dominant group in the EBPR sludge, which was in accordance with previous studies [25]. Table 2 summarizes the clone number of each OTU, the closest sequence

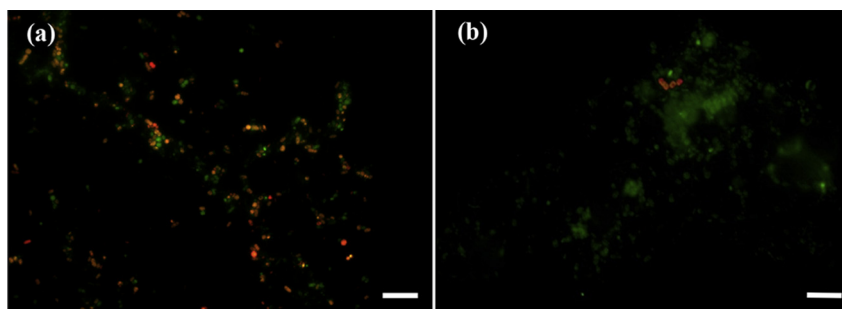


Fig. 2. Overlay FISH images of sludge sample from day 700 of reactor operation showing (a) the PAOs (orange cell clusters) hybridized with both FITC-labelled EUBmix probe (green) and Cy3-labelled PAOmix probe (red); (b) the GAOs (orange cell clusters) hybridized with both FITC-labelled EUBmix probe (green) and Cy3-labelled GB probe (red) (Scale bar = 10 µm).

Table 1

The ratio of anaerobic glycolysis degradation to VFA (Gly/VFA) uptake of a few EBPR systems.

Studies	Carbon source	Gly/VFA	Temperature (°C)
Smolders et al. [20]	Acetate model	0.50	20
This study	Acetate (80% of the total carbon source)	0.69	28
Hesselmann et al. [21]	Acetate	0.60	20
Filipe et al. [22]	Acetate	0.53	22
Lu et al. [23]	Acetate	0.46	20–24

*Gly/VFA expressed in C-mol C-mol⁻¹.**Table 2**

Affiliations of OTUs in the EBPR sludge.

Affiliation	Closest sequences in GenBank	Similarity (%)	OTU	No. of clones
α -Proteobacteria	<i>Rhodobacter</i> sp.	98	SBR1-2_9	3
	<i>Methylocystis</i> sp.	100	SBR1-2_40	2
	<i>Brevundimonas diminuta</i>	97	SBR1-2_86	4
	<i>Mesorhizobium plurifarum</i>	98	SBR1-2_74	2
β -Proteobacteria	<i>Thauera</i> sp.	99	SBR1-2_13	2
	<i>Nitrosomonas</i> sp.	98	SBR1-2_34	4
	<i>Comamonadaceae</i> sp.	98	SBR1-2_47	3
	Uncultured <i>Candidatus</i> Accumulibacter phosphatis SBRA220	99	SBR1-2_27	8
	Uncultured Accumulibacter clone LPU28	98	SBR1-2_110	7
	Uncultured bacterium clone LBP60	95	SBR1-2_1	2
	Uncultured bacterium PHOS-HE 23	94	SBR1-2_91	3
	Uncultured bacterium MO 111_27	99	SBR1-2_22	5
	Uncultured gammaproteobacterium AY172151	97	SBR1-2_11	2
	Uncultured gammaproteobacterium AY172170	99	SBR1-2_76	2
γ -Proteobacteria	Uncultured bacterium PHOS-HE54	99	SBR1-2_61	2
	Uncultured bacterium clone A_SBR_64	94	SBR1-2_6	3
	Uncultured Actinobacteria bacterium	99	SBR1-2_15	5
Actinobacteria	Uncultured <i>Candidatus</i> Microthrix calida strain TNO2-4	94	SBR1-2_105	6
	<i>Runellazeae</i> sp.	99	SBR1-2_10	3
Bacteroidetes	Uncultured Flavobacterium clone HP1A39	95	SBR1-2_25	1
	Uncultured Flexibacteriaceae bacterium	98	SBR1-2_71	2
	Uncultured Bacteroidaceae bacterium	97	SBR1-2_83	2
	Uncultured Sphingobacteriaceae bacterium	96	SBR1-2_53	2
	<i>Bacillus</i> sp.	98	SBR1-2_5	1
Firmicutes	<i>Planctomyces</i> sp. Schlesner 664	99	SBR1-2_52	1
Planctomycetes	Uncultured Acidobacteria bacterium	96	SBR1-2_90	1
Acidobacteria				

found in the NCBI database, and their similarity. The phylogenetic affiliation of the sequences was also analyzed using the neighbor-joining method. Fig. 3 illustrates the phylogenetic tree of the 26 OTUs.

The phylum Proteobacteria encompassed 56 clones forming 18 OTUs. Among the 8 OTUs in the β -proteobacteria, clones SBR1-2_13 and SBR1-2_34 had cultured species *Thauera* sp. and *Nitrosomonas* sp., respectively. Clones SBR1-2_47 and SBR1-2_94 had *Comamonadaceae* sp. as the closest relatives, which is strictly aerobic, non fermentative, capable of accumulating PHB [26] and commonly found in activated sludge and biofilm reactors. The remaining β -proteobacteria affiliated clones were related to sequences of uncultured bacteria represented by SBR1-2_27 and SBR1-2_110, showing > 97.0% similarity with Accumulibacter. We observed a lower abundance of Accumulibacter, 18.7%, by 16S rRNA analysis compared with 32% determined by FISH. This observation reflects the known quantitative bias of clone library and is in accordance with observations in other studies [17].

Four OTUs were found in Gammaproteobacteria. Two of them, viz. SBR1-2_11 and SBR1-2_76, were related to Competibacter-GAOs from the GB lineage. The presence of Competibacter-GAOs in the reactor was also detected through FISH analysis. This correlates well with the earlier claim that the existence of Competibacter is common in lab-scale EBPR process fed with acetate [5,24,27]. Meanwhile, the SBR1-2_61 is closely affiliated with an uncultured bacterium PHOS-HE54 from an aerobic phosphate removal ecosystem [28] and with several uncultured species in the EBPR

systems [5,29]. Among the Alphaproteobacteria, four OTUs were identified. Two had *Rhodobacter* sp. and *Methylocystis* sp. (86.4 and 94.5% similarity, respectively) as their closest relatives. As for the remaining two α -proteobacteria affiliated clones, SBR1-2_86 is closely related to *Brevundimonas diminuta* of little known characteristics, whereas, SBR1-2_74 is closely related to *Mesorhizobium plurifarum*, a common soil bacterium capable of forming nodules at the root and stem of plants.

Several reports claiming that members of *Actinobacteria* accounted for a large proportion of the microbial population in the EBPR process. Although clones of the *Actinobacteria* were detected, they made up only about 14% of the total clones. Both the OTUs identified do not belong to the putative actinobacterial PAO commonly reported [30]. In the phylum Bacteroidetes, one of the OTUs has the cultured bacterium *Runellazeae* as its closest relative with 91.6% similarity; others were related to uncultured environmental clones. Other OTUs clustered to sequences in the families Flavobacteriaceae, Flexibacteriaceae, Bacteroidaceae and Sphingobacteriaceae. The three phyla Firmicutes, Planctomycetes, and Acidobacteria were represented respectively by only one clone namely SBR1-2_5, SBR1-2_52, and SBR1-2_90.

From the clone library, it appears that the only recognized and relatively abundant PAOs exist in our 28 °C EBPR process is Accumulibacter-related. Accumulibacter-PAOs also observed contributing about 36% of total bacteria population according to FISH. Thus, the presence and contribution of Accumulibacter towards the phosphorus removal in the reactor is undeniable. Since

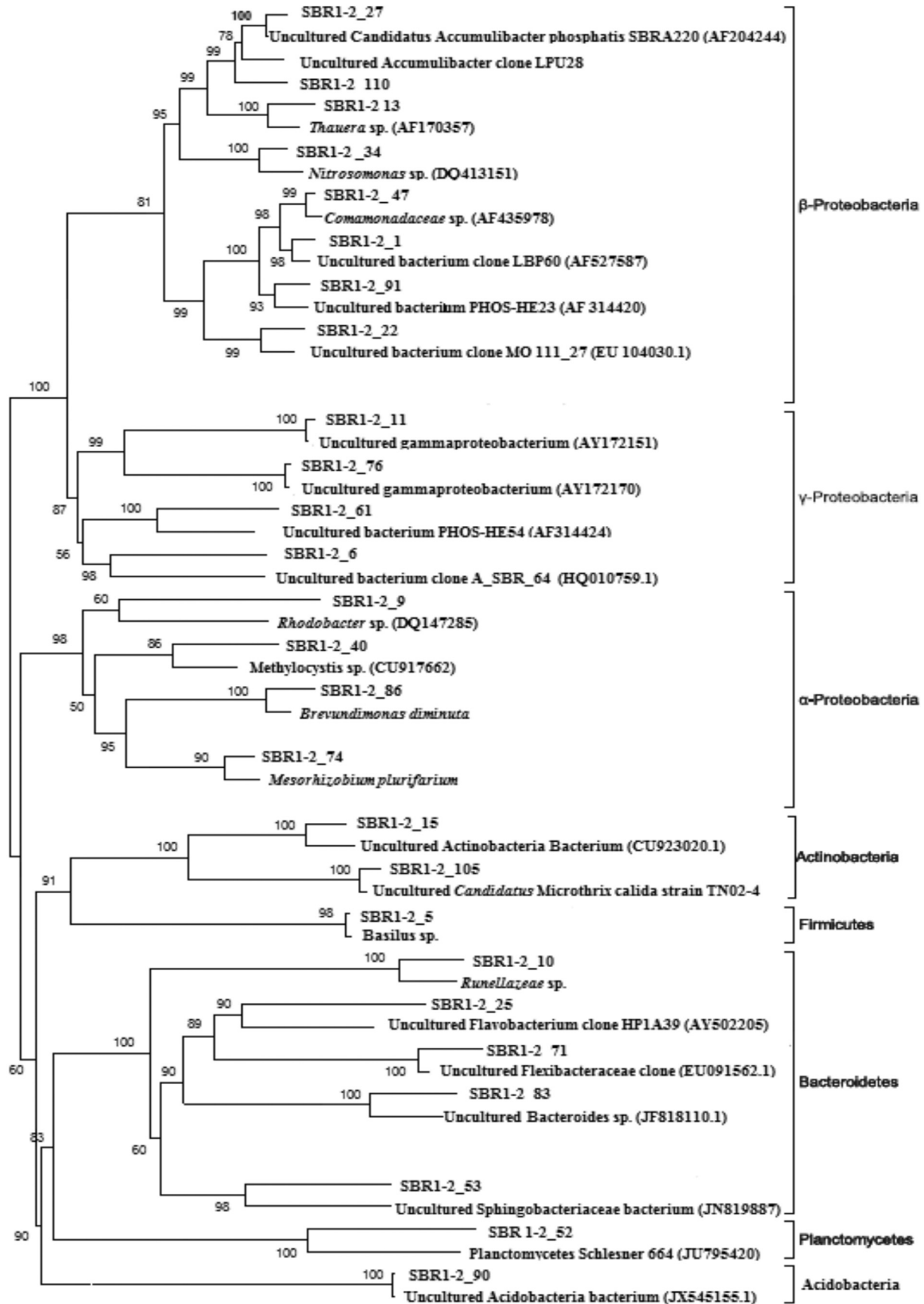


Fig. 3. Neighbor-joining trees deduced from partial sequences of 16S rRNA genes of clones from EBPR sludge sample. Bootstrap confidence values obtained with 1000 replicates are given at the branch plant.

genotypic differences within the *Accumulibacter* lineage are always reported in recent studies using the gene encoding polyphosphate formation, *ppk1* [31–33], it is of great interest to further investigate the subpopulation (or clade) of *Accumulibacter* which contributed to this high-temperature EBPR in the future study. It is also necessary to monitor *Accumulibacter* clade dynamics over time in order to infer and elucidate their effects on the EBPR performance.

4. Conclusions

This work showed the PAOs population and behavior out-competed GAOs by showing good EBPR capacity at 28 °C, a relatively high temperature for EBPR operation. The PAOs to GAOs population balance achieved in this study could have created selection properties to abate the competition between PAOs and GAOs. *Accumulibacter* related PAO could be the only PAO contributed to the 99% of phosphorus removal. Fine scale study of *Accumulibacter* population could further provide more insights into the operational success of this high temperature EBPR process.

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