J. Microbiol. Biotechnol. (2008), **18**(7), 1290–1297

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Fine-Scale Population Structure of Accumulibacter phosphatis in Enhanced Biological Phosphorus Removal Sludge

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Received: December 18, 2007 / Accepted: February 5, 2008

To investigate the diversities of Accumulibacter phosphatis and its polyhydroxyalkanoate (PHA) synthase gene (*phaC*) in enhanced biological phosphorus removal (EBPR) sludge, an acetate-fed sequencing batch reactor was operated. Analysis of microbial communities using fluorescence *in situ* hybridization and 16S rRNA gene clone libraries showed that the population of Accumulibacter phosphatis in the EBPR sludge comprised more than 50% of total bacteria, and was clearly divided into two subgroups with about 97.5% sequence identity of the 16S rRNA genes. PAO *phaC* primers targeting the *phaC* genes of Accumulibacter phosphatis were designed and applied to retrieve fragments of putative *phaC* homologs of Accumulibacter phosphatis from EBPR sludge. PAO *phaC* primers targeting G_{1PAO}, G_{2PAO}, and G_{3PAO} groups produced PCR amplicons successfully; the resulting sequences of the *phaC* gene homologs were diverse, and were distantly related to metagenomic *phaC* sequences of Accumulibacter phosphatis with 75–98% DNA sequence identities. Degenerate NPAO (non-PAO) *phaC* primers targeting *phaC* genes of non-Accumulibacter phosphatis bacteria were also designed and applied to the EBPR sludge. Twenty-four *phaC* homologs retrieved from NPAO *phaC* primers were different from the *phaC* gene homologs derived from Accumulibacter phosphatis, which suggests that the PAO *phaC* primers were specific for the amplification of *phaC* gene homologs of Accumulibacter phosphatis, and the putative *phaC* gene homologs by PAO *phaC* primers were derived from Accumulibacter phosphatis in the EBPR sludge. Among 24 *phaC* homologs, a *phaC* homolog (G1NPAO-2), which was dominant in the NPAO *phaC* clone library, showed the strongest signal in slot hybridization and shared approximately 60% nucleotide identity with the G_{4PAO} group of Accumulibacter phosphatis, which suggests that G1NPAO-2 might be derived from Accumulibacter phosphatis. In conclusion, analyses of the 16S rRNA and *phaC* genes showed that Accumulibacter phosphatis might be phylogenetically and metabolically diverse.

Keywords: EBPR, diversity, *Rhodocyclus*, "*Candidatus* Accumulibacter phosphatis", *phaC*

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Enhanced biological phosphorus removal (EBPR) processes have been widely used to remove phosphate from wastewater; this is done through the accumulation of phosphate inside cells in the form of polyphosphate. A group of bacteria known as polyphosphate-accumulating organisms (PAOs) are enriched through alternating anaerobic/aerobic conditions. Many studies have described the biochemical metabolic mechanisms of these bacteria based mainly on metabolite analyses of the sludge in EBPR systems [5, 14, 16, 22, 27, 31, 39]. The generally accepted hypothesis proposes that PAO first takes up volatile fatty acids (usually assumed to be acetate) during the anaerobic period by using polyphosphate, and stores them inside in the form of polyhydroxyalkanoate (PHA) [30]. The reducing power to form PHA is provided by hydrolysis of glycogen through the glycolytic pathway. The PHA is used as energy and carbon sources for growth in the following aerobic period and to refresh the polyphosphate and glycogen pools. PHA synthase (PhaC), which is encoded by the *phaC* gene and catalyzes 3-hydroxylacyl-acyl CoA to PHA, is considered to be a key enzyme in the PHA synthetic pathway of EBPR metabolism [30, 32, 33]. A significant amount of research efforts have been focused on discovering the identity of PAO, but no PAO showing the characteristics of EBPR mechanisms has been cultivated as a pure culture thus far.

Culture-independent approaches such as 16S rRNA gene-based techniques have identified that an important PAO in

Table 1. Oligonucleotides used for FISH.

Name	Sequence (5'–3')	rRNA target site[a]	Formamide (%)	References
EUB338 I[b]	GCTGCCTCCCGTAGGAGT	388–355	35	[1]
EUB338 II[b]	GCAGCCACCCGTAGGTGT	388–355	35	[7]
EUB338 III[b]	GCTGCCACCCGTAGGTGT	388–355	35	[7]
PAO651[c]	CCCTCTGCCAAACTCCAG	651–668	35	[6]
PAO462[c]	CCGTCATCTACWCAGGGTATTAAC	462–485	35	[6]
PAO846[c]	GTTAGCTACGGCACTAAAAGG	844–863	35	[6]

[a]*E. coli* numbering of 16S rRNA gene
[b]Used as EUBmix
[c]Used as PAOmix

the EBPR processes belongs to the phylogenetically defined *Rhodocyclus* group in the β subclass of the *Proteobacteria* [2, 6, 11, 24, 36]. In addition, fluorescence *in situ* hybridization (FISH) combined with microautoradiography (MAR) has shown the important physiological aspects of the *Rhodocyclus*-related PAO (RPAO) for EBPR in lab-scale reactors, as well as in full-scale plants [6, 20, 35, 40, 41]. Therefore, the *Rhodocyclus*-related PAO was then tentatively named "*Candidatus* Accumulibacter phosphatis" [11]. Recently, some investigations have demonstrated the existence of Accumulibacter phosphatis with different morphotypic and phenotypic traits [3, 10, 20]. In 2006, Garcia Martín *et al.* [9] reported the metagenomic sequencing data of two different activated sludges enriched with Accumulibacter phosphatis, showing that the activated sludges contained two different Accumulibacter phosphatis strains with up to 15% divergence at the nucleotide level.

Therefore, in this study, we investigated the diversity of Accumulibacter phosphatis in EBPR sludge by using PCR-based 16S rRNA gene sequence analysis. In addition, by using PCR primer sets targeting *phaC* genes of Accumulibacter phosphatis as a genetic marker, more resolved population diversities of Accumulibacter phosphatis were studied in order to learn more about the ecology of Accumulibacter phosphatis.

MATERIALS AND METHODS

Reactor Operation

A cylindrical vessel with a 4-l working volume was used for the sequencing batch reactor (SBR); it was operated in 8 h aerobic/anaerobic cycles with 10 days of sludge retention time at 20°C. Microbial inoculum was obtained from an activated sludge treatment plant at POSTECH in the Republic of Korea. Each cycle consisted of 20 min of anaerobic filling, 1 h 40 min of anaerobic reaction, 4 h 20 min of aerobic reaction, 70 min of settling, and a 30 min decanting stage. The feed contained 770 mg/l of sodium acetate, 40 mg/l of NH_4^+-N, and 15 mg/l of PO_4^{3-}-P. The preparation of the synthetic wastewater and the details of the SBR operation have been described elsewhere [17, 19]. The soluble orthophosphate was analyzed using an ICS-1,000 ion chromatograph (Dionex, U.S.A.). Activated sludge samples were collected from the reactor at the end of the aerobic stage.

Fluorescence *In Situ* Hybridization

Fluorescence *in situ* hybridization (FISH) for the analysis of the Accumulibacter phosphatis population was carried out using the method essentially described by Amamm *et al.* [1] and Hugenholtz *et al.* [13]. Generally accepted PAOmix and EUBmix probe sets were used for Accumulibacter phosphatis and eubacteria labeling, respectively (Table 1). The abundance of Accumulibacter phosphatis was determined as the mean image area with a positive signal for both PAOmix and EUBmix relative to the area with a positive signal for EUBmix.

Genomic DNA Extraction and Analysis of 16S rRNA Gene Sequences

Genomic DNA was extracted from the sludge sample using the Fast DNASPIN Kit using a bead-beating procedure (Q-BIO Gene, U.S.A.) according to the instructions of the manufacturer. The effectiveness of the cell lysis procedure was confirmed by microscopic examination of the samples before and after the lysis treatment. The genomic DNA concentration was quantified with an ultraviolet (UV) spectrometer at 260 nm. The PCR reactions of the 16S rRNA gene (~1,500 base-pair product) were performed by using two eubacterial primers, 27f and 1492r [23]. The PCR amplification was performed in a 50-µl thermal cycler (MJ Research, U.S.A.) with 1 µM of primers and 0.1 U of AccuPrime *Taq* polymerase (Invitrogen, U.S.A.). The PCR conditions were modified to minimize the accumulation of the known artifacts (*Taq* errors, chimeras, and heteroduplex molecule) using the following conditions for three replicates for each sample: 3 min (1 cycle); 94°C for 1 min, 55°C for 45 sec, and 72°C for 2 min (18 cycles); 72°C for 5 min (1 cycle) [28, 38]. The three replicates of PCR amplifications were combined, purified using a PCR purification kit (Qiagen, U.S.A.), and resuspended in 40 µl of milliQ water. The resupended PCR products were amplified again by five additional PCR cycles. The product was ligated into the vector pCR2.1 (TOPO TA Cloning; Invitrogen, U.S.A.) by following the protocol recommended by the manufacturer. Clones containing 16S rRNA gene inserts were chosen randomly, and the sequencing of their nucleotides was performed using T7 and M13 reverse sequencing primers.

Phylogenetic Analyses

The almost complete16S rRNA gene sequences obtained (more than 1,450 bases) were compared with available sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the phylogenetic affiliation. These sequences were also checked for their chimeric properties using the Bellerophon program (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) [12]. The 16S rRNA gene sequences were aligned using CLUSTAL W [37] and phylogenic

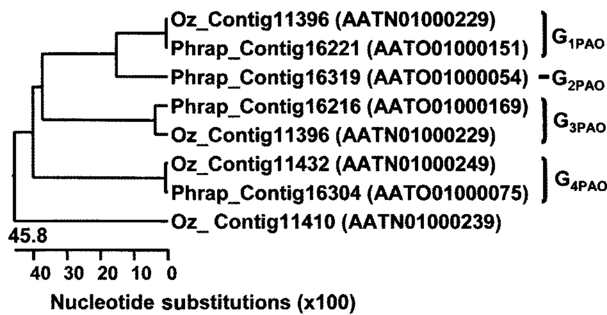

Fig. 1. Phylogenetic analysis of *phaC* gene sequences of Accumulibacter phosphatis by the ClustalV method in the MegAlign program (DNASTAR).

A *phaC* gene sequence belonging to non-Accumulibacter phosphatis bacteria from Oz sludge was used as an outgroup. Sequences of sludge Oz and Phrap included in the tree were metagenomic sequences of Accumulibacter phosphatis. The branch length represents the evolutionary distance.

trees were prepared with DNADIST (Kimura 2-parameter model) in the PHYLIP software, version 3.6 [8], after deleting regions of sequence ambiguity from the analyses.

PCR Amplification of Putative *phaC* Genes

For the PCR amplification of *phaC* gene homologs of Accumulibacter phosphatis (PAO *phaC*), seven whole *phaC* gene sequences of Accumulibacter phosphatis that had been deposited in GenBank were aligned using MegAlign (DNASTAR) software (DNASTAR, U.S.A.), which showed that they could be classified into four groups (Fig. 1). Two oligonucleotide PCR primer sets (PAO *phaC* primers) for each group were designed and named as shown in Table 2. For the PCR amplification of *phaC* gene homologs of non-Accumulibacter phosphatis bacteria (NPAO *phaC*), thirty *phaC* sequences, not including the *phaC* gene sequences of Accumulibacter phosphatis, deposited in GenBank were aligned and four degenerate oligonucleotide primers (NPAO *phaC* primers) were designed (Table 2) according to the method described previously [26]. Homologs of the *phaC* genes were amplified on 100 to 1,000 ng of genomic DNA from EBPR sludge with a touchdown PCR program consisting of an initial 10 min denaturing step at 94°C for 45 sec, 53°C for 45 sec (decreasing 0.5°C per cycle), and 72°C for 1 min. An additional 25 cycles were carried out under the same denaturing and extension conditions, followed by a final 10 min extension at 72°C. The products were ligated into the pCR2.1 vector (TA Cloning; Invitrogen, U.S.A.). Following transformation of plasmids into host cells and blue/white screening, colonies with inserts were verified by PCR with vector-specific primers, ADRAF (5'-GTAACGGCCGCCAGTGTGCT) and ADRAR (5'-CAGTGTGATGGATATCTGCA), that flank the cloning region. Approximately 100 clones from the library were screened by using restriction fragment length polymorphism (RFLP) analysis to identify unique sequence types [18]. The amplicons were digested with HaeIII and HhaI, and the fragment patterns were analyzed on 3% MetaPhore agarose (BioWhittaker, Molecular Applications, U.S.A.) gels with a 100 bp ladder (Bioneer, Republic of Korea) as a size marker. Clones were grouped according to their RFLP patterns, and representative clones containing unique RFLP patterns were sequenced. The retrieved *phaC* gene fragments were compared with available sequences by using the BLASTX network service on the

Table 2. PCR primers targeting *phaC* genes used in this study.

Targets organisms	Primer names	Sequence (5'–3')[a]	Estimated size (bp)	PCR products
Accumulibacter phosphatis	G1PAO1F1	GCTTCCTGCTCAATCAGCAATG	908	+
	G1PAO1R1	CATCAGGTCGAACATCGGTTC		
	G1PAO2F2	GGCCTATCTTGACTGGATG	1,260	+
	G1PAO2R2	GCAATGGATGTTGCTGATCG		
	G2PAO1F1	CTTCTGGATCAGCAGTGGTG	1,040	+
	G2PAO1R1	GCAGTGGATGTTGCTGATCG		
	G2PAO2F2	GCAAGACCATCGATTCGAG	1,276	–
	G2PAO2R2	CCATCTGAAACTTGCGTCG		
	G3PAO1F1	GATTCTTCCCGCTGACGTTGC	867	+
	G3PAO1R1	CAGTTCCTTGAGGTCGACCTTG		
	G3PAO2F2	CAGCAATCTCACCAACCTGAC	804	+
	G3PAO2R2	CAGTTCCTTGAGGTCGACCTTG		
	G4PAO1F1	AAGGCCTTCATGGACGTCTC	927	–
	G4PAO1R1	GCCGGTGTCCGAAAAGTCAA		
	G4PAO2F2	AAGGCCTTCATGGACGTCTC	1,460	–
	G4PAO2R2	GGTAGCACCGCTGAGGAAATC		
Non-Accumulibacter phosphatis bacteria	G1NPAOF	GTSCCGCCSTGSATCAACAAGT	584	+
	G1NPAOR	TCBHGTTCCAGWACAGYAG		
	G2NPAOF	ATCRRCMRRTWCTACMTYYTSGA	444	–
	G2NPAOR	ASAYCAGGTCRTTSGVSCGCA		
	G3NPAOF	CCCTKSATCAACAAGTWCTACAT	780	+
	G3NPAOR	GGRKYGAYGAYGCCGGCGAT		
	G4NPAOF	GGYTAYTGYATKGGTGGTACG	476	–
	G4NPAOR	CCAHRRWGCKATATGSTCTTC		

[a]R=A/G, Y=C/T, M=A/C, K=G/T, S=G/C, W=A/T, H=A/T/C, B=G/T/C.

NCBI Web site. The sequences belonging to the PHA synthase gene homologs were aligned, and their dendrograms were constructed by using the MegAlign method available in the DNASTAR software (DNASTAR, U.S.A.).

Slot-Blot Hybridization

Slot hybridizations were carried out as described previously in detail [17, 29]. Twenty *phaC* gene homologs from clones were amplified using vector-specific primers, ADRAF and ADRAR, as described above. The resultant PCR products were purified using a PCR purification kit (Qiagen, U.S.A.). The purified PCR products were carefully quantified spectrophotometically at 260 nm, and were then adjusted to a concentration of 10 ng/μl. In preparation for blotting, 200 μl of the PCR products were denatured with 4 μl of 10 M NaOH and 35 μl of 20×SSC (3.0 M NaCl and 0.3 M sodium citrate, pH 7.0) for 10 min at 80°C, followed by snap-cooling on ice and the addition of 40 μl of 1 M Tris-HCl (pH 7.5). A Schleicher & Schuell Minifold II slot-blot manifold (Schleicher & Schuell, Germany) was used to apply 250 μl of denatured PCR products per spot to a nylon membrane (Biodyne B 0.45 μ; Pall Corporation, U.S.A.). Genomic DNA from EBPR sludge was used as a positive control. Membranes were baked at 80°C for 2 h, and were prehybridized at 65°C in buffer containing 50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml salmon sperm DNA (Sigma, U.S.A.). One μg of the genomic DNA from EBPR sludge for probe labeling was incubated at 95°C for 2 min, and was then rapidly chilled on ice to denature the doubled DNA strands. Denatured genomic DNA was randomly labeled with ^{32}P using the Prime-a-gene Labeling System Kit (Promega, U.S.A.) according to the instructions of the manufacturer. Southern hybridizations were performed according to standard protocols [21, 34].

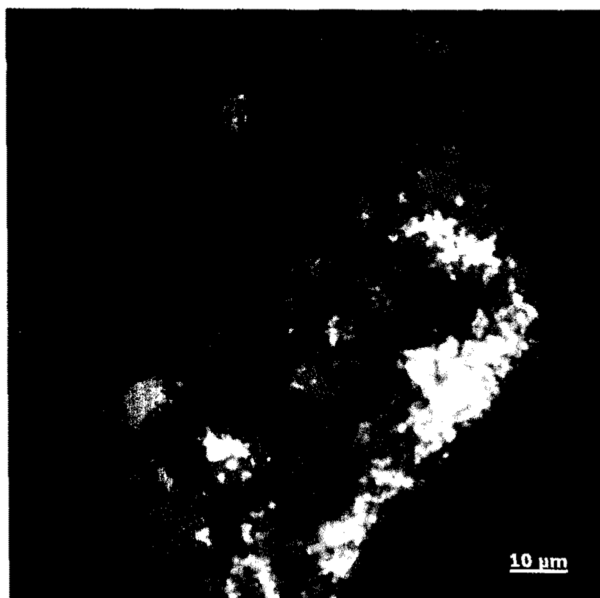

Fig. 2. FISH photomicrograph of the sludge sample showing cells labeled with EUBmix (FITC, green color) and PAOmix (Cy3, red color).

Yellow color indicates Accumulibacter phosphatis. Green color indicates Eubacteria, yellow. Scale bar: 10 μm

RESULTS

SBR Operation and FISH Analysis

Phosphate release during the anaerobic period and phosphate uptake during the aerobic period gradually increased over the SBR operation time, and complete EBPR was accomplished after approximately 60 days (data not shown). The SBR was continuously operated for more than 8 months with acetate as the sole carbon source. The EBPR sludge was sampled at steady state while exhibiting relatively constant mixed liquor suspended solid (MLSS, approx. 3,000 mg/l) and phosphate and carbon compound profiles. Previously developed oligonucleotide probes were used to assess the population density of *Rhodocyclus*-related PAO (Accumulibacter phosphatis) in the EBPR sludge (Table 1). Microorganisms labeled with the PAO probe mix dominated the EBPR sludge, comprising more than 50% of cells labeled with the EUB probe mix (Fig. 2).

Microbial Community Analysis Using an rRNA Gene Clone Library

To further investigate the taxonomic information for these Accumulibacter phosphatis strains, a 16S rRNA gene clone library of the EBPR sludge gene was constructed. Twenty-

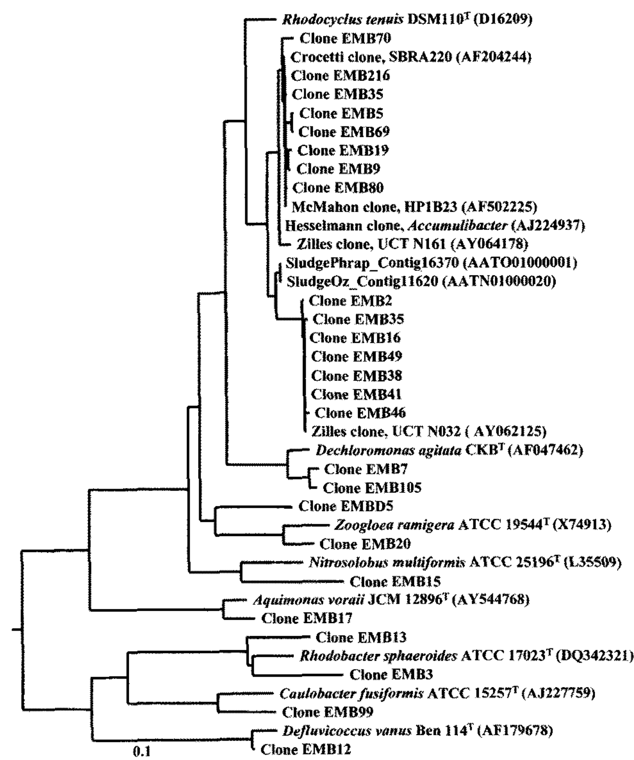

Fig. 3. Phylogenetic analysis of 16S rRNA gene sequences derived from EBPR sludge based on the neighbor-joining method, and showing the diversity of Accumulibacter phosphatis.

Flavobacterium aquatile DSM 1132^T was used as an outgroup (not shown). The scale bar is equal to 0.1 changes per nucleotide position.

five clones with correct DNA inserts were chosen randomly from the clone library, and their almost full 16S rRNA gene sequences were subsequently determined. Their phylogenetic relationships with closely related bacteria are shown in the phylogenetic tree (Fig. 3). Clones affiliated with the sequences of the Accumulibacter lineage were dominant in the EBPR sludge, and were clearly divided into two subgroups, with approximately 97.5% of 16S rRNA gene sequence similarity.

Retrieval of *phaC* Homologs of Accumulibacter phosphatis

With eight primer sets designed to amplify *phaC* homologs of Accumulibacter phosphatis from the EBPR sludge (Table 2), touchdown PCR approaches were applied. Among the eight primer sets, five primer sets were successfully used for the amplification of PCR products with appropriate sizes (Table 2), and these PCR products were used to construct clone libraries. A total of 95 putative *phaC* clones from the libraries were screened through RFLP analysis by double digestion with HhaI and HaeIII, resulting in a total of sixteen clones with distinct RFLP patterns. The representative clones with unique RFLP patterns were sequenced, and their nucleotide sequences were compared with known *phaC* gene sequences of Accumulibacter phosphatis that were identified in Australian and U.S.A. sludge [9] to identify their correlation at the nucleotide level. Sixteen sequenced *phaC* gene homologs were affiliated into three *phaC* clusters of Accumulibacter phosphatis (G_{1PAO}, G_{1PAO}, and G_{3PAO}), and none of the sequences were affiliated with *phaC* homologs of the G_{4PAO} group (Fig. 4). The putative *phaC* gene sequences derived from the EBPR sludge were diverse, and were found to be related to the *phaC* sequences from metagenomic data of Accumulibacter phosphatis [9], with 75–98% nucleotide identities.

Retrieval of PhaC Homologs of Non-Accumulibacter phosphatis Bacteria

To amplify partial *phaC* genes from non-Accumulibacter phosphatis bacteria in EBPR sludge, four degenerate PCR primers (G1NPAO, G2NPAO, G3NPAO, and G4NPAO) were designed on the basis of almost all of the *phaC* sequences deposited in GenBank, not including the *phaC* gene sequences of Accumulibacter phosphatis (Table 2). The primers were then applied to touchdown PCR approaches to amplify

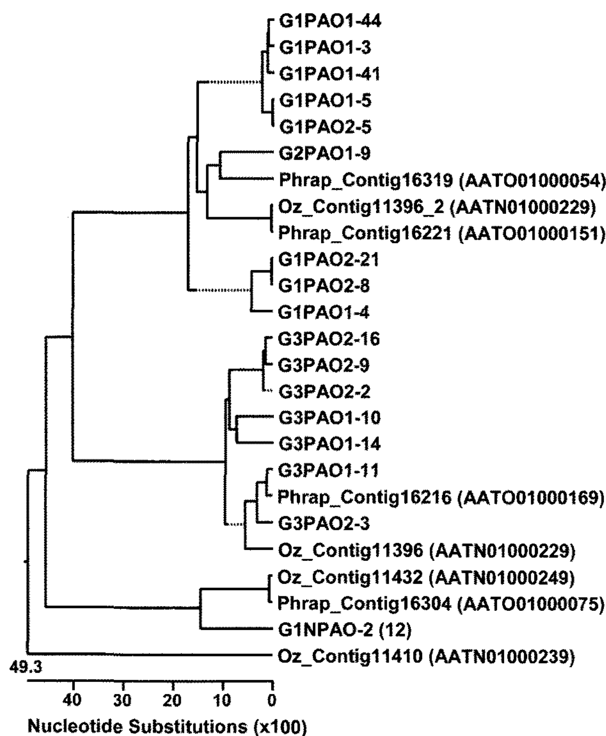

Fig. 4. Phylogram indicating the inferred relatedness of *phaC* gene homologs from the Accumulibacter clade.

Names originated from PAO *phaC* PCR primer sets that were used for library constructions. G1NPAO-2 (12) was a *phaC* gene sequence of clones derived from degenerate PCR primers targeting non-Accumulibacter phosphatis, which was the dominant group and showed the strongest signal in Southern hybridization (Fig. 6). The tree was obtained by the Clustal method using DNASTAR software.

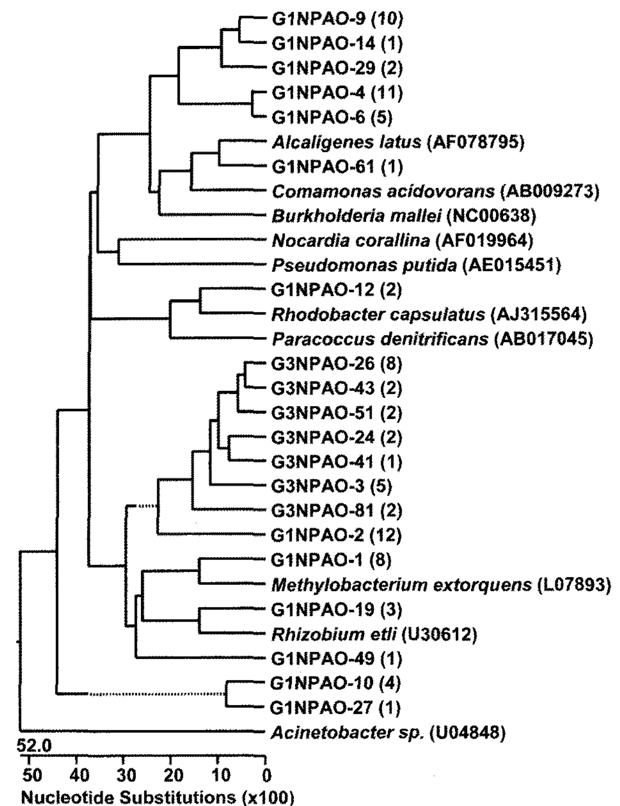

Fig. 5. Phylogram indicating the inferred relatedness of *phaC* gene homologs from the non-Accumulibacter bacteria. Names originated from NPAO *phaC* PCR primer sets for library constructions.

Numbers in parentheses indicate frequencies of colonies exhibiting the same restriction pattern. The tree was obtained by the Clustal method using DNASTAR software.

phaC genes from non-Accumulibacter phosphatis bacteria. Of the four primer sets, G1NPAO and G3NPAO primer sets produced PCR amplicons with predicted sizes of around 580 bp and 780 bp, respectively, and these PCR products were used to construct clone libraries. Sixty-one and 22 *phaC* clones from the libraries using G1NPAO and G3NPAO primer sets, respectively, were randomly chosen by the blue-screening method and screened by RFLP analysis of PAO *phaC* gene homologs of Accumulibacter phosphatis. Twenty-four nucleotide sequences with unique fragment patterns from the libraries were sequenced, and were described as *phaC* gene homologs through a BLAST search in GenBank. Their relationships were subsequently analyzed by the construction of a dendrogram tree using MegAlign (DNASTAR) software (Fig. 5). Two sets of genes were classified as the same type because they shared ≥95% identity at the nucleotide sequence level, resulting in twenty groups of *phaC* gene homologs. The putative *phaC* homologs derived from four degenerate PCR primers targeting non-Accumulibacter phosphatis bacteria shared low identity (less than 75%) to known *phaC* sequences of pure cultured microorganisms from GenBank. The G1NPAO-2 clone was one of the major groups in the clone library, and when twenty *phaC* homologs were compared with those from primer sets targeting Accumulibacter phosphatis, the G1NPAO-2 *phaC* homolog was most closely related to the PAO *phaC* gene sequences (G_{4PAO} group) of Accumulibacter phosphatis, with approximately 60% sequence identity (Fig. 4).

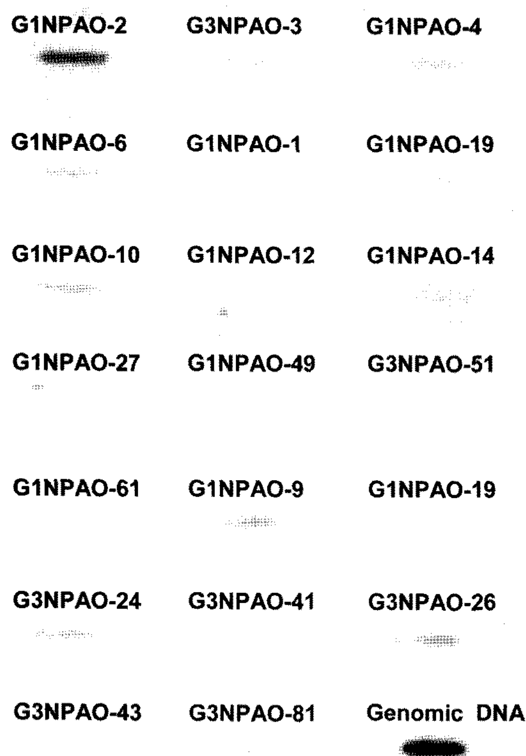

Fig. 6. Slot hybridization of 20 groups of *phaC* gene homologs. Total genomic DNA extracted from EBPR sludge was used as a probe and a positive control.

Slot-Blot Hybridizations

Slot hybridizations were performed to investigate the presence of *phaC* gene sequences belonging to Accumulibacter phosphatis among twenty *phaC* gene homologs derived from four degenerate PCR primers targeting non-Accumulibacter phosphatis in EBPR sludge. Twenty *phaC* gene homologs were PCR-amplified and carefully quantified spectrophotometrically at 260 nm. The quantified PCR amplicons were used as templates, and the probe was the genomic DNA extracted from EBPR sludge enriched with Accumulibacter phosphatis. The results indicated that the G1NPAO-2 clone, which was one of the major groups in the clone libraries and was closely related to the G_{4PAO} group of Accumulibacter phosphatis, showed the strongest signal intensity (Fig. 6). This finding demonstrated that the *phaC* homolog of G1NPAO-2 might be one of the PAO *phaC* gene homologs belonging to Accumulibacter phosphatis.

DISCUSSION

EBPR is more economical and has a lower environmental impact than chemical phosphate removal, but it sometimes happens to unpredictably break down owing to the loss or reduced activity of microbial populations responsible for EBPR [4, 15, 28]. Therefore, elucidation of the microbial communities responsible for EBPR and their function is a prerequisite to understanding the EBPR mechanism and to maintaining stable EBPR processes. Although none of the PAOs has yet been cultured, molecular methods based on 16S rRNA gene sequences such as clone library and FISH approaches have been used to investigate PAO population diversities [2, 25, 40], and have concluded that an important PAO in the EBPR processes is Accumulibacter phosphatis, which belongs to the *Rhodocyclus* group of the *Betaproteobacteria*. However, 16S rRNA gene approaches cannot be used to study the fine-scale population structure of Accumulibacter phosphatis because of the limited phylogenetic resolution of 16S rRNA gene sequences. In fact, although Accumulibacter phosphatis forms a nearly unified group with more than 98.5% 16S rRNA sequence similarities, recent investigations have shown the existence of Accumulibacter phosphatis with different morphotypic and phenotypic traits [3, 10, 20]. For example, Carvalho *et al.* [3] reported that EBPR sludge in SBR contained two microorganisms with distinct morphotypes and physiological properties, which were labeled together by PAOmix probe sets. In addition, metagenomic-sequencing results of EBPR sludge enriched with Accumulibacter phosphatis also showed that two different Accumulibacter phosphatis bacteria were present, with up to 15% divergence at the nucleotide level [9].

Along with polyphosphate, PHA is an important component in relation to polyphosphate accumulating organisms in EBPR systems. PHA is present in numerous eubacteria and members of the family Halobacteriaceae of the *Archaea*. In most of these organisms, the PHA synthase gene (*phaC*) is thought to be the enzyme that is primarily responsible for PHA synthesis. Therefore, in this study, the fine-scale diversity of Accumulibacter phosphatis was investigated using 16S rRNA gene and *phaC* gene sequences as genetic markers.

The libraries of 16S rRNA genes were constructed by using a PCR approach on genomic DNA extracted from EBPR sludge. FISH analysis displayed a high abundance of Accumulibacter phosphatis bacteria in the EBPR sludge. However, analysis of the 16S rRNA gene clone library showed that clones affiliated with Accumulibacter phosphatis in the phylogenetic tree were clearly divided into two clusters, with around 97.5% nucleotide similarity. This indicates that the EBPR sludge contained at least two different Accumulibacter phosphatis bacteria that might have different physiologies and morphologies, as reported previously [3, 10, 20]. Unlike the 16S rRNA gene sequences, the *phaC* genes may be a powerful genetic marker to reveal the fine-scale diversity of Accumulibacter phosphatis bacteria.

PAO *phaC* PCR primer sets targeting *phaC* genes of Accumulibacter phosphatis were designed on the basis of known metagenomic *phaC* gene sequences of Accumulibacter phosphatis [9], and *phaC* gene libraries of the genomic DNA from EBPR sludge were constructed using these primers (Fig. 4). The putative *phaC* homologs were diverse and were related with the *phaC* sequences of Accumulibacter phosphatis, with around 75–98% nucleotide identity, which indicated that Accumulibacter phosphatis bacteria are more diverse than previously thought.

To amplify the *phaC* genes of non-Accumulibacter phosphatis bacteria in EBPR sludge, four degenerate PCR primers (NPAO *phaC* primers; G1NPAO, G2NPAO, G3NPAO, and G4NPAO) were designed on the basis of almost all of the *phaC* sequences deposited in GenBank, except for the *phaC* gene sequences of Accumulibacter phosphatis (Table 2). The putative *phaC* homologs derived from PAO *phaC* primers were completely different from those derived from NPAO *phaC* primers with less than 40% identity, which suggests that the PAO *phaC* primers might be specific to the amplification of *phaC* genes of Accumulibacter phosphatis, and the *phaC* homologs from PAO-*phaC* primers may have been derived from Accumulibacter phosphatis bacteria. In conclusion, we found that EBPR sludge contained at least two different Accumulibacter phosphatis bacteria based on 16S rRNA gene sequences. In addition, analysis of PAO *phaC* genes showed that Accumulibacter phosphatis might be more phylogenetically and metabolically diverse. This finding is important for obtaining a better understanding of the microbiodiversity of Accumulibacter phosphatis and EBPR processes.

Acknowledgments

This study was supported by grants from the MOST/KOSEF to the Environmental Biotechnology National Core Research Center (Grant No.: R15-2003-012-02002-0) and to the 21C Frontier Microbial Genomics and Application Center Program (Grant No.: MG05-0104-4-0), Ministry of Science and Technology, Korea.

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