Identification of a novel subgroup of uncultured gammaproteobacterial glycogen-accumulating organisms in enhanced biological phosphorus removal sludge

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INTRODUCTION

Enhanced biological phosphorus removal (EBPR) has been applied in many wastewater treatment plants to decrease the amount of phosphorus that causes eutrophication in surface waters. EBPR is achieved through the enrichment of a group of polyphosphate-accumulating organisms (PAO), generally known as ‘Candidatus Accumulibacter phosphatis’ (Accumulibacter), in activated-sludge systems using alternating anaerobic and aerobic cycles. According to the commonly accepted EBPR model proposed by Mino et al. (1987), short-chain volatile fatty acids, usually acetate or propionate, are taken up and converted to polyhydroxyalkanoates (PHA) by PAO during the anaerobic phase. In the subsequent aerobic phase, with oxygen as an electron acceptor and in the absence of external carbon sources, the internally stored PHA is used for cell growth and maintenance as well as polyphosphate accumulation in excess of levels normally required to satisfy the metabolic demand for growth (Oehmen et al., 2007; Seviour & McClroy, 2008).

Another group of micro-organisms, glycogen-accumulating organisms (GAO), often coexist with PAO, and their presence has been hypothesized to be a cause of deterioration in EBPR processes (Cech & Hartman, 1993; Bond et al., 1995; Liu et al., 1996; Mino et al., 1998; Seviour...
et al., 2003). Like PAO, GAO proliferate under alternating anaerobic and aerobic conditions. However, they use glycogen as their primary energy source for anaerobic volatile fatty acid uptake and PHA synthesis. Also, aerobic PHA oxidation in these organisms leads only to biomass growth and glycogen replenishment, not to polyphosphate storage (Liu et al., 1994; Satoh et al., 1994; Mino et al., 1995). These GAO are diverse and have been found in numerous lab-scale and full-scale EBPR processes. Some uncultured members of Alphaproteobacteria and Gammaproteobacteria have been proposed as GAO candidates, showing biochemical properties consistent with typical GAO metabolisms. Alphaproteobacterial GAO are closely related to the genera Sphingomonas and Defluvicoccus and have been assumed to play an important role in EBPR deteriorations (Wong et al., 2004; Meyer et al., 2006; Burrow et al., 2007; McLrroy et al., 2010).

Gammaproteobacterial GAO (GB) known as ‘Candidatus Competibacter phosphatis’ (Competibacter) have been studied intensively and are widely present in lab- and full-scale EBPR processes (Nielsen et al., 1999; Crocetti et al., 2002; Kong et al., 2002, 2006). All GB reported up to now share a similar morphology of Gram-negative large coccobacilli of 2–4 μm, although they are phylogenetically very diverse (Crocetti et al., 2002; Kong et al., 2002, 2006; Schroeder et al., 2009). Kong et al. (2002) showed that GB could be clustered into seven subgroups using 14 existing and 18 newly retrieved 16S rRNA gene sequences. They designed a set of fluorescent in situ hybridization (FISH) probes targeting the GB subgroups at different hierarchical levels. In this study, a new subgroup (GB8) of GB that was not hybridized by the known GB-targeting FISH probes was discovered. FISH probes targeting GB8 and all GB subgroups including GB8 were designed, and the ecophysiology and distributions of these organisms in lab- and full-scale plants were investigated using FISH and/or microautoradiography (MAR) approaches.

**METHODS**

**Operation of the sequencing batch reactor.** A cylindrical vessel with a 4 l working volume was used for the sequencing batch reactor (SBR) operation. Activated sludge for the microbial inoculum was obtained from a wastewater treatment plant at the campus of POSTECH, Kyungbuk, Korea. Synthetic wastewater was used in the experiment. The feed of the synthetic wastewater contained 770 mg sodium acetate l⁻¹, 40 mg NH₄Cl-N l⁻¹, 15 mg PO₄-P l⁻¹, and trace element mixtures (Jeon & Park, 2000). The SBR was operated in 8 h anaerobic/aerobic cycles at 20 °C. More details of SBR operation have been given previously (Jeon et al., 2003a). In brief, each cycle consisted of 20 min of anaerobic filling, 1 h 40 min of anaerobic reaction, 4 h of aerobic reaction, 90 min of settling and 30 min of decanting. Two litres of clarified supernatant was withdrawn at the end of the settling phase. Mean sludge retention time was maintained at approximately 10 days by withdrawing a small portion of sludge from the reactor at the end of the aerobic phase. Soluble orthophosphate was analysed using an ICS-1000 ion chromatograph (Dionex).

**DNA extraction and phylogenetic analysis.** Genomic DNA from sludge samples of the SBR was extracted using a FastDNA SPIN Kit (Qbiogene) according to the manufacturer’s instructions. Bacterial 16S rRNA gene clone libraries were constructed as described previously (Kim et al., 2010). Briefly, modified two-step PCR amplification was used to reduce chimeric products using primers 271f and 1492r (Lane, 1991; Lu et al., 2006; Wang et al., 2008). PCR products were purified and ligated into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) according to the manufacturer’s instructions. After blue–white screening of the colonies, inserted 16S rRNA genes of 114 clones were amplified using vector-specific primers that flank the cloning region (Jeon et al., 2003b) and were analysed according to restriction fragment length polymorphism (RFLP) analysis after HaeIII and HhaI double digestions using previously described methods (Kim et al., 2008). All representative clones (44 clones) which showed unique fragment patterns were partially sequenced with the M13 reverse primer of the TOPO cloning kit. Resulting sequences were submitted to GenBank for BLASTN searches. If representative sequences were related to Gammaproteobacteria, the clones were more completely sequenced to determine their potential diversities. The chimeric properties of the 16S rRNA gene sequences were checked using the Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl, DeSantis et al., 2006). The resulting sequences were compared with available Competibacter 16S rRNA gene sequences of Gammaproteobacteria from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were aligned using the SILVA server (http://www.arb-silva.de, Pruesse et al., 2007). A phylogenetic tree using the neighbour-joining (NJ) algorithm was constructed by the PHYLIP software (version 3.6, Felsenstein, 2002). The resulting tree topology was evaluated using bootstrap analysis based on 1000 resamplings. Maximum-likelihood analysis was also performed using RAxML-HPC on ABe (version 7.2.6) of the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; Stamatakis et al., 2005) at the San Diego Supercomputer Center.

**FISH probe design and evaluation.** Oligonucleotide FISH probes and competitors targeting the new GB subgroup (GB8) and whole GB subgroups were designed using the probe design tool based on comparative analyses of reliable sequences with >1200 bp and our clone sequences in the ARB software package (Ludwig et al., 2004). Specificities of the FISH probes were subsequently confirmed using Probe Match on the website of the Ribosome Database Project (RDP Release 10, Cole et al., 2009). The designed oligonucleotides were synthesized and labelled at the 5′ end with FITC or 3-Iodocyanine dye (Cy3) by Thermo (Ulm, Germany). Paraformaldehyde (PFA)-fixed sludge samples were used to optimize formamide (FA) concentrations in FISH experiments because GB cannot be cultured. FA concentrations for optimum probe stringency were determined empirically by performing a series of FISH experiments at 5 % FA increments from 15 % to 70 % FA at set hybridization and wash temperatures (Hugenholz et al., 2002). Subsequently, these designed probes were simultaneously and/or independently used with generally accepted FISH probes of PAOmix, EUBmix, GB, GB_G1 and GB_G2, and appropriate competitors. The FISH probes and competitors used in this study are listed in Table 1.

**FISH analyses.** For FISH analyses of GB subgroups, sludge samples collected from the SBR at the end of the anaerobic phase were fixed with 4 % paraformaldehyde/PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.2) for 4 h at 4 °C. Fixed sludge samples were washed twice in PBS, resuspended in PBS (ethanol (1 : 1, v/v)), and then stored at −20 °C until FISH analyses. Fixed sludge samples were homogenized using a homogenizer (Dispergierantrieb Top10 basic; IKA, Germany) at 45 s for three cycles at maximum speed prior to the FISH experiments in order to remove cell aggregates. Fish experiments were performed on poly-l-lysine-coated slides (Sigma).
GTTAGCTACGGCACTAAAAGG
CCCTCTGCCAAACTCCAG
CCGTCATCTACWCAGGGTATTAAC

sodium
anaerobic phases were anaerobically and aerobically incubated with
samples collected from the SBR at the ends of the aerobic and

1999; Nielsen

The abundance of the probe-targeted cells was
images, and Nomarski photographs were also collected simultaneous to
AxioVs40 V 4.7.1.0 (Zeiss) by the combination of the three captured

camera (Zeiss). Enhanced FISH images were obtained using
HBO100 mercury vapour short-arc lamp, filter-sets no. 10 and no. 20
using a Zeiss Axiophot epifluorescence microscope equipped with an
of three images per specimen were captured at
analyses were mounted in Citifluor (Citifluor, UK) and viewed. A total
specific oligonucleotide binding (Amann
et al.

FISH/MAR analyses. To evaluate the abilities of GB8 to take up
acetate and phosphorus, combined FISH and MAR experiments were
 carried out according to previously described procedures (Lee et al.,
1999; Nielsen et al., 2003; Kong et al., 2004; Kim et al., 2010). Sludge
samples collected from the SBR at the ends of the aerobic and
anaerobic phases were anaerobically and aerobically incubated with
sodium [1-14C]acetate (Amersham) and 32P (Perkin Elmer), each at a
concentration of 10 μCi (370 kBq) per mg suspended solids (Lee
et al., 1999; Kim et al., 2010). All anaerobic preparations of the sludge
samples were carefully flushed with O2-free N2. For FISH/MAR, FISH
hybridization methods were the same as those described for FISH
analyses except for the use of coverslips instead of poly-L-lysine-
hybridization methods were the same as those described for FISH

Table 1. Information relevant to the FISH oligonucleotides designed and used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence (5′−3′)</th>
<th>Target organisms</th>
<th>Competitors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GB429*</td>
<td>CCCCACCTAAAGGGCTTTT</td>
<td>GB8</td>
<td>GB429_C1 and 2</td>
<td>This study</td>
</tr>
<tr>
<td>GB429_C1†</td>
<td>CCCCACCTAAAGGGCTTTT</td>
<td>GB1−7</td>
<td>GB429_C1</td>
<td>This study</td>
</tr>
<tr>
<td>GB429_C2†</td>
<td>CCCCACCTAAAGGGCTTTT</td>
<td>GB1−7</td>
<td>GB429_C2</td>
<td>This study</td>
</tr>
<tr>
<td>GB742*</td>
<td>CTACGCTCAGTGGTGGCC</td>
<td>GB1−7</td>
<td>GB_C</td>
<td>Kong et al. (2002)</td>
</tr>
<tr>
<td>GB</td>
<td>CATTCTCTAGGGCCTAC</td>
<td>GB1−7</td>
<td>GB_C</td>
<td>This study</td>
</tr>
<tr>
<td>GB_C†</td>
<td>CATTCTCTAGGGCCTAC</td>
<td>GB8</td>
<td>GB_C</td>
<td>Kong et al. (2002)</td>
</tr>
<tr>
<td>GB_G1‡</td>
<td>TCCCTCAGGATGTCAGGCC</td>
<td>GB1−4, 6</td>
<td>GB_G1_C</td>
<td>Crocetti et al. (2002)</td>
</tr>
<tr>
<td>GB_G1_C‡</td>
<td>TCCCTCAGGATGTCAGGCC</td>
<td>GB1−4, 6</td>
<td>GB_G1_C</td>
<td>Crocetti et al. (2002)</td>
</tr>
<tr>
<td>GB_G2</td>
<td>TCCCTCAGGATGTCAGGCC</td>
<td>GB5 and 7</td>
<td>GB_G2_C</td>
<td>Crocetti et al. (2002)</td>
</tr>
<tr>
<td>GB_G2_C‡</td>
<td>TCCCTCAGGATGTCAGGCC</td>
<td>GB5 and 7</td>
<td>GB_G2_C</td>
<td>Crocetti et al. (2002)</td>
</tr>
<tr>
<td>EUB338§</td>
<td>GCTCCGCTCGGCTAGGAGT</td>
<td>Eubacteria</td>
<td></td>
<td>Amann et al. (1995)</td>
</tr>
<tr>
<td>EUB338I§</td>
<td>GCAGGAGCCTATAGGCTG</td>
<td>Eubacteria</td>
<td></td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB338II§</td>
<td>GCTCCGCTCGGCTAGGCTG</td>
<td>Eubacteria</td>
<td></td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>NonEub338</td>
<td>CGAGGAGGCGATCCTCA</td>
<td>Nili</td>
<td></td>
<td>Amann et al. (1995)</td>
</tr>
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<td>PAO651§</td>
<td>CCCCCTGCCAACCTCCAG</td>
<td>Accumulibacter</td>
<td></td>
<td>Crocetti et al. (2000)</td>
</tr>
<tr>
<td>PAO662§</td>
<td>CGCTGCTACWCAGGGTATTAAAC</td>
<td>Accumulibacter</td>
<td></td>
<td>Crocetti et al. (2000)</td>
</tr>
<tr>
<td>PAO846§</td>
<td>GTTGGTACGCAGCCTAAAAGG</td>
<td>Accumulibacter</td>
<td></td>
<td>Crocetti et al. (2000)</td>
</tr>
</tbody>
</table>

拍卖 previously described methods (Amann et al., 1995; Hugenholtz et al.,
2002). Non-probe controls were prepared to examine auto-
fluorescent bacteria and the NonEUB338 probe was used for non-
specific oligonucleotide binding (Amann et al., 1995). Slides for FISH
analyses were mounted in Citifluor (Citifluor, UK) and viewed. A total
of three images per specimen were captured at ×1000 magnification
using a Zeiss Axiophot epifluorescence microscope equipped with an
HBO100 mercury vapour short-arc lamp, filter-sets no. 10 and no. 20
for fluorescein and Cy3, respectively, and an AxioCam MRm digi-
tal camera (Zeiss). Enhanced FISH images were obtained using
AxioVs40 V 4.7.1.0 (Zeiss) by the combination of the three captured
images, and Nomarski photographs were also collected simultaneous to
the FISH images. The abundance of the probe-targeted cells was
quantified using more than 20 FISH images per sample.

RESULTS AND DISCUSSION

Reactor operation

An anaerobic/aerobic SBR for EBPR was continually
operated for more than 8 months under the same conditions,
supplied with sodium acetate as the sole carbon
source. After initiation of the SBR by an inoculation of
activated sludge seed, phosphate (PO4-P) release during
the anaerobic phase and phosphate accumulation during
the subsequent aerobic phase gradually increased with
operation time. Complete PO4-P uptake at the end of the
aerobic phase was accomplished after about 2 months of
operation. After approximately 4 months of operation,
soluble PO4-P values reached more than 90 mg l−1 at the
end of the anaerobic phase. FISH analysis showed that cells
(Accumulibacter) labelled with PAOmix probes constituted
more than 55 % of the total cells labelled with the EUBmix
probes. However, after 6 months of operation, PO4_P
release in the anaerobic phase decreased to below 55 mg
l−1, and the population of Accumulibacter decreased to less
than 25 % of the total cells detected with EUBmix probes,
although complete PO4-P uptake was still accomplished
during the aerobic phase (data not shown). At this time,
Phylogenetic analysis of the new GB subgroup

To analyse the phylogeny of the predicted new GB, a 16S rRNA gene clone library was constructed using genomic DNA from a sludge sample containing cells with large cocccobacilli morphologies. A total of 114 bacterial 16S rRNA gene clones were selected randomly and were evaluated via RFLP analysis using HaeIII and HhaI double digestion of PCR amplicons. Representative clones (43 clones) with unique RFLP patterns were partially sequenced (approx. 700 nt), and the resulting sequences were submitted to GenBank for BLASTN searches. Accumulibacter-related 16S rRNA gene clones (nine of 43 representative clones) were equivalent to approximately 17.5 % of the total clones (20 of 114 total clones) and Gammaproteobacteria-related 16S rRNA gene clones (six of 43 representative clones) made up approximately 28.1 % (32 of 114 clones) of the total clones. However, no 16S rRNA gene sequence related to the genera Defluviicoccus and Sphingomonas of Alphaproteobacteria was identified from the clone libraries. The six representative 16S rRNA gene clones related to Gammaproteobacteria were further sequenced (>1430 nt) and analysed phylogenetically. Among them, four representative 16S rRNA gene clones making up approximately 21.9 % (25 of 114) of the total clones were closely related to members of the known GB subgroups. The 16S rRNA gene sequences of GB retrieved from this study formed a clearly distinct phylogenetic lineage, designated GB8, from those of the known GB, namely GB1–GB7 (Kong et al., 2002), with relatively low sequence identities of 89.2 to 92.2%, suggesting a novel putative subgroup of GB (Fig. 1). However, the phylogenetic analysis showed that the GB8 sequences of this study formed a phylogenetic lineage with some other 16S rRNA gene sequences (e.g., Jiang clones 71 and 91) from previous studies with high bootstrap values (99–100 %) (Fig. 1). Although they had relatively low sequence identities (<97.0%), bacteria containing 16S rRNA gene sequences of Jiang clones 71 and 91 also might be members of the GB8 subgroup of Competibacter because the 16S rRNA gene sequences were retrieved from an anaerobic/aerobic cycled reactor for PHA production (Jiang et al., 2009). The maximum-likelihood tree also showed that clades of each GB subgroup comprised identical members of 16S rRNA gene sequences to those of the neighbour-joining tree with basically the same topology (data not shown). Because the 16S rRNA gene sequences of GB8 were quite different from those of already known GB subgroups and they did not hybridize with any of the known GB-targeting probes (Kong et al., 2002), oligonucleotide FISH probes and competitors targeting the new GB subgroup (GB8) and all of the GB subgroups including GB8 were designed.

FISH probe designs and evaluation

To apply newly designed FISH probes to environmental samples, optimal conditions of binding stringency to avoid non-specific results should be determined; this is generally achieved by the use of controls such as cultured organisms or clones with zero or one mismatch within the rRNA probe targeting sequences (Hugenholtz et al., 2002; Schramm et al., 2002). However, this specificity of any single probe to the target group is not guaranteed since most of the target organisms are unculturable, and the environmental samples include a large proportion of unknown organisms. A solution to this limitation is the design and application of specific probes for multiple phylogenetic groups that encompass the phylogenetic hierarchies of the uncultured target group. Therefore, FISH probes targeting uncultured GB subgroups have been designed at different hierarchical levels and applied to sludge samples. We evaluated the specificities of existing GB FISH probes for the GB8 members and designed a GB8-specific FISH probe and a new FISH probe targeting all of the GB subgroups including GB8 for ecophysiological studies of GB8.

First, the specificities of the existing GB-targeting FISH probes in Table 1 were evaluated using the sludge samples containing the new GB subgroup, GB8. Analysis of 16S rRNA gene sequences of all GB subgroups of Fig. 1 showed that known FISH probes targeting existing GB subgroups (GB1–GB7) at different hierarchical levels (Kong et al., 2002) did not adequately match with the 16S rRNA gene sequences of GB8. However, our FISH experiments revealed that the FISH probe GB (Table 1), which targeted all existing GB subgroups (GB1–GB7), produced weak positive signals for the sludge samples even with 70 % FA. These FISH signals generally overlapped with the GB429 FISH probe-targeted cells (GB8-specific FISH probe designed in this study; see the following results of the GB429 FISH probe design). There were two mismatches between the GB probe sequence (5′-CGATCCTCTAGCCCCACT-3′) and the GB probe-targeting positions (5′-AGTGGGCTAGGGACTG-3′) of GB8 members in the comparisons of the GB probe sequence with the 16S rRNA gene sequences of GB8 members, suggesting that the weak positive signals of the FISH probe GB might be produced by members of GB8 in the sludge samples. Therefore, a GB competitor, GB_C (5′-CAGTCTCCTAGCCCAC-3′), was designed for GB probe-targeting sequences of GB8 members. Because optimal FA concentrations for the GB FISH probe were 65–70 % (Kong et al., 2002), FISH experiments were performed with the sludge samples with 70 % FA, using the GB FISH probe with non-labelled GB_C. In these experiments the weak positive signals...
disappeared, and only a few clear GB probe-positive signals were present, which suggests that the FISH probe GB with the GB competitor GB_C did not hybridize with GB8 members. The GB_G1 and GB_G2 probes of Table 1 were also tested with the sludge samples, but these probes did not produce positive signals for GB429-targeted cells (data not shown), indicating that the GB_G1 and GB_G2 probes have sufficient specificities for their target bacteria, at least with respect to the newly reported GB8 group.

A specific oligonucleotide FISH probe, GB429, which targeted members of GB8 was designed using the probe

**Fig. 1.** Phylogenetic analysis of Gammaproteobacteria-related 16S rRNA gene sequences from EBPR sludge. Sequences retrieved in this study (bold) were compared with those of reference sequences from GenBank. Bootstrap values >50% are shown in percentages of 1000 replicates. *Defluviicoccus vanus* Ben 114^T^ (AF179678) was used as an outgroup. The asterisks indicate 16S rRNA gene sequences that have been classified as members of the order Chromatiales of Gammaproteobacteria in ‘RDP classifier’. The scale bar indicates the number of changes per nucleotide position.
design tool in the ARB software package (Table 1), and was confirmed using the ‘Probe Match’ tool of the Ribosomal Database Project (Cole et al., 2009). An optimal FA concentration for the GB429 FISH probe for FISH analysis was determined using increments of FA for PFA-fixed sludge since no pure culture was available. The optimal FA concentration of the GB429 FISH probe was approximately 35%. FISH analysis using the FISH probe GB429 always produced positive clear fluorescent signals as well as a small number of weak signals for our sludge samples (data not shown). Therefore, two sets of GB429 competitors, GB429_C1 and GB429_C1, which targeted the other seven GB subgroups (GB1–GB7) were also designed (Table 1). In FISH experiments using the GB429 FISH probe with two competitors, GB429_C1 and GB429_C2, the weak signals disappeared and only clear FISH signals were produced (Fig. 2). Probe GB429-targeted cells (members of GB8) were Gram-negative large coccobacilli about 2–4 μm in size (Figs 2 and 3) and had morphological characteristics distinct from those of other EUBmix-positive cells (Fig. 2a).

A new FISH probe, GB742, which targets all of the GB subgroups, including members of GB8, was also designed. However, confirmation of the GB742 FISH probe by the ‘Probe Match’ tool of the Ribosomal Database Project indicated that the sequence of this probe matched several 16S rRNA gene sequences classified as the order Chromatiales of the Gammaproteobacteria. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these sequences are also members of GB subgroups that have been misclassified as Chromatiales sequences from other activated sludge samples (Fig. 1), indicating that the GB742 probe can target all GB subgroups of Compatibacter. Moreover, GB742-positive cells were completely matched with cells hybridized by GB + GB429 probe mixture (Fig. 2c), which also supported the sufficient specificity of the GB742 FISH probe. Members of the two more distantly related 16S rRNA gene sequences (FJ002855 and FJ002854) to the seven GB subgroups were also reported to show typical phenotypic properties of GB such as acetate uptake as PHA (Schroeder et al., 2009). However, they were excluded in the design of the GB742.

**Fig. 2.** FISH images of cells which hybridized with different FISH probes. (a) FISH images hybridized with GB429 (Cy3, red) and EUBmix (FITC, green). (b) FISH images hybridized with GB429 (Cy3, red) and GB742 (FITC, green) of lab-scale SBR sludge (from Kyungpook National University). The image indicates that the sludge sample contained members of other GB subgroups in addition to members of GB8. (c) FISH images hybridized with GB429 + GB (Cy3, red) and GB742 (FITC, green) of lab-scale SBR sludge (from Kyungpook National University). The image indicates that GB742-positive cells were completely matched with cells hybridized by the mixture of GB and GB429 probes. (d) FISH images hybridized with GB429 (Cy3, red) and EUBmix (FITC, green) of full-scale EBPR sludge (from Tancheon wastewater treatment plant). In all panels, yellow cells (overlay of red and green) indicate members of GB8 that were simultaneously hybridized with GB429 (Cy3, red) and other probes (FITC, green). Scale bars (all panels): 10 μm.
A FISH probe which targeted all of the GB subgroups because they have not been detected in most EBPR plants or other activated-sludge processes, indicating that they may not be distributed widely in EBPR processes (Schroeder et al., 2009). The optimal FA concentration of the GB742 FISH probe was determined using increments of FA concentration for PFA-fixed sludge, and was found to be approximately 35%. Although the target site of the GB742 probe was located in region IV of the Escherichia coli 16S rRNA gene sequence with relatively low accessibility (Fuchs et al., 2000), FISH hybridization using the GB742 probe yielded sufficiently strong fluorescent signals with a 35% FA concentration (Fig. 1), indicating that helper probes were not necessary for the increase of signal intensity.

**Ecophysiology analysis of GB8 using FISH/MAR analyses**

Complete PO₄-P uptake at the end of the aerobic phase was accomplished during 8 months of SBR operation. However, FISH analyses showed that members of GB8 labelled with the GB429 FISH probe coexisted together with members of *Accumulibacter* in sludge samples that were labelled with PAOmix probes (data not shown), and that the population densities of GB8 (5–25% of EUBmix-
Table 2. Distribution of GB subgroups in sludge samples from two lab-scale reactors and three full-scale plants for EBPR.

<table>
<thead>
<tr>
<th>Sludge sample*</th>
<th>Sample source†</th>
<th>GB-targeted cells (GB1–7) (%)‡</th>
<th>GB429-targeted cells (GB8) (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAU</td>
<td>Lab-scale</td>
<td>11.8</td>
<td>88.2</td>
</tr>
<tr>
<td>KNU</td>
<td>Lab-scale</td>
<td>39.1</td>
<td>60.9</td>
</tr>
<tr>
<td>Tancheon</td>
<td>Full-scale</td>
<td>19.3</td>
<td>80.7</td>
</tr>
<tr>
<td>POSTECH</td>
<td>Full-scale</td>
<td>38.5</td>
<td>61.4</td>
</tr>
<tr>
<td>Jungnang</td>
<td>Full-scale</td>
<td>29.8</td>
<td>70.1</td>
</tr>
</tbody>
</table>

*CAU and KNU represent sludge samples from Chung-Ang University and Kyungpook National University, respectively.
†Percentages of cells targeted by each probe for all GB cells labelled with the GB742 probe.

binding cells) and Accumulibacter (25–55 % of EUBmix-binding cells) fluctuated greatly with operation time although the reactor operation was constant and showed good EBPR. With increases in GB429-targeted cell densities, both PAOmix-targeted cell densities and PO₄-P release during the anaerobic phase decreased, suggesting that GB429-targeted cells competed with Accumulibacter for acetate uptake during the anaerobic phase.

FISH/MAR combinational analysis demonstrated that bacterial cells responding to the GB429 FISH probe (members of GB8) were able to take up acetate as PHA during the anaerobic phase (Fig. 3b), but they had no ability to accumulate orthophosphate during the subsequent aerobic phase (Fig. 3d), consistent with in situ phenotypic properties of GB in the biochemical model (Satoh et al., 1994; Liu et al., 1996; Jeon et al., 2001). Also, they out-competed Accumulibacter for acetate uptake during the anaerobic phase.

Additional FISH analysis on sludge samples taken from an SBR of another lab (Kyungpook National University, Korea) displaying EBPR failure showed that GB429-targeted cells predominated (61 % of total GB cells labelled with GB742 probe), suggesting that these cells could be important GAO. Subsequently, GB429 and GB742 probes were applied to several sludge samples from two other lab-scale and three full-scale EBPR processes. Probe GB429-positive cells (members of GB8) were detected from all sludge samples in different cell densities (5–25 % at lab-scale SBR and 1–5 % at full-scale EBPR plants), suggesting that members of GB8 might be widely distributed in EBPR processes (Fig. 2b, d). Probe GB742-positive cells (all GB) without hybridization signals of the GB429 FISH probe (GB8) were also detected (Fig. 2b), indicating that other members of the GB subgroups except for GB8 also existed in the sludge samples. The quantitative analysis of GB subgroups in sludge samples from two lab-scale reactors and three full-scale plants showed that the probe GB429-positive cells (GB8) were more dominant than the probe GB-positive cells (GB1–7) in all sludge samples, as shown in Table 2, indicating that the GB8 subgroup might be more significant than subgroups GB1–7 in EBPR sludge.

Conclusions

The presence of GAO has been hypothesized to be a cause of deterioration in EBPR processes due to their ability to out-compete PAO (Accumulibacter). In this study, a new subgroup (GB8) of GB (Compatibacter) with a distinct phylogenetic lineage based on 16S rRNA gene sequences was discovered in SBR sludge. The subgroup showed morphological and physiological properties (Gram-negative large cocccobacilli about 2–4 μm in size, acetate uptake under anaerobic conditions without subsequent polyphosphate accumulation under aerobic conditions) consistent with those of previously reported GB. Because no existing GB-targeting FISH probes matched the 16S rRNA gene sequences of GB8 members, a GB8-specific probe and a FISH probe which targeted all GB subgroups including GB8 were designed. FISH/MAR approaches showed that members of GB8 have typical properties of GAO with abilities to take up acetate under anaerobic conditions and without accumulation of polyphosphate under the subsequent aerobic conditions, and they were distributed widely in several lab- and full-scale EBPR processes as a significant GB subgroup. Therefore, designs of a set of FISH probes which hybridize all GB subgroups at different hierarchical levels will allow for monitoring of the distributions of GB and studies into their ecophysiology in lab- or full-scale EBPR plants.

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REFERENCES


resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 35, 7188–7196.


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