Abundance of Candidatus ‘Accumulibacter phosphatis’ in Enhanced Biological Phosphorus Removal Activated Sludge Acclimatized with Different Carbon Sources

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In the present study, the abundance of Candidatus ‘Accumulibacter phosphatis’ and the accumulation of polyphosphate were investigated in five enhanced biological phosphorus removal (EBPR) activated sludge reactors operated with different carbon sources. Fluorescence in situ hybridization (FISH) in combination with 4’,6-diamidino-2-phenylindole (DAPI) staining for polyphosphate granules confirmed the accumulation of polyphosphate by Candidatus ‘Accumulibacter phosphatis’ in all the reactors. The abundance of Candidatus ‘Accumulibacter phosphatis’ was determined from the FISH images. When EBPR activity was high and phosphorus content made up around 9% or more of mixed liquor volatile suspended solids (MLVSS), Candidatus ‘Accumulibacter phosphatis’ accounted for over 20% of the eubacteria in the reactors acclimatized with acetate, aspartate, or glucose. Whereas this value was as low as around 10% in the reactors acclimatized mainly with yeast extract, peptone, or glutamate. In these reactors, bacteria affiliated with Actinobacteria were found to accumulate polyphosphate and to contribute to phosphorus removal. Candidatus ‘Accumulibacter phosphatis’ takes part in the removal of phosphorus by using various carbon sources, but its abundance varies according to the type of carbon source.

Key words: EBPR, PAOs, Candidatus ‘Accumulibacter phosphatis’, carbon source, FISH

Phosphorus is one of the causes of the eutrophication of enclosed water bodies. There is a strong need for the development of a reliable and economical method to remove phosphorus from wastewaters. The enhanced biological phosphorus removal (EBPR) activated sludge method is a way of removing phosphorus from wastewater used all over the world. EBPR process is characterized by the circulation of activated sludge through anaerobic and aerobic phases, coupled with the introduction of influent wastewater into the anaerobic phase4.5. With this anaerobic-aerobic configuration, bacteria that accumulate polyphosphate are selected and achieve dominance in the process. Phosphorus is removed from the wastewater by withdrawing excess sludge enriched with these polyphosphate accumulating organisms (PAOs)17–23. However, there is not yet enough information about PAOs because the PAOs responsible for EBPR in full-scale systems have not been isolated despite tremendous research efforts. It is therefore important that we gain a better understanding of PAOs so that we can understand the reactor performance from a microbiological viewpoint.

Hesselmann et al.9) and Crocetti et al.10) obtained the phylogenetic identity of dominant PAOs in laboratory-scale
EBPR reactors fed with acetate by constructing clone libraries of 16S rRNA gene fragments and by using fluorescence in situ hybridization (FISH) with newly designed oligonucleotide probes. Both research groups concluded that almost the same bacteria, closely related to *Rhodocyclus* (a member of the Betaproteobacteria), were responsible for phosphorus removal in their reactors. Hesselmann et al. reported that the dominant PAO was a coccobacillus and tentatively proposed the name *Candidatus Accumulibacter phosphatis*. Crocetti et al. reported that *Candidatus Accumulibacter phosphatis* occupied over 80% of the eubacterial population in a laboratory-scale EBPR reactor fed acetate, and accumulated polyphosphate granules in its cells. In a laboratory-scale EBPR reactor fed propionate, *Candidatus Accumulibacter phosphatis* occupied over 30% of the eubacterial population. *Candidatus Accumulibacter phosphatis* has also been found (4–18% of eubacteria) in full scale plants treating domestic sewage and accumulated polyphosphate. It is now regarded as one of the key PAOs species in EBPR processes. To date, however, the abundance of *Candidatus Accumulibacter phosphatis* in EBPR processes acclimatized to carbon sources other than acetate and propionate has not been evaluated.

Therefore, to investigate the abundance of *Candidatus Accumulibacter phosphatis* in EBPR processes acclimatized to different carbon sources, we operated four laboratory-scale EBPR activated sludge reactors with (a) yeast extract and peptone, (b) glutamate, (c) aspartate, and (d) glucose respectively as the main or sole carbon sources. In addition, we also operated a laboratory-scale EBPR activated sludge reactor with (e) acetate as a control. We used two methods of microscopic analyses. One was FISH in combination with 4’,6-diamidino-2-phenylindole (DAPI) staining to confirm the accumulation of polyphosphate in situ by *Candidatus Accumulibacter phosphatis*. The other was a quantitative analysis by FISH, where the abundance of *Candidatus Accumulibacter phosphatis* was determined and compared with phosphorus content in activated sludge during the course of acclimatization. Furthermore, an attempt was made to identify PAOs other than *Candidatus Accumulibacter phosphatis*.

**Materials and Methods**

**Reactor operation**

The operation of the laboratory-scale EBPR activated sludge reactors was almost the same as that described by Onuki et al. Each of the reactors was a sequencing batch reactor (SBR) with a working volume of 10 L (Fig. 1). The SBR cycle was 6-h in total comprising a 0.5-h feeding phase, a 1.5-h anaerobic phase, a 2.5-h aerobic phase, a 1-h settling phase, and a 0.5-h discharge phase. During the feeding phase, dissolved oxygen was removed by N₂ purge. In each cycle, a sterilized stock feed solution and tap water were added as synthetic wastewater in the feeding phase, and 6 L of supernatant was withdrawn after the settling phase. Excess sludge was withdrawn as mixed liquor at the end of the aerobic phase. The sludge retention time (SRT) and the hydraulic retention time (HRT) were about 8 days and 10 hours, respectively. The pH was controlled between 7.0 and 7.2 by a pH controller. The temperature was maintained around 20°C.

Five runs—R5–R9—were operated under the same conditions except for the differences in carbon sources and seed sludges. The feeding solution for R5 contained mainly peptone (40% of carbon) and yeast extract (50%) as the main carbon sources with a minor amount of acetate (10%). The feeding solution for R6 contained mainly glutamate (94% of carbon) with some yeast extract (6%). For R7, R8, and R9, acetate, aspartate, and glucose was the sole carbon source, respectively. The composition of the synthetic wastewater is shown in Table 1. The carbon concentration in each run was approximately 40 mgC/L. Allylthiourea was added to inhibit nitrification. The seed sludge was obtained from the same full-scale wastewater treatment plant but on different days.

**Sampling and chemical analyses**

The performances of the reactors were monitored by measuring dissolved organic carbon (DOC), dissolved orthophosphate (PO₄-P), mixed liquor suspended solids...
(MLSS), mixed liquor volatile suspended solids (MLVSS),
and the phosphorus content of the sludge (P content) at the
end of the aerobic phase. Determination of the total phos-
phorus (TP) concentration in the mixed liquor by the ascor-
bic acid method after persulfate digestion, and the determi-
nation of MLSS, and MLVSS were performed as specified
in the Standard Methods. For the analysis of the compo-
nents in the supernatant, the mixed liquor was centrifuged
immediately after the sampling to remove solids. DOC was
measured by a TOC analyzer (TOC-500 or TOC-V, Shi-
madzu, Kyoto, Japan), and PO₄-P was measured by a capil-
lar ion analyzer (CIA, Waters, Milford, MA, USA). The P
content of sludge was calculated by (TP—PO₄-P)/MLVSS
at the end of the aerobic phase.

For the microbial community and microscopic analyses,
sludge samples were taken and stored immediately in liquid
nitrogen.

FISH in combination with DAPI staining for polyphosphate

FISH analyses were performed in combination with DAPI staining to confirm the accumulation of polyphosphate by the target microorganisms. Table 2 lists the oligo-
nucleotide probes, their specificities, and the concentrations
of formamide used for FISH in this study. The targets of
PAOMIX probe, HGC69A, actino_1011, and NOC.1032

Table 1. Composition of the synthetic wastewater

<table>
<thead>
<tr>
<th></th>
<th>mg/L</th>
<th>R5</th>
<th>R6</th>
<th>R7</th>
<th>R8</th>
<th>R9</th>
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<tr>
<td>Peptone</td>
<td>34.6</td>
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<td>Yeast Extract</td>
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<td>5.0</td>
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<td></td>
<td>124.7</td>
<td></td>
<td></td>
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<td>Acetic Acid</td>
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<td></td>
<td></td>
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<td>Sodium Acetate, 3-hydrate</td>
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<td></td>
<td></td>
<td>22.7</td>
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<td>Monosodium L-Aspartate, 1-hydrate</td>
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<td>(+)-Glucose</td>
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<td>Potassium Chloride</td>
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<td>Ammonium Sulfate</td>
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<tr>
<td>Potassium Dihydrogen Phosphate</td>
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<tr>
<td>Allythioure</td>
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</table>

Table 2. Information relevant to the FISH probes used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Probe sequence (5'-3')</th>
<th>Reported specificity</th>
<th>FA (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
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<td>PAO462&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CCGTATCCTACWCAGGTTATTAAC</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>PAO651&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CCGTATCCTACWCAGGTTATTAAC</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35</td>
<td>6</td>
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<tr>
<td>PAO846&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CCGTATCCTACWCAGGTTATTAAC</td>
<td>Candidatus ‘Accumulibacter phosphatis’</td>
<td>35</td>
<td>6</td>
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<tr>
<td>HGC69A</td>
<td>TATAGTTACCACCGCGGT</td>
<td>Actinobacteria</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>actino&lt;sub&gt;1011&lt;/sub&gt;</td>
<td>TTAGTTACCACCGCGGT</td>
<td>Actinobacteria</td>
<td>25</td>
<td>22</td>
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<tr>
<td>Noc.1032</td>
<td>CCACCTGTACACCCCAAA</td>
<td>Nocardioles-related bacteria (Actinobacteria)</td>
<td>20</td>
<td>This study</td>
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<tr>
<td>EUB338&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GCTGCCCGCTCCGGCTGCCGT</td>
<td>Most Bacteria</td>
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<td>EUB338-II&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Planctomycetes</td>
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<tr>
<td>EUB338-III&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GCTGCCACCCGTAGGTGT</td>
<td>Verrucomicrobials</td>
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<td>8</td>
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</tbody>
</table>

<sup>a</sup> Concentration of formamide

<sup>b</sup> PAO462, PAO651, and PAO846 were used in the mixture called PAOMix.

<sup>c</sup> EUB338, EUB338-II, and EUB-III were used in the mixture called EUBmix.
are Candidatus ‘Accumulibacter phosphatis’, Actinobacteria, bacteria closely related to Tetrasphaera, and bacteria closely related to Nocardioides, respectively. These probes were commercially synthesized and 5’ labeled with Cy3 (Proligo, Kyoto, Japan). Cells stained with DAPI at 50 µg/mL have been reported to emit blue fluorescence from DNA molecules and yellow fluorescence from polyphosphate granules(10).

There are two ways to combine FISH and DAPI staining for polyphosphate: staining followed by FISH(13), and vice versa. The latter method is simpler, but the FISH procedure can affect the detection of polyphosphate granules. In the present study, the former method was employed to detect polyphosphate granules with higher confidence. Yet, the latter method was employed for the detection of polyphosphate granules in HGC69A and NOC.1032-positive cells.

FISH analyses using PAOMIX and actino_1011 probes in combination with DAPI staining were performed as follows; DAPI staining first followed by FISH. The activated sludge samples were taken at the end of the aerobic phase, fixed with 4% paraformaldehyde (for PAOMIX-positive cells) or 50% ethanol (for actino_1011-positive cells) in phosphate-buffered saline, and then held at 4°C for 30 min. The fixative solution was then removed. Fixed samples were immobilized on gelatin-coated glass slides by air-drying followed by dehydration in 50%, 80%, and 99% ethanol (vol/vol) successively for a few seconds each. Then, for staining polyphosphate granules, DAPI solution (50 µg/mL of sterilized water) was added to the sample, and then DAPI and FISH images were observed under the fluorescence microscope.

Quantitative analysis by FISH
For the quantification of Candidatus ‘Accumulibacter phosphatis’, FISH was performed with a Cy3-labeled PAOMIX probe and an FITC-labeled EUBMIX probe (see Table 2). The fixed cells were sonicated for 60 s with an ultrasonic processor (VibraCell, Sonics) at a pulse of 5 s and an output power of 4 W so that all the cells in a microscopic field are located on the same plane and are focused simultaneously, avoiding erroneous area-measurements caused by blurring from cells out of focus. At least 20 photographs were taken of each sample. The areas of each image that were positive for the PAOMIX and EUBMIX probes were measured with image analysis software (Qwin, Leica, Cambridge, UK).

DNA extraction and construction of the 16S rRNA gene clone library
DNA was extracted from the activated sludge samples of R6 by using a FastDNA SPIN kit for Soil (Qbiogene, Vista, CA, USA), in accordance with the instructions provided by the manufacturer. Primers Hgc-1, targeting Actinobacteria(13), and 1492r, targeting most eubacteria and archaeabacteria(14), were used to selectively amplify the 16S rRNA gene of bacteria affiliated with Actinobacteria in the DNA extracted from the sample. The nucleotide sequences were as follows: Hgc-1, 5’-TTCGGGTTGTAAACCTCT-3’; 1492r, 5’-GGCTACCTTGTTACGACTT-3’. PCR amplification was performed by using Ampli Taq Gold DNA polymerase (PE Applied Biosystems, CA, USA), and a T3 Thermocycler (Biometra, Göttingen, Germany), in accordance with the instructions provided by the manufacturer. Samples were preincubated at 94°C for 10 min. The PCR cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 90 s of extension at 72°C. This cycle was repeated 30 times and followed by incubation at 72°C for 10 min for the final extension. With the PCR products, a 16S rRNA gene clone library was constructed using QIAGEN PCR Cloning kit (QIAGEN, Valencia, CA, USA), in accordance with the instructions provided by the manufacturer. 16S rRNA gene sequence commissioned Macrogen, Inc. (Korea). The 16S rRNA gene sequences
obtained were compared with those in the GenBank database by using a Basic Local Alignment Search Tool (BLAST) to retrieve similar sequences and phylogenetically related species.

**Design and optimization of oligonucleotide probes**

The 16S rRNA-targeted oligonucleotide probe NOC.1032 was designed using the probe design function in ARB. No pure cultures were available to be used as positive or negative controls. Also, the NOC.1032-positive cells were expected to have a distinct morphology. Therefore, the formamide concentration was optimized using activated sludge samples from Run 6. The same approach has been used when neither positive nor negative controls are available.

**Results**

**Reactor performance**

Five runs (R5–R9) were operated with six different carbon sources, and the changes in the relevant parameters during the SBR cycles were monitored. The temporal changes in P content are shown in Fig. 2. In all the reactors, P content was around 3% on day 0, increased to 8.1% to 14.9%, and then, decreased to less than 6%. In the periods when P content was higher, PO₄-P release and DOC uptake in the anaerobic phase and PO₄-P uptake under the aerobic phase were observed clearly (data not shown).

![Fig. 2. Temporal changes of P content in five runs (R5–R9). The P content of sludge was calculated by (TP—PO₄-P)/MLVSS at the end of the aerobic phase. The feeding solution for R5 contained peptone (40% of carbon), yeast extract (50%), and acetate (10%). The feeding solution for R6 contained glutamate (94% of carbon) and yeast extract (6%). In R7, R8, and R9, acetate, aspartate, and glucose were fed as the sole carbon source, respectively.](image-url)
Polyphosphate accumulation by Candidatus ‘Accumulibacter phosphatis’

Activated sludge samples were taken at the end of the aerobic phase of each of the runs in the period when significant EBPR was observed, and subjected to FISH using the PAOMIX probe in combination with DAPI staining to observe the accumulation of polyphosphate by Candidatus ‘Accumulibacter phosphatis’. Almost all of the PAOMIX-positive coccoid cells in the samples from all runs gave bright yellow fluorescence when stained with DAPI (Fig. 3), indicating the accumulation of polyphosphate granules. The presence of Candidatus ‘Accumulibacter phosphatis’ and the accumulation of polyphosphate were confirmed in all of the runs.

Quantification of Candidatus ‘Accumulibacter phosphatis’

The ratios of PAOMIX-positive cells (Candidatus ‘Accumulibacter phosphatis’) to eubacterial cells during the operation of the five reactors are shown in Fig. 4. In R5 and R6, PAOMIX-positive cells accounted for around 10% or less. In R7, R8, and R9 (Fig. 4), PAOMIX-positive cells accounted for more than 20% of eubacterial cells when phosphorus-removal activity was high, but these ratios decreased when phosphorus-removal activity deteriorated.

Polyphosphate accumulation by bacterial cells other than Candidatus ‘Accumulibacter phosphatis’

In R5 and R6, the accumulation of polyphosphate by bacterial cells other than Candidatus ‘Accumulibacter phosphatis’ was observed by FISH in combination with DAPI staining.

In R5, bacterial cells targeted by the probe actino_1011 were found in abundance (more than one tenth of eubacteria) and FISH in combination with DAPI staining showed that these cells accumulated polyphosphate.

In R6, small coccoid cells stained yellow with DAPI were found in abundance (more than one tenth of eubacteria) when EBPR activity slightly recovered after its deterioration (day 151). These cells were positive for the HGC69A probe targeting Actinobacteria, and were confirmed to accumulate polyphosphate by FISH first followed by DAPI. For the detection of these cells by FISH, lysozyme treatment was needed. A clone library was constructed targeting the 16S rRNA sequence of Actinobacteria. A total of 55 clones were selected from the library and classified into 3 different sequences after partial sequencing (approximately 500 bp). The 16S rRNA partial sequences of these clones showed highest levels of similarity to Nocardioides aquiter-rae AF529063 (97%), Mycobacterium moriokaense AY859686 (99%), and Propionivibrio dicarboxylicus Y17601 (97%), respectively. The sequences from these clones were reported to GenBank with accession numbers AB289556, AB289557, and AB289558.

A probe NOC.1032 for bacteria affiliated with Nocardioides was designed and the formamide concentration was optimized. When hybridization was performed at formamide concentrations above 20%, fluorescence signal was not detected. On the other hand, when the formamide concentration was 20% or less, only small coccoid cells as described above were detected. Therefore, the optimal formamide concentration was determined to be 20%. FISH using the NOC.1032 probe in combination with DAPI staining for polyphosphate revealed that most of the NOC.1032-positive cells were stained yellow with DAPI (Fig. 5). These cells were in abundance (around or more than one tenth of eubacteria) when EBPR activity recovered slightly.

Fig. 3. Microscopic images of PAOMIX-positive bacteria: A: Phase-contrast image; B: Epifluorescent image of sludge stained with DAPI at the phosphate-probing concentration; C: Epifluorescent image of sludge hybridized with the PAOMIX probe. Arrows indicates PAOMIX-positive bacteria. Almost all of the PAOMIX-positive coccoid cells gave bright yellow fluorescence when stained with DAPI (B), indicating the accumulation of polyphosphate granules.
Discussion

In this study, the abundance of *Candidatus ‘Accumulibacter phosphatis’* and the accumulation of polyphosphate were investigated in five EBPR activated sludge reactors operated with different carbon sources. While the *in situ* anaerobic substrate uptake patterns of *Candidatus ‘Accumulibacter phosphatis’* have been already reported by using
the MAR-FISH technique\textsuperscript{12}\textsuperscript{19}, there has been no report on
the abundance Candidatus ‘Accumulibacter phosphatis’ in
EBPR processes acclimatized with different carbon sources.

Five runs—Runs R5–R9—were operated with different
carbon sources and effective enhanced biological phospho-
rus removal was achieved in all cases. That is, while the
phosphorus content of activated sludge without EBPR is
around 3% or less, the phosphorus content of these reactors
was 6% or higher during a significant part of their operation.
Then, EBPR activity deteriorated in all cases despite
no operational error, for some unknown reason. A similar
deterioration with no operational error has been reported by
Okunuki et al.\textsuperscript{19}.

Candidatus ‘Accumulibacter phosphatis’ existed in all
the runs, and was found to accumulate polyphosphate by
FISH using the PAOMIX probe for Candidatus ‘Accumulibacter
phosphatis’ in combination with DAPI staining for
polyphosphate granules.

In R7 (acetate), R8 (aspartate), and R9 (glucose), Candida-
utus ‘Accumulibacter phosphatis’ accounted for over 20%
of the eubacterial population when EBPR activity was high,
as can be seen in Fig. 2 and Fig. 4. As for acetate, Crocetti et
al.\textsuperscript{5} also reported that Candidatus ‘Accumulibacter phos-
phatis’ was observed in abundance in a reactor acclimatized
with acetate. Yet, the occurrence and abundance of Candida-
utus ‘Accumulibacter phosphatis’ in EBPR reactors fed
with aspartate or glucose have not been reported before.

Kong et al.\textsuperscript{12} and Chua et al.\textsuperscript{17} reported that Candidatus
‘Accumulibacter phosphatis’ can take up acetate under anaerobic conditions. Concerning aspartate, Chua et al.\textsuperscript{17}
reported that Candidatus ‘Accumulibacter phosphatis’ took
it up under anaerobic conditions, while Kong et al.\textsuperscript{12}
reported the opposite. At least some of Candidatus ‘Accumul-
ibacter phosphatis’ can take up aspartate anaerobically.
In the case of glucose, Kong et al.\textsuperscript{12} reported that it could
not directly be assimilated by Candidatus ‘Accumulibacter phosphatis’,
but it was easily fermented and the fermentation
products were subsequently taken up. In R9, the occur-
rence and disappearance of lactate, propionate, and acetate
were observed during the anaerobic period. It is assumed
that Candidatus ‘Accumulibacter phosphatis’ assimilated
these fermentation products. Candidatus ‘Accumulibacter phosphatis’ utilizes acetate, aspartate, and glucose directly or indirectly: these carbon sources can lead to the predomi-
nance of Candidatus ‘Accumulibacter phosphatis’ to be as
high as or higher than 20% of the eubacterial population.

In R6, the proportion of Candidatus ‘Accumulibacter phosphatis’ among eubacteria slightly increased from 4% to
10% and P content increased from around 3% to more than
9% in the period from day 0 to day 67. Yet, while P content
on day 67 in R6 (10.0%) was as high as or even higher than
the maximum in R8 (8.4%) and R9 (8.2%), the ratio of Can-
didatus ‘Accumulibacter phosphatis’ to eubacteria in R6 on
this day (10%) remained only around half of that in R8
(26%) and in R9 (20%) when maximum P content was
achieved. Kong et al.\textsuperscript{12} and Chua et al.\textsuperscript{17} reported, based on
MAR-FISH experiments, that Candidatus ‘Accumulibacter phosphatis’ took up glutamate anaerobically. However, the
abundance of Candidatus ‘Accumulibacter phosphatis’ in
R6 was much lower than that in R7, R8, or R9.

The abundance of Candidatus ‘Accumulibacter phospha-
tis’ in R5 (acetate, yeast extract and peptone) was much less
than that in R6, R7, R8, or R9. In R5, the ratio of Candida-
utus ‘Accumulibacter phosphatis’ to eubacteria was signific-
antly lower on day 40 (3.2%) than day 0 (8.1%), while P
content increased from 3.8% to 5.5% in this period. In R5
on day 61, P content was 8.4% while the proportion of Can-
didatus ‘Accumulibacter phosphatis’ among eubacteria was
1.3%. This value is less than one tenth of that in R7 (20% to
28%) when P content was 13% to 15%. Since the feed for
R5 contained a small amount of acetate (one tenth of that in
R7), it is assumed that Candidatus ‘Accumulibacter phospha-
tis’ utilizes only acetate in R5.

As in R5 and R6 the amounts of Candidatus ‘Accumuli-
bacter phosphatis’ were much lower than those in R7, R8,
and R9 while EBPR performance was comparable, the
existence of PAOs other than Candidatus ‘Accumulibacter phosphatis’ can be expected. Indeed, bacterial cells that
were positive for actino_1011 (Tetrasphaera-related) and
NOC.1032 (Nocardioides-related) were found to accumu-
late polyphosphate in R5 and R6, respectively. Concerning the
actino_1011-positive cells, some studies have demon-
strated their polyphosphate accumulation by FISH in com-
bination with DAPI staining\textsuperscript{15}. Kong et al.\textsuperscript{13} reported that
these cells could take up orthophosphate aerobically if, in
the preceding anaerobic phase, they had taken up casamino
acids as determined with a MAR-FISH analysis using radio-
labeled orthophosphate. These results imply that actino_1011-positive cells may be able to accumulate poly-
phosphate utilizing peptone, since casamino acids and pep-
tone are hydrolyzed by casein. On the other hand, there has
so far been no report on bacterial cells affiliated to Nocar-
dioides that accumulate polyphosphate. The NOC.1032-
positive cells in R6 were abundant when EBPR activity
slightly recovered. These results suggested that the
actino_1011- and NOC.1032-positive cells contributed to
the removal of phosphorus in R5 and R6 together with Can-
didatus ‘Accumulibacter phosphatis’.
Consequently, our results revealed that *Candidatus 'Accumulibacter phosphatis'* plays an active part in EBPR, but its abundance varies according to the type of carbon source. Furthermore, when its abundance is low, other bacteria may contribute to the removal of phosphorus.

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**References**