Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorus removal systems

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Abstract Polyhydroxyalkanoate (PHA)- and polyphosphate-accumulating traits of different taxonomic tetrad-forming organisms (TFOs) in two anaerobic-aerobic sequential batch reactors (SBRs) were characterized by the simultaneous use of fluorescence in-situ hybridization, PHB stain and DAPI stain. The two SBRs with glucose as the main carbon source were operated under different P:total organic carbon feeding ratios for more than 300 days, but both exhibited no enhanced biological phosphorus removal (EBPR) activity. Microscopic observations on sludge samples taken at various times from those two SBRs revealed that TFOs consistently accounted for more than 50% of total cells, and were mostly affiliated with the β- and γ-subclasses of Proteobacteria and the high G+C phylum of Gram-positive bacteria (HGC).

Those TFOs from the β-Proteobacteria exhibited PHB stain positive and DAPI stain negative, indicating that they could utilize compounds other than polyphosphate (i.e. glycogen) as reducing power for PHA synthesis from glucose. In contrast, two types of TFOs within the HGC group showed negative PHB stain and positive DAPI stain, indicating their capacity to accumulate polyphosphate without the synthesis and degradation of PHA. This metabolic trait was different from the widely accepted biochemical model of EBPR and non-EBPR metabolisms. Other TFOs within the HGC group and γ-Proteobacteria showed negative responses to both PHA and DAPI stains, and their function in the deteriorated EBPR system need to be further clarified. Overall findings suggested that the phylogenetic and physiological heterogeneity of TFOs in anaerobic-aerobic activated sludge systems were diverse and greatly exceeded the current understanding.

Keywords DAPI; EBPR systems; fluorescence in situ hybridization; PHA; polyphosphate; tetrad-forming organisms

Introduction
Enhanced biological phosphorus removal (EBPR) systems, which selectively enrich microorganisms to remove soluble phosphate from discharged wastewater, sometimes experience deteriorated EBPR activity (Cech and Hartman, 1990, 1993; Liu et al., 1994). During deterioration, proliferation of non-phosphate-accumulating organisms is often reported. These bacteria are initially named as the “G-Bacteria” for their dominance in acetate-fed reactors supplemented with glucose. The G-Bacteria are subsequently named as glycogen accumulating organisms for the ability of utilizing glycogen instead of polyphosphate as reducing power and energy to synthesize polyhydroxyalkanoate (PHA) under anaerobic stages (Liu et al., 1996, 1997), and are thought of as competitors of polyphosphate-accumulating organisms (PAOs), which are responsible for EBPR activity (Cech and Hartman, 1990, 1993; Liu et al., 1994). G-Bacteria consist of several different morphotypes (Liu et al., 1996; Nielsen et al., 1999; Seviour et al., 2000). Among them, cocci arranged distinctively in tetrads or sheets are the most commonly observed and predominant ones, and are referred to as tetrad-forming organisms (TFOs) in this study.

A number of TFOs have been isolated from deteriorated EBPR systems. These include members of α-Proteobacteria (i.e. *Amaricoccus* gen.) (Maszenan et al., 1997) and Gram-positive high G+C phylum [HGC] (i.e. *Tesaracoccus* spp. and *Tetrasphaera* sp., *Friedmanniella* sp., and *Micropruina* sp.) (Maszenan et al., 1999a, 1999b and 2000a; Shintani et al., 2000): the former can store PHA but not polyphosphate, and the latter can store...
polyphosphate or glycogen under aerobic conditions but is not studied for its PHA accumulation ability. Nevertheless, physiological roles of these bacteria in the deteriorated EBPR systems have not been demonstrated under repetitive anaerobic-aerobic conditions. Using fluorescence in situ hybridization (FISH), TFOs are identified as members of the α and β subdivisions of the Proteobacteria in reactors fed with low influent P/C ratios (Sudiana et al., 1998) or with deteriorated EBPR activity (You et al., 2000). However, little is known about the physiological roles (e.g. PHA and polyphosphate accumulation) of these TFOs.

Comparison of results between FISH studies and traditional isolation clearly suggests that the phylogeny and physiology of TFOs in deteriorated EBPR systems are still fragmental. This finding suggests possibilities that TFOs in deteriorated EBPR systems may include members from different phylogenetic groups, and the traits of polyphosphate and PHA accumulation can vary among different TFOs. To clarify this, a study on the taxonomic affiliations and functional traits of TFOs in deteriorated EBPR systems is necessary. In this study, a method that combined DAPI stain, PHB stain and FISH was developed to simultaneously determine the physiological traits of a phylogenetic group in deteriorated EBPR reactors fed mainly with glucose.

Materials and methods

Activated sludge processes

Two anaerobic-aerobic sequential batch reactors were operated with glucose as the main carbon source under two different influent P:total organic carbon ratios (i.e. 2/100 and 6/100). Each cycle consisted of an anaerobic phase (5 hr), an aerobic phase (5 hr), and a settling period (2 hr). The organic loading was 0.083 kg COD/m³/day from day 1 to day 240, and was increased to 0.166 kg COD/m³/day after that. Although the influent P/C ratios were different, both reactors showed poor EBPR activities and the sludge phosphorus content in both SBRs ranged from 2.5 to 3% of dry sludge weight. Sludge samples were taken at days 30, 203, 241, and 363, and used for the characterization of microbial diversity of TFOs.

Combine analysis of FISH, DAPI staining, and PHB staining

Simultaneous use of DAPI staining, PHB staining and FISH was developed to investigate the polyphosphate- and PHA-accumulating traits within certain phylogenetic groups in the deteriorated SBRs. Initially, microbial cells in sludge samples were fixed in 4% paraformaldehyde after harvesting from the end of the anaerobic and aerobic stages of both reactors. After fixation, a freeze and thaw process (Sekiguchi et al., 1999) or lysozyme treatment (Kawaharasaki et al., 1999) was applied to improve the penetrability of probes. Then the cells were fixed on poly-L-lysin coated slides (Poulsen et al., 1993). For each sample, the microbial community was dual-hybridized with EUB 338 and one of those rRNA-targeted oligonucleotide probes at different taxonomic levels (Table 2).

After final washing in the FISH procedure, slides were immersed in a freshly prepared DAPI (4',6-diamidino-2-phenylindol) solution (0.2 mM) for a period of 1–5 min, rinsed thoroughly with milli Q water, and air-dried. The DAPI signal of stained cells was examined using a Zeiss Axioskop-II epifluorescence microscope (Carl Zeiss) equipped with a 12-bit cooled CCD camera (Quantex, Photometrics) under filter set 1 (Carl Zeiss). Under the epifluorescence microscope, the polyphosphate granules were bright yellow while the other parts of the cells remained pale blue. However, the DAPI images were photographed by a mono-CCD so that the poly-P granules would appear brightly white on the photographs. For FISH analysis, the same field examined for DAPI was examined under the corresponding filters sets for fluorescence labels cy3 and cy5. When quantification was performed, at least 20 views were photographed for individual samples under respective epifluorescent filters at the same site. The percentage of cells targeted by group-specificity
to the universal probe EUB338 was counted by image analyzing software, V-FOR-WIN-DOWS (Photometrics).

For PHB staining, the immersed oil on the slide was carefully removed by washing the slide with pure ethanol, and then air-dried. The slide was then stained with PHB staining solutions as described by Jenkins et al. (1986). The same microscopic fields examined for DAPI and FISH were observed by the epifluorescence microscope under bright field mode. PHB-staining micrographs were taken using a color digital camera (Hitachi,) with image analyzing software (Imaq Vision Builder, National Instrument Co.).

Results and discussion

During the course of the operation, both SBR reactors showed deteriorated EBPR characteristics, i.e. little EBPR activity and low intracellular phosphate accumulation (Lin and Liu, unpublished results). Microscopic observations suggest that different P/C feeding ratios did not significantly influence the microbial communities in both reactors at the same loading stage. However, the microbial community in both reactors was more complex under a low loading stage (0.083 kg COD/m3*day) than a high loading stage (0.166 kg COD/m3*day).

Diversity of TFOs in those two deteriorated EBPR SBRs was classified based on their morphology, physiological traits (i.e. polyphosphate and PHA accumulation) and phylogenetic affiliation. At least six different types of TFOs were observed during those 363 days of operation (Table 2). Four were affiliated with the HGC group (HGC-TFO1, HGC-TFO2, HGC-TFO3, HGC-TFO4), one with the β-Proteobacteria (β-TFO1), and the last one with the γ-Proteobacteria (γ-TFO1). None of them showed simultaneous accumulation of polyphosphate and PHA granules. Two contained polyphosphate but not PHA granules. Only one type of TFOs (β-TFO1) exhibited the PHA accumulating trait of G-Bacteria described previously (Liu et al., 1996). Detailed description of each type at different organic loading rates was provided as follows.

Under an organic loading rate of 0.083 kg COD/m3*day, four predominant types were frequently observed in both reactors during the first 240 days of operation. Cells of type HGC-TFO1 (Figures 1 A–D) were big, dark tetrads (1.5 to 2 µm in diameter) usually appearing in blocks. These cells apparently contained a thick cell wall or exopolysaccharides, because lysozyme treatment was required to improve the penetration of oligonucleotide probes on these fixed cells during FISH analysis. Cells showed negative responses to both DAPI and PHB stains (Figure 1 C and D), suggesting that Type HGC-TFO1 did not play a significant role in EBPR activity. Type HGC-TFO2 usually aggregated as big, regular or irregular packets (0.5 to 1 µm in diameter) under phase contrast observation (data not shown), and showed DAPI stain positive or negative in reactors with a high or a low P/C-feeding ratio, respective-

<table>
<thead>
<tr>
<th>Table 1 Oligonucleotide probes used in this study</th>
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<tbody>
<tr>
<td><strong>Standardized probe name</strong></td>
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<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>S-D-Arch-0915-a-A-20</td>
</tr>
<tr>
<td>L-Mo-ph-19-a-A-17</td>
</tr>
<tr>
<td>L-Mo-ph-1027-a-A-17</td>
</tr>
<tr>
<td>L-Mo-ph-1027-a-A-17</td>
</tr>
<tr>
<td>L-Mo-ph-5402-a-A-18</td>
</tr>
<tr>
<td>L-Mo-HGC-1901-a-A-18</td>
</tr>
<tr>
<td>L-Sc-CF-319-a-A-18</td>
</tr>
<tr>
<td>S-Sc-HGC-1011-a-A-19</td>
</tr>
</tbody>
</table>

*Percent formamide (FA) in hybridization during hybridization at 37°C
PHA-accumulating traits of type HGC-TFO2 differed from those predominant populations commonly observed in efficient and deteriorated EBPR processes.

Types $\beta$-TFO1 and $\gamma$-TFO1 (Figure 1, panels M–P) were white, large cocci (1–1.2 µm in diameter), and showed negative response to DAPI stain. Furthermore, type $\beta$-TFO1 showed positive to PHB staining, exhibiting a similar metabolic trait to G-Bacteria that could utilize energy compounds other than polyphosphate (e.g. glycogen) for PHA synthesis from glucose under anaerobic conditions (Liu et al., 1996). Type $\gamma$-TFO1 exhibited a negative response to PHB stain. Since $\gamma$-TFO1 was not directly related to EBPR or non-EBPR biochemical reaction, its role in the deteriorated EBPR systems required further verification.

After the increase in influent COD loading at day 240, the predominant microbial populations in both reactors shifted to types HGC-TFO3 (Figure 1, panels E–H) and HGC-TFO4 (Figure 1, panels I–L). Both types were large cocci (1.5–2 µm in diameter) with dark background under phase contrast observation, and appeared in sheet-like aggregates but loosely packed. Unlike type HGC-TFO2, type HGC-TFO3 cells were easily hybridized by oligonucleotide probes in FISH analyses. Type HGC-TFO3 showed DAPI stain positive and PHB stain negative (Figure 1, panels G and H). Type HGC-TFO4 showed both DAPI and PHB stain negative (Figure 1, panels K and L), and its role in the deteriorated EBPR systems remained unclear.

Recently, a predominant rod-shaped bacterial group with polyphosphate accumulation was identified using phylogenetic and FISH analyses as a member of the HGC group (Liu et al., 2001). This group was closely related to T. japonica based on 16S rDNA sequence, but differed from T. japonica in morphology. Since two of the TFOs found in the HGC group could accumulate polyphosphate, their relation with the rod-shaped PAO was determined using FISH with an oligonucleotide probe, action_1011, specifically targeting the rod-shaped HGC group. None of the cells in the activated sludge from those two reactors was hybridized by probe action_1011, suggesting the absence of the rod-shape POA in our reactors.

Furthermore, our findings and previous studies strongly suggest that members of the HGC group are important microbial populations in EBPR systems (Maszenan et al., 1997, 1999a, 1999b and 2000a; Shintani et al., 2000; Wagner et al., 1994). Identification of four different TFOs for the first time from the HGC groups in this study supported a previous finding (Liu et al., 1998, 2001) that the microbial diversity of HGC groups in EBPR and non-EBPR groups was more diverse than our present understanding (Liu et al., 2001; Seviour et al., 2000). None of those TFOs from the HGC group could accumulate PHA in the deteriorated EBPR. This observation suggests that these TFOs may use a different substrate metabolism as described for the G-Bacteria to take up glucose under anaerobic conditions (Liu et al., 1996). Since the G-Bacteria are not capable of accumulating polyphosphate, the polyphosphate-accumulating traits observed with two of those four TFOs in the HGC group could exclude them from the G-Bacteria.

Table 2  Morphology, phylogenetic affiliation and physiological traits of TFOs

<table>
<thead>
<tr>
<th>Name</th>
<th>Morphological traits</th>
<th>Phylogenetic affiliation</th>
<th>Accumulation of polyphosphate</th>
<th>Accumulation of PHA</th>
<th>Existence in reactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGC-TFO1</td>
<td>Large tetrad, dark cells</td>
<td>HGC</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>HGC-TFO2</td>
<td>Small compact, dark cells in tetrad or packets</td>
<td>HGC</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HGC-TFO3</td>
<td>Large and black cells forming sheet</td>
<td>HGC</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>HGC-TFO4</td>
<td>Large and black cells forming sheet</td>
<td>HGC</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>$\beta$-TFO1</td>
<td>Large and white cells</td>
<td>$\beta$-Proteobacteria</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>$\gamma$-TFO1</td>
<td>Large and white cells</td>
<td>$\gamma$-Proteobacteria</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

*a"TFO" means tetrad forming organisms

b $+$ means stain positive and $-$ means negative
c $++$ indicates abundant, "$++$ means usually seen, "$-" presents rarely observed.
In summary, the combined use of DAPI staining, PHB staining and FISH has provided a rapid means to explore the physiological characteristics and phylogenetic diversity of an interested morphological group, TFOs, in deteriorated EBPR processes. At least six different types of TFOs from different phylogenetic groups were discovered, and some of them exhibited different physiological traits from those microbial populations observed for PAOs and the G-Bacteria in anaerobic-aerobic activated sludge systems. The overall results suggest heterogeneity in the phylogenetic and physiological traits among those TFOs in deteriorated EBPR processes.

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References


