Microbial Community Composition of Polyhydroxyalkanoate-Accumulating Organisms in Full-Scale Wastewater Treatment Plants Operated in Fully Aerobic Mode

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The removal of biodegradable organic matter is one of the most important objectives in biological wastewater treatments. Polyhydroxyalkanoate (PHA)-accumulating organisms (PHAAOs) significantly contribute to the removal of biodegradable organic matter; however, their microbial community composition is mostly unknown. In the present study, the microbial community composition of PHAAOs was investigated at 8 full-scale wastewater treatment plants (WWTPs), operated in fully aerobic mode, by fluorescence in situ hybridization (FISH) analysis and post-FISH Nile blue A (NBA) staining techniques. Our results demonstrated that 1) PHAAOs were in the range of 11–18% in the total number of cells, and 2) the microbial community composition of PHAAOs was similar at the bacterial domain/phylum/class/order level among the 8 full-scale WWTPs, and dominant PHAAOs were members of the class Alphaproteobacteria and Betaproteobacteria. The microbial community composition of α- and β-proteobacterial PHAAOs was examined by 16S rRNA gene clone library analysis and further by applying a set of newly designed oligonucleotide probes targeting 16S rRNA gene sequences of α- or β-proteobacterial PHAAOs. The results demonstrated that the microbial community composition of PHAAOs differed in the class Alphaproteobacteria and Betaproteobacteria, which possibly resulted in a different PHA accumulation capacity among the WWTPs (8.5–38.2 mg-C g-VSS⁻¹ h⁻¹). The present study extended the knowledge of the microbial diversity of PHAAOs in full-scale WWTPs operated in fully aerobic mode.

Key words: activated sludge, fluorescence in situ hybridization, microbial community composition, polyhydroxyalkanoates-accumulating organisms, wastewater treatment plants

Polyhydroxyalkanoates (PHA) are polyesters found in microbial cells as carbon and energy storage materials (5, 29). To date, PHA accumulation by microorganisms has been identified in various natural and engineered ecosystems such as soil (28, 53), compost (53), river biofilm (17), marsh microbial mats (53), algal mat (10), estuarine sediment (16, 21, 45), freshwater (36, 60) and activated sludge processes (45, 53, 63).

A variety of PHA-accumulating organisms (PHAAOs) have been identified in activated sludge processes so far. It is well known that the members of polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) take up organic matter and concomitantly synthesize PHA under anaerobic conditions (42). As a member of PAOs or GAOs, PHA accumulation has been identified in Candidatus “Accumulibacter phosphatis” (18, 22, 46), Candidatus “Competibacter phosphatis” (44) and Defluvicoccus-related microorganisms (32, 64). Other than PAOs or GAOs, filamentous bacteria such as Sphaerotilus natans have also been identified as PHAAOs in activated sludge; however, the outline of microbial community composition of PHAAOs is still unknown in activated sludge processes since previous studies have just focused on the individual members of PHAAOs.

The present study was conducted to investigate the microbial community composition of PHAAOs in activated sludge. For this purpose, activated sludge samples were collected from 8 full-scale wastewater treatment plants (WWTPs) operated in fully aerobic mode. The activated sludge samples were incubated with the addition of acetate to allow PHAAOs to accumulate PHA, and then subjected to the following microbial community structure analysis. First, the abundance of PHAAOs was enumerated using microscopy subsequent to dual staining with 4',6-diamidino-2-phenylindole (DAPI) and Nile blue A (NBA), a specific fluorescence dye for PHA granules (50). Next, microbial community compositions of PHAAOs were investigated by fluorescence in situ hybridization (FISH) analysis with bacterial domain/phylum/class/order-specific oligonucleotide probes. The accumulation of PHA in the cells hybridized...
with an oligonucleotide probe was subsequently identified by post-FISH NBA staining technique (27, 56). Moreover, 16S rRNA gene cloning libraries were generated from the activated sludge samples, 16S rRNA gene sequences determined, and 4 and 12 oligonucleotide probes designed, targeting α- and β-proteobacterial PHAAs, respectively. The designed oligonucleotide probes were applied to the activated sludge samples to further examine the microbial community composition of α- or β-proteobacterial PHAAs.

Materials and Methods

Activated sludge

Activated sludge samples were collected from 8 municipal WWTPs in Japan. These WWTPs were operated in fully aerobic mode, where the reaction tank was mixed with air aeration. The concentration of dissolved oxygen in the reaction tanks was maintained at the range of 1 to 3 mg O₂ L⁻¹ to maintain the aerobic condition. The operational conditions of the 8 WWTPs are summarized in Table S1. One liter of biomass suspension was collected from the end of the aeration tank and taken to the laboratory with cooling on ice. The activated sludge samples obtained from a WWTP to H WWTP are hereafter referred as AS-A to AS-H, respectively.

Batch experiments

The activated sludge samples were washed twice with inorganic media (CaCl₂ 2H₂O 44 mg L⁻¹, MgCl₂ 6H₂O 453.5 mg L⁻¹, KCl 210 mg L⁻¹, NH₄Cl 88 mg L⁻¹, (NH₄)₂SO₄ 108 mg L⁻¹, KH₂PO₄ 90 mg L⁻¹, and KH₂PO₄ 70 mg L⁻¹), and the concentration of mixed liquor volatile suspended solids (MLVSS) was set at 500 mg L⁻¹. The biomass suspension was aerobically incubated at 22°C for 6 hours with the addition of acetate at the final concentration of 100 mg-C L⁻¹. The aerobic condition was maintained by supplying air using an air pump at a flow rate of 0.5 L min⁻¹ and the biomass suspension was continuously mixed with a magnetic stirring bar at 150 rpm. The pH was controlled to pH 8.0–8.2 during incubation by adding 1 N H₂SO₄ or 1 N NaOH. A liquid sample was collected every hour and the acetate concentration was determined. Supplementary acetate was added when the acetate concentration dropped below 40 mg-C L⁻¹. The biomass suspension after 6 h of incubation was subjected to microbial analysis and the determination of PHA concentration.

Dual staining with NBA and DAPI

Dual staining with NBA and DAPI was performed as previously described (47). Briefly, the biomass was sonicated at 3 watt for 4 min on ice with a Branson Sonifier 250D (Branson Ultrasonics, Danbury, CT, USA), and placed on glass slides (HTC super cured glass, Danbury, CT, USA), and fixed in either 4% paraformaldehyde for Gram-negative organisms or 50% ethanol for Gram-positive organisms, sonicated at 3 watt for 4 min, and placed on the glass slides. After hybridization with the oligonucleotide probes shown in Table 1, the specimen was subjected to microscopy. After microscopy, post-FISH NBA staining was performed as previously described (56). The cover slip was carefully removed, the microorganisms on the slide were stained with NBA (0.1% w/v ethanol solution, certified dye content 81%, Kodak, NY, USA) for 30 min (31), and then with 2 μg mL⁻¹ DAPI for 5 min.

FISH analysis and post-FISH NBA staining

Fixation of biomass and in situ hybridization of oligonucleotide probes were performed as previously described (3, 30). The biomass was fixed in either 4% paraformaldehyde for Gram-negative organisms or 50% ethanol for Gram-positive organisms, sonicated at 3 watt for 4 min, and placed on the glass slides. After hybridization with the oligonucleotide probes shown in Table 1, the specimen was subjected to microscopy. After microscopy, post-FISH NBA staining was performed as previously described (56). The cover slip was carefully removed, the microorganisms on the slide were stained with NBA, and the same microscopic field was relocated and examined. The oligonucleotide probes used in the present study were synthesized and labeled at the 5’ end with the indocarbocyanine dye (Cy3) from the SIGMA Genosys (Ishikawa, Japan).

PCR-cloning-sequencing analysis

Total genomic DNA was extracted from AS-A, AS-B, AS-E and AS-G by a FastDNA SPIN DNA extraction kit (MP Biomedicals LLC, OH, USA) following the instruction manual. The nearly full-length 16S rRNA gene sequence was amplified with 27f and 1492r (34) primer sets in 50-μL reaction tubes. Each reaction tube contained 1 × AmpliTaq Gold PCR reaction buffer, 0.2 mM deoxynucleotidetriphosphate, 1.25 U AmpliTaq Gold (Life Technologies, CA, USA), 0.2 μM primer, and 50 ng extracted DNA. Thermal conditions of PCR amplification were as follows: initial denaturation at 95°C for 10 min, 20 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 2 min, and final elongation at 72°C for 10 min. Fragment size of the amplicon was checked by 1.0% agarose gel electrophoresis. The amplicon was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), ligated into a PMD20-T vector by a Mighty cloning kit (Takara Bio, Otsu, Japan), and transformed into the cells of Escherichia coli K12 DH5α. Clones were randomly picked up and subjected to sequencing analysis using 357f primer (34) and an Applied Biosystems 3730 DNA analyzer (Life Technologies). The nucleotide bases assigned a Phred quality score lower than 15 were trimmed by the software Paracel Filtering Package (Paracel, CA, USA), and the sequence reads more than 300 bp were subjected to phylogenetic analysis. The phylogenetic affiliation was examined using the blastn search program (2) with the database of all non-redundant nucleotide sequences in the National Center for Biotechnology Information. Clones with greater than 95% sequence similarity were grouped into operational taxonomic units (OTUs), and the diversity indices including Shannon, Simpson and Chao1 and sampling coverage indices were calculated for each clone library by the software Mothur (55). For OTU-1–OTU-27, which were affiliated to class α- or Betaproteobacteria, nearly full-length 16S rRNA gene sequences were determined from representative clones of each OTU. If OTU contained clones derived from different activated sludge samples, representative clones from each activated sludge sample were selected and subjected to sequencing analysis. Sequencing analysis was conducted using M13 sequencing primers (forward and reverse) and 357f, 968f, 1099f, 518r, 907r and 1114r primers (34), the sequence reads were assembled by the software AutoAssembler (Life Technologies), and the vector sequences and annealing sites of 27f and 1492r primers were trimmed manually. The phylogenetic tree was developed using ARB software (37). First, the nucleotide sequences were imported into the SILVA database (SSURef_102_SILVA_12_02_10), aligned by the Integrated Aligners tool with the default parameters, and alignments were refined manually. The phylogenetic tree was constructed by the maximum parsimony (Phylip, DNAPARS), neighbor-joining (Jukes-Cantor model) and maximum likelihood (RAxMX) methods. Bootstrap resampling analysis was performed using 1,000 replicates for the neighbor-joining method and 100 replicates for the maximum parsimony and maximum likelihood methods to estimate the confidence of tree topologies.

Oligonucleotide probe design and optimization of formamide concentration

Oligonucleotide probes were designed using the Design probes tool in ARB software with the default parameters, and the coverage and specificity were first examined by comparative analysis of all sequences in the ARB database, which is composed of both the SILVA database and our clone sequences. The coverage and specificity of the oligonucleotide probes were further examined by the Probe Match tool in the Ribosomal Database Project (12).

The optimal formamide (FA) concentrations of oligonucleotide probes were determined by hybridization with a set of reference pure cultures or activated sludge samples, shown in Table S2. In this experiment, FA concentrations were changed in 5% increments, starting at 0% FA. The FA concentration, at which the fluorescence of the oligonucleotide probe was confirmed by microscopy from
the biomass of a positive control but not of a negative control, was chosen as the optimal FA concentration.

Microscopy
An Olympus BX51, equipped with a CCD camera DP70 (Olympus, Tokyo, Japan), was used for microscopy. DAPI fluorescence signals were observed through a WU filter, and NBA fluorescence signals were observed through a WIG filter (Olympus). The Cy3 and NBA fluorescence signals before or after post-FISH NBA staining were observed through WIG and NIBA filters, respectively. At least ten randomly selected fields containing more than one thousand total cells were used for the enumeration of PHAAOs. The numbers of total cells and the cells of PHAAOs were counted manually on the captured images.

Chemical analysis
The concentration and monomer compositions of PHA in activated sludge were determined by gas chromatography (59). Briefly, the biomass was lyophilized, PHA was extracted and derivatized by acidic methanol, and the monomeric units of PHA were determined by gas chromatography. Sodium 3-hydroxybutyrate (Tokyo Chemical Industry, Tokyo, Japan) and a copolymer composed of 81% 3-hydroxybutyrate and 19% 3-hydroxyvalerate (Sigma-Aldrich) were used as the standards for 3-hydroxybutyrate and 3-hydroxyvalerate units, respectively.

The concentration of acetate was monitored by ion chromatography (ion chromatograph DX-A1100 equipped with an AS-9HC column; Thermo Fisher Scientific, MA, USA). The liquid sample was filtered through a 0.2 μm pore-size cellulose acetate membrane (Millipore, MA, USA) and the filtrates were injected to ion chromatography.

The concentration of MLVSS was determined according to the standard methods of the American Public Health Association (4).

Linear regression analysis
Linear regression analysis was performed to identify the parameters that possibly caused the difference in the PHA accumulation rate among the activated sludge samples. The correlations were examined using Microsoft Excel 14.0.0.

Nucleotide sequence accession numbers
Sequence data of 16S rRNA gene were deposited in the DDBJ nucleotide sequence database under accession numbers AB515437–AB516239.

Results
PHA accumulation by activated sludge
The activated sludge samples were aerobically incubated

### Table 1. Oligonucleotide probes used in the study. Phylogenetic affiliations of the OTUs defined in the study are shown in Fig. 2.

| Probe name | Specificity | Sequence (5'-3') | FA | Reference |
|------------|-------------|------------------|----|-----------|
| EUB338a | Most Bacteria | GCTGCCCTCCCGTACGGTGTT | 35% | (3) |
| EUB338-III | Planctomycetes | GACGACACCACCGTACGGTGTT | 35% | (14) |
| EUB338-III | Verrucomicrobiales | GCTGCCACCCGCTACGGTGTT | 35% | (14) |
| ALF968 | Alphaproteobacteria | GFTAAAGTCTCCTCGCGAAGT | 20% | (43) |
| BET42a | Betaproteobacteria | GCTTCCTCACCAGCTCTTGGT | 35% | (38) |
| GAM42a | Gammaproteobacteria | GCTTCCCCACAGCTATTGGT | 35% | (38) |
| HGC69a | Actinobacteria | TATAGGTTACACACCGCGTGTT | 35% | (52) |
| LGC354A | Firmicutes | TGGAAAGTCTCCTCCTGCTGCT | 35% | (41) |
| LGC354B | Firmicutes | CGAAAGTCTCCTCCTGCTGCT | 35% | (41) |
| CF319a | Bacteroidetes | TGTCCTGCTGCTCAGTACGCT | 35% | (39) |
| CFX1223 | Chloroflexi | CCATGTACGCTGCTGCTGCT | 35% | (7) |
| ARR994 | OTU-1, 2, 3 | GTGCCCGGCTACTCCAGCTGCT | 40% | This study |
| Comp994 | OTU-4, 5, 6 | CTCCACCTCCTCTGCTGCTCCT | 50% | This study |
| ARP653 | OTU-7 | TGGCAAGGCTGGAATCGCTGCT | 30% | This study |
| Comp653 | OTU-8 | CAATTGGACTGCGAGCTGCTGCT | 55% | This study |
| AB1302 | OTU-9, 10, 11, 12 | CTGCCTCCTCCTGCTGCTGCT | 40% | (19) |
| AHS56 | OTU-13, 14 | GTGAGTGACTGCGGCTGCTGCT | 35% | (13) |
| BRDA454 | OTU-15 | CCGTGTAATAGGAGAGCGCTGCT | 10% | This study |
| ZOO834 | OTU-16 | CTTCAAGTGGCTCTCTCGCTGCT | 50% | (47) |
| OTU1-427 | OTU-17 | CCGCGATACTAAGCGCTGCTGCT | 30% | (47) |
| OTU1-472 | OTU-17 | CTCCGATTACCTAAGAGCTGCTGCT | 30% | (47) |
| BCC1212 | OTU-18 | GTTCTACGCTGCTGCTGCTGCT | 40% | This study |
| BCO395 | OTU-19, 20, 21 | TTTGTCCCGCTACGCGCTGCTGCT | 30% | This study |
| Comp395 | OTU-22, 23 | TTATCCTCTGCTGCTGCTGCTGCT | 30% | This study |
| BCD1422 | OTU-24, 25 | ACCAATGGTGGCTGCTGCTGCT | 30% | This study |
| BCAT1010 | OTU-26 | CTCCGAGTCTCCTGCTGCTGCT | 40% | This study |
| Comp1010 | OTU-27 | ACGCGCTGCTCCTGCTGCTGCT | 10% | This study |
PHA Accumulation in Activated Sludge

with the addition of acetate. PHA accumulated concurrently with the consumption of acetate, whereas no PHA accumulation was observed when the activated sludge samples were incubated without the addition of acetate. As shown in Table 2, the PHA accumulation rate and the conversion ratio of acetate into PHA were in the range of 8.5–38.2 mg-C g-VSS\(^{-1}\) h\(^{-1}\) and 29–64%, respectively. The increase of biomass concentration (excluding the amounts of PHA) during the batch experiment was less than 5%, indicating that the growth of microorganisms would be negligible.

Abundance of PHAAOs in activated sludge

The abundance of PHAAOs in biomass was examined by microscopy after dual staining with NBA and DAPI. A typical microscopic image after dual staining with NBA and DAPI is shown in Fig. 1. The following three types of particles were observed in the biomass: i) particles exhibiting only the DAPI signal; ii) particles exhibiting both DAPI and NBA signals; and iii) particles exhibiting only the NBA signal. These particles were referred to as non-PHAAOs, PHAAOs, and NBA particles, respectively. In the present study, the total number of cells was defined as the sum of cells exhibiting the DAPI signal, which included the population of non-PHAAOs and PHAAOs. On the other hand, the abundance of NBA particles was ignored in the enumeration of PHAAOs and total cells.

We identified the population of PHAAOs from all activated sludge samples. The abundance of PHAAOs in the total number of cells ranged between 11% (AS-D) and 18% (AS-F), as presented in Table 3.

Microbial community compositions of PHAAOs

The microbial community composition of PHAAOs was investigated by FISH analysis and post-FISH NBA staining techniques. A set of bacterial domain/phylum/class/order-specific oligonucleotide probes was first applied to outline the microbial community composition of PHAAOs. As shown in Table 4, the PHAAOs hybridized with the EUB mix probe accounted for 80% to more than 95% in the entire population of PHAAOs. The abundance of \(\alpha\) - and \(\beta\)-proteobacterial PHAAOs was 13–40% and 53–83% in the entire population of PHAAOs, respectively. Members of PHAAOs affiliated to the class Gammaproteobacteria, the phylum Bacteroidetes, Actinobacteria or Firmicutes were also detected, while their abundance was less than 6% in the entire population of PHAAOs.

The above community structure analysis revealed that dominant PHAAOs are members of the class Alphaproteobacteria or Betaproteobacteria, leading us to further

### Table 2. PHA accumulation rates and conversion rates of acetate into PHA.

| Activated sludge sample | PHA accumulation rate (mg-C g-VSS\(^{-1}\) h\(^{-1}\)) | PHA conversion ratio (mg-C mg-C\(^{-1}\) × 100) |
|-------------------------|-----------------------------|---------------------------------|
| AS-A                    | 8.5                         | 39%                            |
| AS-B                    | 10.9                        | 29%                            |
| AS-C                    | 11.5                        | 41%                            |
| AS-D                    | 11.6                        | 35%                            |
| AS-E                    | 19.7                        | 49%                            |
| AS-F                    | 20.1                        | 40%                            |
| AS-G                    | 25.3                        | 45%                            |
| AS-H                    | 38.2                        | 64%                            |

### Table 3. Abundance of PHAAOs in activated sludge samples.

| Activated sludge sample | PHAAOs/Total cells |
|-------------------------|---------------------|
| AS-A                    | 15 ± 5%             |
| AS-B                    | 13 ± 3%             |
| AS-C                    | 12 ± 3%             |
| AS-D                    | 11 ± 2%             |
| AS-E                    | 13 ± 2%             |
| AS-F                    | 18 ± 5%             |
| AS-G                    | 16 ± 2%             |
| AS-H                    | 14 ± 3%             |
Table 4. Microbial community composition of PHAAOs in the 8 full-scale WWTPs. Activated sludge samples were examined by FISH with the oligonucleotide probes described in Table 1 and post-FISH NBA staining analysis. Abundance of individual PHAAOs refers to the entire population of PHAAOs. n.d.: not detected

| Target                      | AS-A | AS-B | AS-C | AS-D | AS-E | AS-F | AS-G | AS-H |
|-----------------------------|------|------|------|------|------|------|------|------|
| **Most bacteria**  
Alphaproteobacteria        |   >95% | 89 ± 14% | 88 ± 11% |   >95% |  >95% |  >95% |  >95% | 80 ± 8% |
| OTU-1, 2, 3               |   <1% |      —   |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| OTU-4, 5, 6               | n.d. |   <1%  |      —  |      —  | 2 ± 1%  |      —  |      —  |      —  |
| OTU-7                      | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| OTU-8                      | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| **Betaproteobacteria**     | 70 ± 6% | 56 ± 12% | 64 ± 14% | 83 ± 13% | 74 ± 14% | 71 ± 15% | 60 ± 16% | 53 ± 10% |
| OTU-9, 10, 11, 12          | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| OTU-13, 14                 | n.d. |   <1%  |      —  |      —  | 9 ± 7%  |      —  |      —  |      —  |
| OTU-15                     | n.d. | 2 ± 1%  |      —  |      —  | 15 ± 6%  |      —  | 3 ± 2%  |      —  |
| OTU-16                     | 5 ± 3% | 4 ± 3%  |      —  |      —  | 7 ± 2%  |      —  | 3 ± 2%  |      —  |
| OTU-17                     | 8 ± 3% | 4 ± 2%  |      —  |      —  | 2 ± 1%  |      —  |   <1%  |      —  |
| OTU-18                     | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| OTU-19, 20, 21             | 5 ± 4% | 7 ± 4%  |      —  |      —  | 13 ± 6%  |      —  | 4 ± 2%  |      —  |
| OTU-22, 23                 | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| OTU-24, 25                 | n.d. | 5 ± 3%  |      —  |      —  | 4 ± 2%  |      —  | 1 ± 1%  |      —  |
| OTU-26                     | 16 ± 5% | <1%    |      —  |      —  |   <1%   |      —  |      —  |      —  |
| OTU-27                     | n.d. |      —  |      —  | n.d.  |      —  | n.d.  |      —  |      —  |
| **Gammaproteobacteria**    | 3 ± 2% | 4 ± 2%  | 2 ± 1%  | 4 ± 2%  | 3 ± 1%  | 1 ± 1%  | 6 ± 2%  | 5 ± 2%  |
| Bacteroidetes              | 3 ± 1% | 5 ± 3%  | <1%    | 2 ± 1%  | 3 ± 1%  | n.d.  | 5 ± 2%  | <1%    |
| Actinobacteria             | n.d. |      —  | <1%    | 2 ± 2%  | 2 ± 2%  | n.d.  |      <1%  | n.d.    |
| Firmicutes                 | n.d. |      —  | n.d.   | 2 ± 1%  | n.d.    | n.d.  |      n.d.  | n.d.    |
| Chloroflexi                | n.d. |      —  | n.d.   | n.d.   | n.d.    | n.d.  |      n.d.  | n.d.    |

* Hybridized with the EUB mix probe

investigate the microbial community structure of α- or β-proteobacterial PHAAOs. For this purpose, 16S rRNA gene clone libraries were constructed from AS-A, AS-B, AS-E, AS-G and AS-G that exhibited low (8.5 and 10.9 mg-C g-VSS⁻¹ h⁻¹ for AS-A and AS-B, respectively), medium (19.7 mg-C g-VSS⁻¹ h⁻¹ for AS-E) and high (38.2 mg-C g-VSS⁻¹ h⁻¹ for AS-H) PHA accumulation rates in the batch experiments (Table 2). In total, 335 clones were randomly picked up from the four clone libraries and the 16S rRNA gene sequences were partially determined. The number of clones, OTUs, Shannon, Simpson, Chao1 and coverage indices of the four clone libraries are summarized in Table S3. The coverage indices of the four clone libraries are between 63 and 78%. We identified 8 and 19 OTUs in the class Alphaproteobacteria or Betaproteobacteria, respectively, and nearly the full length of 16S rRNA gene sequences was determined from the representative clones in each OTU. Phylogenetic relationships of the OTUs in class Alphaproteobacteria or Betaproteobacteria are presented in Fig. 2.

To identify PHA accumulation by microorganisms affiliated to the OTUs in class Alphaproteobacteria or Betaproteobacteria, 12 oligonucleotide probes were designed as follows: ARR994 for OTU-1, 2, and 3, ARP653 for OTU-4, 5, and 6, ABU1302 for OTU-7, an AHS576 for OTU-8, BRDA454 for OTU-15, BCC1212 for OTU-18, BCOS395 for OTU-19, 20 and 21, BCAD1422 for OTU-22 and 23, BCAT1010 for OTU-24 and 25, BCR622 for OTU-26 and BC1823 for OTU-27. The concentration of FA for each designed oligonucleotide probe is shown in Table 1 and was determined using the reference pure cultures or the activated sludge samples (Table S2). In addition, the oligonucleotide probes DEN441 for OTU-9, 10, 11 and 12, PAO846 for OTU-13 and 14, ZOO834 for OTU-16 and OTU1 mix for OTU-17 were also employed from previous reports (13, 19, 47). Even though ARR994, ARP653, DEN441, PAO846, BCOS395, BCAD1422 and BCAT1010 probes are not specific to a single OTU but cover multiple OTUs, these oligonucleotide probes are useful for comparison of the microbial community composition of PHAAOs among the activated sludge samples.

The microbial community composition of PHAAOs was investigated by FISH analysis with a set of newly designed and selected oligonucleotide probes and post-FISH NBA staining techniques. As shown in Table 4, PHA accumulation was confirmed for the following OTUs: OTU-1, 2, 3, 4, 5 and 6 in the class Alphaproteobacteria and OTU-13, 14, 15, 16, 17, 19, 20, 21, 24, 25 and 26 in the class Betaproteobacteria. Their abundance ranged from <1% to 16%, which was quite different across the activated sludge samples. For instance, the PHAAOs in OTU-26 accounted for 16% in the entire population of PHAAOs in AS-A, whereas they were less than 1% in AS-B and AS-E, and even not detected in AS-G. This outcome clearly demonstrated that the microbial community compositions of α- and β-proteobacterial PHAAOs were different among the WWTPs.

**Discussion**

The removal of biodegradable organic matter is the prime...
objective of biological wastewater treatment. Previous studies revealed that up to 45% of easily biodegradable organic matter in sewage was removed and tentatively stored in microbial cells as PHA granules (11, 48). In the present study, we incubated activated sludge samples with the addition of acetate and determined the PHA conversion ratio. As shown in Table 2, the PHA conversion rates obtained in the present study were in the range of 29–64%, and an even higher PHA conversion rate was determined from the activated sludge sample collected from a laboratory scale sequencing batch reactor (i.e. 66–almost 100%) (6). These outcomes indicate that large amounts of easily biodegradable organic matter can be removed by PHAAOs in activated sludge. On the other hand, PHAAOs were not as abundant in the activated sludge processes throughout the world.

The objective of biological wastewater treatment is to remove biodegradable organic matter, their abundance and microbial community composition in activated sludge are still poorly understood, especially in full-scale WWTPs. So far, only one report is available, which described the microbial community composition of PHAAOs in full-scale WWTPs operated in AO and A2O mode (47). However, the 16S rRNA gene-based clone library (58, 62), PCR-denaturing gradient gel electrophoresis (DGGE) (9), and quinone profiling (23, 26) analysis have revealed that the microbial community composition in full-scale WWTPs operated in AO or A2O mode differed from that in fully aerobic mode; therefore, we investigated the microbial community compositions of PHAAOs in full-scale WWTPs operated in fully aerobic mode, the most common configuration mode of activated sludge processes throughout the world.

The outcome of FISH analysis and post-FISH NBA staining revealed that the population of PHAAOs in full-scale WWTPs was dominantly composed of the members of α- and β-proteobacterial PHAAOs. The dominance of β-proteobacterial PHAAOs was also reported in a lab-scale aerobic sequencing batch reactor (SBR) fed with acetate (15, 35, 57). Dionisi et al. (15) examined microbial community composition in activated sludge exhibiting high PHA-accumulating capacity (i.e. 392 mgCOD gCOD⁻¹ h⁻¹) by 16S rRNA gene-based clone library. Their outcome pointed out that the clones affiliated to the genera *Thauera*, *Alcaligenes* and *Comamonas* (Betaproteobacteria) were dominant and accounted for 76% in the clone library. In addition, the combination of FISH analysis and post-FISH NBA staining revealed the dominance of β-proteobacterial PHAAOs affiliated to the genus *Azoarcus* (23.3–45.9% in the entire population of PHAAOs) or *Thauera* (41.1–49.4%) (35, 57). Moreover, we recently reported the dominance of β-proteobacterial PHAAOs in full-scale WWTPs operated in AO or A2O mode (39–60% in the entire population of PHAAOs) (47). The dominance of α-proteobacterial PHAAOs affiliated to the genus *Amaricoccus* (61% in the entire population of PHAAOs) has been also identified in activated sludge collected from a lab-scale SBR fed with propionate (35). On the other hand, the occurrence of γ-proteobacterial PHAAOs has been reported previously from an aerobic SBR with relatively short biomass retention time (i.e. 1 day) (27). In addition, the capability for the synthesis of intracellular PHA granules has been confirmed using microorganisms affiliated to the phylum *Firmicutes* (29), *Actinobacteria* (40) and *Chloroflexi* (33); however, PHAAOs affiliated to class *Gamma-proteobacteria*, phylum *Firmicutes*, *Actinobacteria* and *Chloroflexi* were minor in full-scale WWTPs operated in fully aerobic mode, as shown in Table 4 (<6% in the entire population of PHAAOs).

Unexpectedly, NBA particles were found in activated sludge as the outcome of NBA staining, and were stained with NBA but not DAPI. Recently, the authors examined the
characteristics of NBA particles, and concluded that they
were not PHA-accumulating cells but PHA granules for the
following reasons: (i) no fluorescent signal was obtained from
NBA particles even after staining with nucleic acid-staining
dyes, including DAPI, SYBR Green I or SYBR Gold
(Invitrogen, OR, USA), (ii) no cellular structure was observed
from NBA particles under electron microscopy and (iii) the
buoyant density of NBA particles (1.21–1.23 g ml⁻¹) was
significantly higher than that of microbial cells in activated
sludge (1.16–1.18 g ml⁻¹) but comparable with that of pure
PHA granules (1.21–1.23 g ml⁻¹) (Oshiki, et al., unpublished
data). These observations support the idea that the NBA
particles were not PHA-accumulating cells but probably PHA
granules, and thus the abundance of NBA particles was
ignored in the enumeration of PHAAOs or total cells in the
present study.

In contrast to the similarity of the microbial community
composition of PHAAOs at domain/phyllum/class/order
level, the microbial community compositions of α- and β-
proteobacterial PHAAOs differed among AS-A, AS-B, AS-
E and AS-G. Such a difference was expected because a broad
range of PHA accumulation rate (8.5–25.3 mg C g VSS⁻¹ h⁻¹)
was found in the batch experiment even though the
abundance of PHAAOs in these activated sludge samples
was not significantly different (13–16% in the number of
total cells). Therefore, it was speculated that the microbial
community structure of α- and β-proteobacterial PHAAOs
was different, which resulted in different PHA accumulation
rates among the activated sludge samples. Various previous
studies support this hypothesis as they have reported a wide
range of PHA accumulation capability from pure isolates
of PHAAOs (20, 61). For instance, PHA accumulation
rates of Cupriavidus necator, Azohydromonas lata, Azohydromonas australica, Burkholderia cepacia and Pelomonas saccharophila have been reported to be in the
range of 0.001–0.105 g-PHA g-cell-dry weight⁻¹ h⁻¹ (20).

Environmental conditions (i.e. wastewater composition and
operational conditions) must affect the niche speciation of
PHAAOs in full-scale WWTPs. As shown in Table S1, the
wastewater composition and operational conditions differed
among the WWTPs. For instance, the values of SRT ranged
between 3 and 8 days among the 8 WWTPs. The influence
of SRT on the microbial community structure in activated
sludge has been investigated previously by 16S rDNA
terminal restriction fragment length polymorphism (T-RFLP)
(54) or DGGE (1) analysis, indicating that the bacterial
diversity in activated sludge was influenced by the SRT
values in the range of 2–8 days (54) or 2.6–10.4 days (1),
which is comparable to the present study (3–8 days). The
specific influence of SRT on the microbial community
composition of PHAAOs is still unclear, while it could be
a cause for the occurrence of different microbial
community composition of PHAAOs in WWTPs. In addition,
physiological characteristics of PHAAOs are another factor
for deciding their niche speciation. Different physiological
characteristics (i.e. optimal temperature for growth, maxi-
mum growth rate, substrate specificity and PHA production
capability) have been demonstrated from the 6 pure cultures
of PHAAOs, although the similarities of 16S rDNA nucle-
otide sequences implied that they were affiliated with the
same species (25); however, further investigation is required
to elucidate the specific influence of environmental conditions
or physiological characteristics on the niche speciation of
PHAAOs.

Interestingly, a weak but significant correlation was found
between the values of SRT and PHA accumulation rates (Fig.
S1, \( R^2=0.61, p=0.05 \)). The activated sludge sample collected
from the WWTP operated with shorter SRT showed a
higher PHA accumulation rate. In addition, the authors
found a significant correlation between the population of
α-proteobacterial PHAAOs in total PHAAOs and the
PHA accumulation rates (Fig. S1, \( R^2=0.66, p=0.05 \)). These
observations suggested that the operation at short SRT
induced the proliferation of α-proteobacterial PHAAOs who
had higher PHA accumulation capacity. The increase of PHA
accumulation capacity of activated sludge under short SRT
operational conditions was previously observed in the
laboratory scale sequencing batch reactor, while the dominant
PHAAOs were affiliated to Gammaproteobacteria and not
Alphaproteobacteria (27). In the present study, the authors
defined α-proteobacterial PHAAOs that were affiliated to
OTU-1, 2, 3, 4, 5 or 6, while they were minor in the population of
α-proteobacterial PHAAOs (Table 4). Previously, α-
proteobacterial PHAAOs affiliated to the genus Amaricoccus
have been identified from activated sludge (32) while their
abundance was less in our activated sludge samples since
tetrad-forming microorganisms, which are the typical cell
morphology of the genus Amaricoccus, were not found under
microscopy. Further investigation is essential to clarify the
phylogeny of α-proteobacterial PHAAOs in full-scale
WWTPs.

FISH analysis and post-FISH NBA staining allowed us to
identify the members of PHAAOs affiliated to the following
OTUs: OTU-1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 19, 20, 21,
24, 25 or 26. As for the members affiliated to OTU-13, 14,
15, 16 or 17, we previously identified their PHA accumulation
in a similar manner in the present study from activated sludge
samples collected from full-scale WWTPs operated in AO
or A2O mode (47). This finding suggested that the members
affiliated to OTU-13, 14, 15, 16 or 17 are distributed in
full-scale WWTPs operated in fully-aerobic, AO and A2O
mode. On the other hand, PHA accumulation by members
affiliated to OTU-1, 2, 3, 4, 5, 6, 19, 20, 21, 24, 25 or 26 has
de not been demonstrated, while the PHA accumulation
capacity by members affiliated to OTU-1, 2, 3 can be
speculated from the knowledge of previous pure culture
studies. The nucleotide sequences affiliated to OTU-1, 2 or
3 shared similarities with those of Rhodobacter sphaeroides
(Accession number: X53853), which accumulate intercellular
PHA granules (8). It should be noted that the distribution of
PHAAOs in natural or man-made ecosystems is still poorly
understood, although the accumulation of PHA has been
demonstrated in various ecosystems such as soil (28, 53),
compost (53), river biofilm (17), marsh microbial mats (53),
algal mat (10), estuarine sediment (16, 21, 45) and freshwater
(36, 60). In order to examine the distribution of PHAAOs
identified in the present study, future studies are required.

Consequently, the present study demonstrated the distri-
bution of PHAAOs in full-scale WWTPs operated in fully
aerobic mode and also their significant contribution to the
removal of acetate. The population of PHAAOs was mostly composed of α- and β-proteobacterial PHAAOs, whereas their microbial community compositions differed among the WWTPs. This study shed light on the microbial diversity of PHAAOs in full-scale WWTPs, and further studies are required to investigate the niche specification of PHAAOs in activated sludge.

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