

Characterizations of Denitrifying Polyphosphate-accumulating Bacterium *Paracoccus* sp. Strain YKP-9

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A denitrifying polyphosphate-accumulating bacterium (YKP-9) was isolated from activated sludge of a 5-stage biological nutrient removal process with step feed system. This organism was a Gram-negative, coccus-shaped, facultative aerobic chemoorganotroph. It had a respiratory type of metabolism with oxygen, nitrate, and nitrite as terminal electron acceptors. The 16S rRNA gene sequence of strain YKP-9 was most similar to the 16S rRNA gene sequence of *Paracoccus* sp. OL18 (AY312056) (similarity level, 97%). Denitrifying polyphosphate accumulation by strain YKP-9 was examined under anaerobic-anoxic and anaerobic-oxic batch conditions. It was able to use external carbon sources for polyhydroxyalkanoates (PHA) synthesis and to release phosphate under anaerobic condition. It accumulated polyphosphate and grew a little on energy provided by external carbon sources under anoxic condition, but did neither accumulate polyphosphate nor grow in the absence of external carbon sources under anoxic condition. Cells with intracellular PHA cannot accumulate polyphosphate in the absence of external carbon sources under anoxic condition. Under oxic condition, it grew but could not accumulate polyphosphate with external carbon sources. Based on the results from this study, strain YKP-9 is a new-type denitrifying polyphosphate-accumulating bacterium that accumulates polyphosphate only under anoxic condition, with nitrate and nitrite as the electron acceptors in the presence of external carbon sources.

Keywords: Denitrifying polyphosphate-accumulating bacterium, PHA synthesis, polyphosphate granule

The activated sludge wastewater treatment process relies on the selective enrichment of microorganisms with the capacity to remove or remediate specific pollutants. Microorganisms with the ability to take up and store polyphosphate are exploited

for removing phosphorus from wastewaters in a process known as enhanced biological phosphorus removal (EBPR). The EBPR system is based on enrichment of so-called polyphosphate-accumulating organisms (PAOs) through recycling of the sludge between anaerobic and aerobic zones [21, 31, 33]. In the last few years, several studies have shown that biological phosphate removal can take place under anoxic denitrifying conditions [6, 18, 19, 22]. Denitrifying phosphate-accumulating organisms (dPAOs) have received attention owing their to possible cost savings of lower aeration. Low-molecular-weight organic compounds such as acetate are converted to polyhydroxyalkanoates (PHA), whereas polyphosphate and glycogens are degraded and then phosphate is released under anaerobic conditions. PHA is converted to glycogen, phosphate is assimilated, and polyphosphate is accumulated under aerobic or anoxic conditions [29]. Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-oxic sequencing batch reactors have been found to belong to the species *Paracoccus denitrificans*, *Agrobacterium tumefaciens*, *Aquaspirillum dispar*, and *Agrobacterium radiobacter* based on phenotypic characterizations [5, 27]. However, the information on dPAOs is still unclear, and as such, a better understanding of dPAOs in EBPR systems is required for stable operation.

In this study, a polyphosphate-accumulating bacterium (YKP-9) was isolated from a 5-stage biological nutrient removal process with a step feed system [21], which showed a very stable organic carbon and nutrient removal efficiency (87% COD, 79% nitrogen, and 87% phosphorus) for 2 years. This strain was capable of polyphosphate accumulation under anoxic condition, with nitrate and nitrite as the electron acceptor. Until the physiology and taxonomic status of this strain are resolved in pure culture, interpretation of the function of this strain in activated sludge systems will remain unclear. Therefore, this study improves the understanding of the role of dPAOs and will contribute to the understanding of the BNR processes of nitrogen and phosphorus removal to obtain successful plant performance.

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MATERIALS AND METHODS

Isolation of Denitrifying Polyphosphate accumulating Bacteria

Activated sludge (1 g) of a 5-stage biological nutrient removal process with step feed system [21] was washed twice with phosphate-buffered saline (135 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 [pH 7.4]), suspended, and serially diluted in sterile distilled water. Diluted activated sludge samples were directly spread on sludge agar medium (prepared by mixing 17 g of bacto agar per liter of sewage supplement). The sludge agar medium was incubated at 30°C for 7 days and, on the basis of pigment, shape, size, surface texture, and opacity, the morphologically distinct colonies were isolated by transferring single colonies to the fresh sludge agar medium [10]. The denitrifying bacteria of pure colonies were initially selected by detection in the presence of nitrite or nitrate followed by the addition of Griess-Ilosvay reagent and zinc powder in butyl rubber-stoppered tubes sparged with argon before the inoculation that held 10 ml of nitrate-containing liquid sludge medium for each isolate, and then N_2 -producing bacteria in the initially selected bacteria were isolated. Polyphosphate accumulating bacteria in N_2 -producing bacteria were confirmed by using DAPI stain as PAO [25] in anoxic-condition with nitrite. Sudan Black, in particular, was utilized as a presumptive test for the presence of PHA even though it stains all lipophilic storage materials [10, 17].

DNA Preparation, Sequencing, and Analysis of 16S rRNA Gene Sequencing Data

Genomic DNA of strain YKP-9 was purified by the procedure of Hallin and Lindgren [12]. Amplification of 16S rRNA from chromosomal DNA was carried out in a DNA thermal cycler (Model 480; Perkin-Elmer, Norwalk, CT, U.S.A.) with universal bacterial primers, 27F and 1492R [7, 16]. The PCR temperature program began with an initial 5-min denaturation step at 94°C; 30 cycles of 94°C for 1 min, 57°C for 2 min, 72°C for 2 min; and a final 10-min extension step at 72°C. The PCR product (approximately 1.5 kb) was confirmed by agarose gel electrophoresis of the amplified mixture using a gel extraction kit (NucleoGen, Seoul, Korea) [8].

The PCR product was purified with a QIAquick PCR purification kit (Qiagen, Germany), cloned into pGEM-T Easy vector system-I according to the manufacturer's instruction (Promega, Madison, WI, U.S.A.) [30]. The 16S rRNA gene sequence of the cloned products was determined from plasmid DNA preparations using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI310 Sequencers (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturers instructions. Vector primer T7 and SP6 were used for the sequencing reactions.

The 16S rRNA gene sequence of strain YKP-9 was compared with those in the National Center for Biotechnology Information nucleotide database by using BLAST searching [1] and was compared with sequences in the RDP 8.1 database [26]. Sequences closely similar to the 16S rRNA gene sequence of strain YKP-9 as well as selected reference sequences were downloaded and manually aligned and analyzed by using BioEdit (version 5.0.9) [11]. A distance matrix and a neighbor-joining tree were constructed by using 1,000 bootstrap replicates and the PHYLIP v. 3.6 software package (J. Felsenstein, University of Washington, Seattle, 2005). A phylogenetic tree was created from the neighbor output by using the program TreeView. The 16S rRNA gene sequence of *Paracoccus denitrificans* (CP000489) was used as an outgroup reference on this tree. The robustness of inferred

tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data, and only values of >70% were shown on the trees.

Morphological Characteristics

Gram staining was performed as described by Gerhardt *et al.* [10]. Morphology was examined by phase-contrast microscopy, scanning electron microscopy, and transmission electron microscopy. For scanning electron microscopy, cultured cells were harvested by centrifugation, washed, and suspended in 20 mM phosphate buffer (pH 7.0). The suspended cells were fixed with 2.5% glutaraldehyde and postfixed with 2% osmium tetroxide. The cells were dehydrated using acetone. After critical point drying, samples were sputter-coated with gold and observed with a model JSM-840A scanning electron microscope (JEOL, Ltd., Tokyo, Japan). For transmission electron microscopy, a centrifuged cell pellet was fixed with 2.5% glutaraldehyde and 2% osmium tetroxide. Ultrathin sections of the sample embedded in epoxy resin were prepared with a Reichert Ultracut E or S ultramicrotome, stained with uranyl acetate and lead citrate. Samples were examined with a model JEM-1200EX transmission electron microscope (JEOL, Ltd., Tokyo, Japan). The energy-dispersive X-ray (EDS) spectra were recorded with a KEVEX-X 7000 analytical spectrometer (Kevex Co., Foster City, CA, U.S.A.).

Physiological and Biochemical Characteristics

Screening of the carbon substrates utilized was performed with the Gram Negative Identification Card (bioMérieux VITEK, Inc., Hazelwood, MO, U.S.A.). Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine dihydrochloride on filter paper [10]. Catalase activity was determined with a 3% hydrogen peroxide solution. The liquid samples were centrifuged at 12,000 rpm and 4°C for 15 min. Then, the supernatant was filtered through a 0.22- μm porosity filter. The supernatants were diluted as required by the different analytical methods. $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{PO}_4\text{-P}$ were analyzed with an Ion Chromatograph (DIONEX model DX-500; U.S.A.). Acetate was analyzed by HPLC (high-pressure liquid chromatography) with a Bio-Rad Aminex HPX-87H column and with 5 mM sulfuric acid as the eluent; the absorbance was monitored at 210 nm. All other analytical procedures followed the Standard Methods [2]. The quinones were extracted from the cell pellet washed with 50 mM phosphate buffer (pH 7.0) using chloroform/methanol (2:1, v/v), and analyzed by thin-layer chromatography (TLC) on a Kieselgel 60F₂₅₄ plate (Merck, Darmstadt, Germany) using a mixture of hexane:diethyl ether (85:15, v/v) as the developing solvent. The quinone bands were detected under UV wavelength, scraped from the TLC plate, and recovered in acetone. High-performance liquid chromatography (HPLC) was used to determine the isoprenoid quinone composition. The analytical conditions were as follows column, Spherisorb 5 μm ODS2 (4.6 \times 250 mm; Waters Associates, Milford, MA, U.S.A.); eluent, methanol/isopropyl ether (3:1, v/v) for ubiquinones, methanol/isopropyl ether (4:1, v/v) for menaquinones; flow rate, 1 ml/min. The ubiquinones and menaquinones were detected by monitoring at 275 nm and 270 nm, respectively, using a UV detector.

Culture Conditions of Phosphate Release and Uptake

Strain YKP-9 was tested for phosphate release and uptake efficiency. The cells were pregrown aerobically at 30°C overnight in preculture medium containing the following: 15 g/l peptone, 15 g/l yeast extract, 1.5 g/l KNO_3 , and 3.6 g/l $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$ (adapted to NO_3^- preculture). When the cells were in the logarithmic growth phase, they

were harvested by centrifugation (6,000 rpm, 10 min, 4°C), washed twice with 0.9% NaCl solution, and then suspended in phosphate limitation medium for anaerobic culture at 30°C for 48 h in butyl rubber-stoppered tubes and sparged with argon. The composition of phosphate limitation medium was (per liter): 3.23 g $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$, 23 mg $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, 152.8 mg NH_4Cl , 81.12 mg $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 17.83 mg K_2SO_4 , 11 mg $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 7 g HEPES buffer, and 2 ml of the trace mineral solution [15]. The pH was adjusted to 7 with 1 N NaOH. After anaerobic incubation, the cells were harvested and washed with 0.9% NaCl solution for shift anoxic or oxic incubation. Strain YKP-9 was inoculated in the phosphate uptake medium (24.5 mg PO_4/l) of the following composition (per liter): 3.23 g $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$, 25 mg KH_2PO_4 , 305.52 mg NH_4Cl , 91.26 mg $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 25.68 mg $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 8.5 g PIPES buffer, and 2 ml of the trace mineral solution. KNO_3 (1.5 mg/l) was added to a phosphate uptake medium for anoxic-condition. Air (DO concentration: 2–3 mg/l) was added to a phosphate uptake medium for oxic condition. The pH was adjusted to 7 with 1 N NaOH. Triple cultures were incubated at 30°C for 48 h. Then, each bacterial suspension was filtered through a 0.22- μm porosity filter, and the supernatant was analyzed for phosphate. Bacterial cells were counted by the conventional plate counting method by using R2A (Difco Laboratories) [10].

Analysis and Quantification of PHA in Cells

Strain YKP-9 was pregrown aerobically at 30°C overnight in preculture medium. When the cells were in the logarithmic growth phase, they were harvested by centrifugation and washed twice with 0.9% NaCl solution. The cultures were then grown on phosphate limitation medium for 48 h before being harvested for PHA analysis. PHA content was quantified by gas chromatography-mass spectrometry (Hewlett-Packard 5890 gas chromatograph [HP-5MS column] equipped with a Hewlett-Packard 5972 mass spectrophotometer) analysis of dichloroethane extracts of dried cell material subjected to propanolysis [20]. The cells were harvested by centrifugation, dried overnight at 101°C, washed six times with warm methanol (65°C), and redried. Dry cell material (50 mg) was then incubated in a boiling water bath for 2 h in a mixture of 0.5 ml of dichloroethane plus 0.5 ml of acidified propanol.

Quantification of Polyphosphate Content in Cells

Strain YKP-9 was cultured in the phosphate uptake medium with NO_3^- as the electron donor after the cells were pregrown aerobically at 30°C overnight in preculture medium. For the extract of polyphosphate, aliquots of cells were collected at appropriate times during the anoxic and aerobic phases and centrifuged at 4°C for 2 min. Polyphosphate was extracted from cell extracts by using Glassmilk from harvested cells and then assayed by the reverse reaction of *E. coli* polyphosphate kinase with ADP excess [4]. The concentration of ATP was quantified by using an ATP assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA, U.S.A.) as described in the manufacturer's instructions. The assay was based on the luciferase-catalyzed oxidation of D-luciferin in the presence of an ATP-magnesium salt and oxygen to produce light. Luminescence was measured with an LS6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA, U.S.A.).

Nucleotide Sequence Accession Number

The 16S rRNA gene sequence of strain YKP-9 has been deposited in the Genbank, EMBL, and DDBJ nucleotide sequence database under Accession No. AY256516.

RESULTS AND DISCUSSION

Morphological Characteristics

Morphological characteristics of strain YKP-9 can be described as a Gram-negative, coccus-shaped, nonmotile bacterium. It is 1.0 to 1.8 μm in diameter and occurred singly or in pairs (Fig. 1). Cell size depended on the growth stage; cells were larger at the early stage of a culture than at the late stationary phase. Filamentous and rod-shaped cells were not observed at any stage of growth. Strain YKP-9 did not grow as flocs in the liquid medium either.

Physiological and Biochemical Characterizations

Utilization of sole carbon source and some enzyme activities for strain YKP-9 are summarized in Table 1. Based on Table 1, strain YKP-9 was a facultative aerobic chemoorganotroph that had a respiratory type of metabolism with oxygen, nitrate, and nitrite as the terminal electron acceptors. The temperature range for growth was 5 to 37°C, and the optimum temperature was 25 to 30°C. Strain YKP-9 did not grow over 40°C. The pH range for growth was 5 to 9, and maximum growth occurred at pH 7. Strain YKP-9 did not grow under pH 4 and over pH 10. Catalase and oxidase activities were positive, but those of lysine, ornithine, and arginine decarboxylase were negative.

Strain YKP-9 had ubiquinone 10 (Q-10) as its major quinone component. Respiratory quinones have been used as markers in both laboratory-scale and full-scale EBPR systems. Ubiquinone profiles have been reported in EBPR biomass samples [22, 32] and Q-8 and Q-10 were more dominant than Q-9. Ubiquinone Q-8, diagnostic of the β -*Proteobacteria*, was the abundant quinone in both EBPR and non-EBPR plant biomass samples from both full-scale and laboratory-scale systems, and not Q-9 associated with the γ -*Proteobacteria*, which include *Acinetobacter* spp. [3, 13]. The *Actinobacteria* and α -*Proteobacteria* possessing

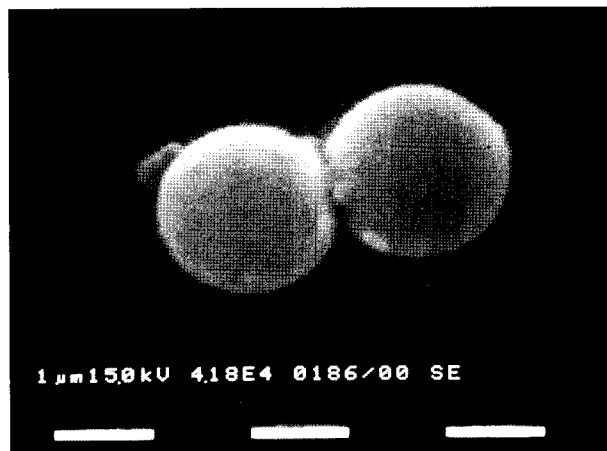


Fig. 1. Scanning electron micrograph of strain YKP-9. Strain YKP-9 shows coccoid cells. Bar, 1 μm .

Table 1. Physiological and biochemical characteristics of YKP-9.

Characteristics	Results
Gram reaction	—
Morphology	Cocci
Gas production	+
Oxidase	+
Catalase	+
Capsule	—
Indole test	—
Decarboxylase	
Lysine	—
Ornithine	—
Arginine	—
Utilization as sole carbon source	
Maltose	+
Lactose	+
Malonate	+
Acetate	+
<i>p</i> -Coumaric	—
Polymyxin B	—
Tryptophan	+
Esculin	—
Mannitol	+
Raffinose	—
Sorbitol	+
Galactose	+
Rhamnose	—
Inositol	+
Sucrose	+
Acetamide	—
L-Arabinose	+
Xylose	+
Citrate	—
Glucose oxidative	+
Glucose fermentation	+
Acid production from carbohydrate	—
ONPG fermentation	+
H ₂ S production	—
Anaerobic growth with nitrate	+
NO ₃ ⁻ reduced to NO ₂ ⁻ , NO, N ₂ O, and N ₂	+
Intracellular poly-β-hydroxybutyrate present	+
Polyphosphate accumulating	+
Optimum temperature	25–30°C
pH range for growth	5–9
β-Galactosidase	—
Pigment produced on nutrient agar	—

+, positive, —, negative

ubiquinone Q-10 are also thought to be present in large numbers in the EBPR systems [23, 24, 27]. Strain YKP-9 had Q-10 associated with the α -Proteobacteria that has been shown to dominate in the EBPR sludge [23, 24, 27].

Phylogenetic Analysis

The 16S rRNA gene sequence of strain YKP-9 was sequenced. The total length of the fragments, excluding the PCR primer

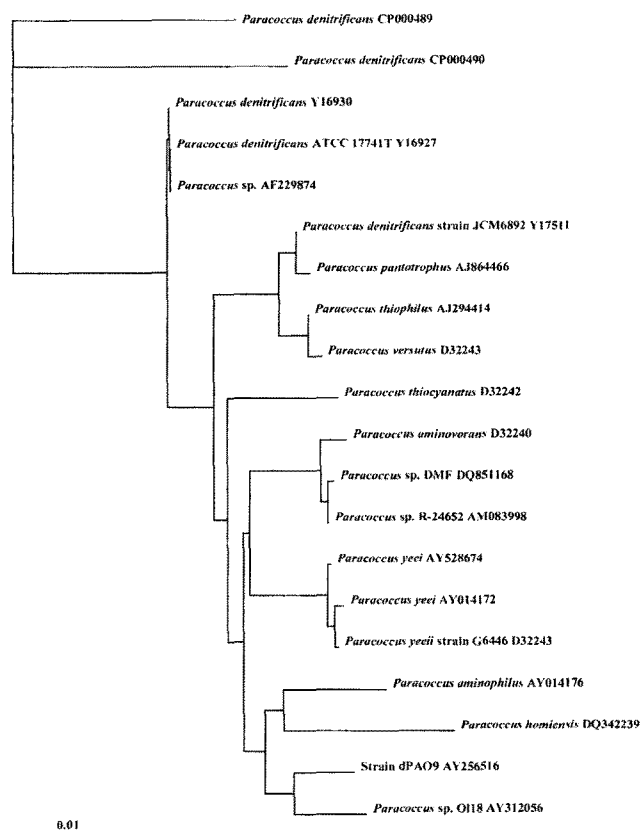


Fig. 2. Phylogenetic tree showing strain YKP-9 together with selected reference strains based on 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method. The scale bar indicates 0.01 estimated substitution per nucleotide.

annealing regions (positions 8 to 27 and 1492 to 1510), was 1,401 nucleotide base pairs. Phylogeny after 16S rRNA analysis using the GenBank database and the BLAST programs indicates strain YKP-9 belongs to the α -subclass of Proteobacteria in the domain bacteria. Strain YKP-9's closest relative is the genera *Paracoccus* in the family *Rhodobacteraceae*. The 16S rRNA gene sequence of strain YKP-9 was most similar to the 16S rRNA gene sequence of *Paracoccus* sp. OL18 (AY312056) (similarity level, 97%). Fig. 2 shows the neighbor-joining phylogenetic tree. Strain YKP-9 formed a lineage distinct from members of the α -subclass of Proteobacteria. *Paracoccus denitrificans* (CP000489), *Paracoccus yeii* (AY528674), and *Paracoccus aminophilus* (AY014176) were not closely related to the lineage containing our isolate. The 16S rRNA gene sequence of *Paracoccus* sp. OL18 (AY312056) was the closest phylogenetic neighbor.

Comparison of the Kinetics of Polyphosphate Removal Between Anaerobic-Anoxic and Anaerobic-Oxic Incubation Conditions

Fig. 3 shows the cell growth of strain YKP-9, acetate consumption, and phosphate release during anaerobic culture

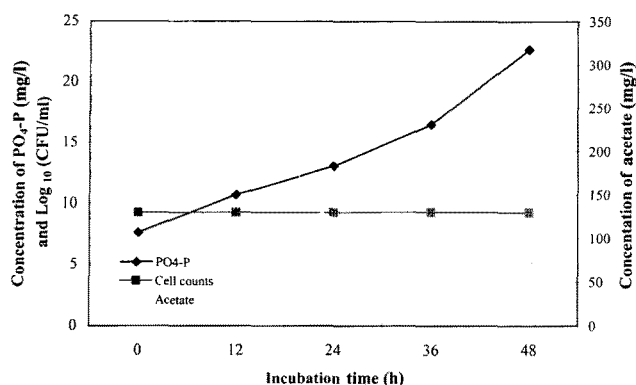


Fig. 3. Phosphate release by strain YKP-9 under anaerobic condition.

Strain YKP-9 was harvested in the late logarithmic phase, washed, and suspended in the PBS buffer at a concentration $\log_{10}^{9.28}$ (CFU/ml) and then a mixture of phosphate limitation medium was added to the suspension under anaerobic conditions.

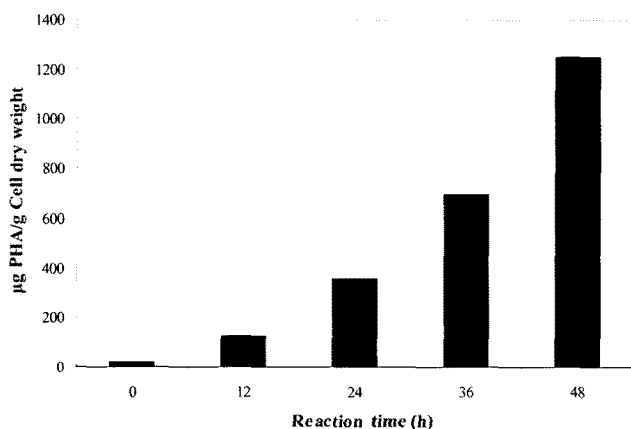


Fig. 4. PHA content in dried biomass of strain YKP-9 at various times during anaerobic culture.

incubation. The phosphate concentration released during the anaerobic phase was approximately 15.1 mg PO₄-P/l for 48 h. The acetate consumption by strain YKP-9 was approximately 76.3% of the initial concentration during anaerobic incubation. However, the number of cells did not increase.

The concentrations of PHA in dry cells of strain YKP-9 were quantified during anaerobic culture. Fig. 4 shows the time profiles of the PHA concentration. The PHA concentrations could be increased, but the number of bacteria was the almost same during the culture period (Fig. 3). The PHA concentration of the initial sample (0 h) was 21.3 µg of PHA/g of dry cell, whereas the PHA concentration of the last sample (48 h after inoculation) was 1,248.2 µg of PHA/g of dry cell. These results mean that the PHA was accumulated as intracellular storage during anaerobic culture. For confirmation of intracellular PHA in strain YKP-9, Sudan Black staining and transmission electron microscopy

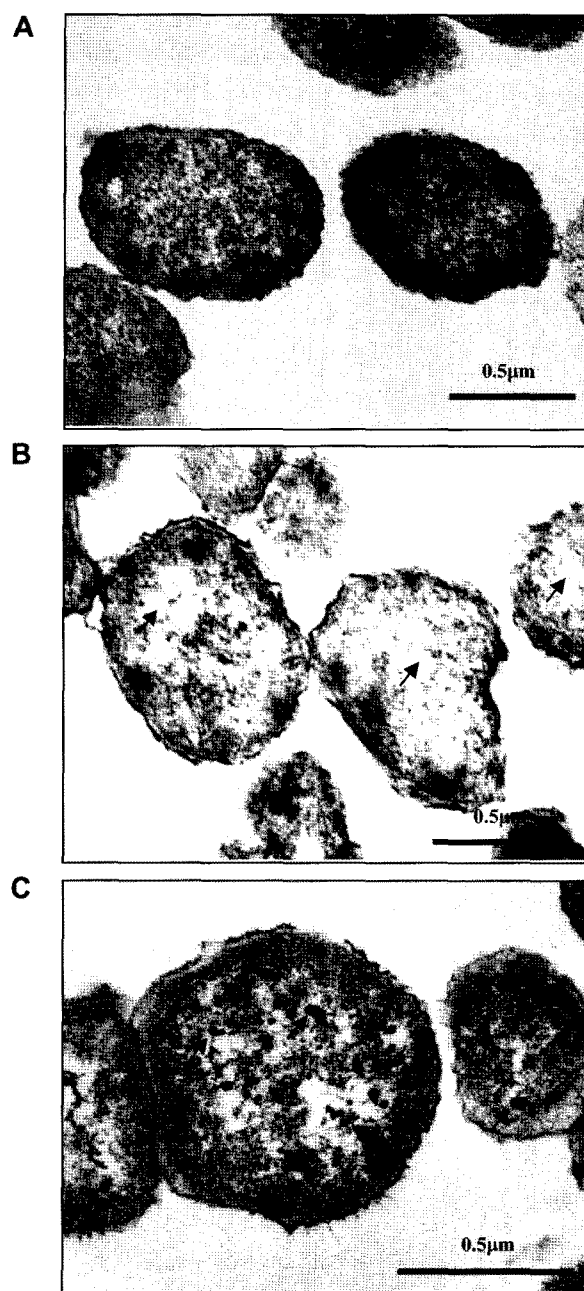


Fig. 5. Transmission electron micrographs of strain YKP-9.

A. Cells observed in the preculture medium under aerobic culture. B. White areas seen in cells are attributed to PHA granule (arrows) under anaerobic culture. C. The dark spots are polyphosphate granules (arrows), and white areas indicate PHA granules under anoxic culture.

were carried out. Sudan Black staining showed that the cell inclusions contained poly-PHA, which is a black-blue granule in a clear or light pink background (data not shown). moreover, PHA granules were observed in cells by using transmission electron microscopy (Fig. 5B). The PHA granules were not shown (Fig. 5A) and were not stained by Sudan Black in cells of oxic culture. Additionally, the cells of anoxic culture include PHA granules in the body (Fig. 5C).

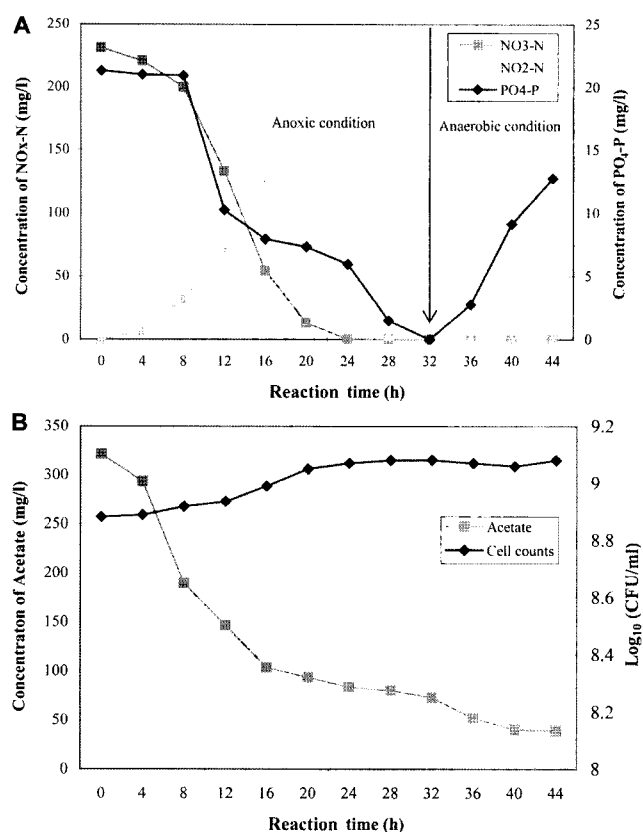


Fig. 6. Polyphosphate uptake and nitrate removal (A), and growth rate and acetate culture by strain YKP-9 under anoxic-culture (B).

Fig. 6A shows profiles of $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{PO}_4\text{-P}$ concentration, and Fig. 6B shows cell growth and acetate concentration during the anoxic batch incubation in the presence of acetate. NO_3^- , $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{PO}_4\text{-P}$ were completely removed in 32 h (Fig. 6A). Acetate was consumed during polyphosphate accumulation and denitrification (Fig. 6B). Cell growth did not increase, but the acetate concentration decreased while polyphosphate was accumulated as intracellular storage under anoxic culture (Fig. 6B).

While polyphosphate was accumulated by strain YKP-9 under anoxic culture (Fig. 7), PHA synthesized by strain YKP-9 under anaerobic culture did decrease, and intracellular polyphosphate was shown in cells (Fig. 5C). Energy-dispersive X-ray spectroscopy was used to identify the dark granules in the cell (Fig. 5C). Elemental analysis was performed by the comparison of phosphorus k-peaks at 2.01 keV (data not shown). EDS analysis revealed that the electron-dense granules contained large amounts of phosphate; so intracellular granules in cell were an accumulation of polyphosphate. Additionally, this strain can use lactose malonate, tryptophan, sucrose, and citrate as sole carbon sources for polyphosphate accumulation and denitrification under anoxic condition.

The poly-P concentration in dry cells (1 g) was determined during anoxic incubation. The poly-P concentration in dry

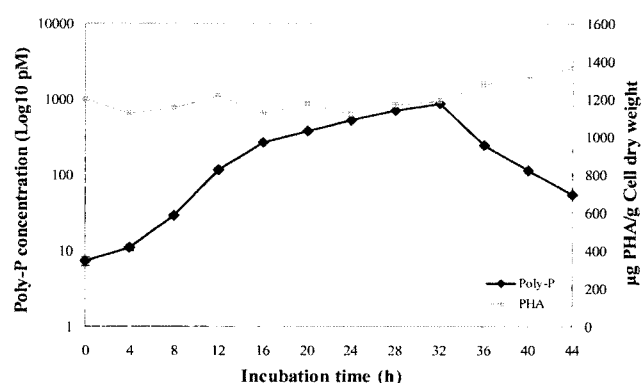


Fig. 7. Polyphosphate and intracellular PHA concentrations of strain YKP-9 at various times during anoxic culture.

cells increased initially during anoxic condition and then decreased after 32 h because of depletion of oxygen (Fig. 7). Fig. 5C shows poly-P granules within the cell at 32 h. At the same time, PHA was quantified during the anoxic incubation time. The concentration of PHA was not affected during anoxic culture (Fig. 7). The PHA granules were shown in cells of anoxic-incubation (Fig. 5C). These results indicate that this strain can use acetate as an electron donor but cannot use PHA granules in cells for denitrification and polyphosphate accumulation. At high concentrations (50 mg/ml) of DAPI staining for polyphosphate granules [31], DAPI-DNA fluorescence is blue-white, whereas for both DAPI-poly-P and DAPI-lipid the fluorescence is yellow. Discrimination between the two storage compounds is by the intensity of the fluorescent response; the lipid fluorescence is weak and fades in a few seconds whereas polyphosphate granules appear bright yellow [31]. Strain YKP-9 was stained bright yellow (data not shown).

When grown in the phosphate uptake medium during aerobic condition (Fig. 8), strain YKP-9 consumes acetate as an electron donor. However, phosphate concentration in this medium scarcely decreases. In addition, intracellular poly-P of strain YKP-9 was not detected, and the concentration of intracellular poly-P was lower than 7.02 pM/g dry cell in all samples. These results indicate that strain YKP-9 could not accumulate the intracellular poly-P under aerobic incubation (Fig. 5A and Fig. 8). Additionally, this result shows that strain YKP-9 is able to respire using oxygen for cell division but is unable to accumulate polyphosphate in cells.

Recent research classifies PAOs under PAO and dPAO [28]. The PAOs use an external carbon source for PHA synthesis and release phosphate under anaerobic condition and then produce polyphosphate by degradation of PHA under aerobic condition. They also grow in the absence of an external carbon source on PHA. Notably, an external carbon source might inhibit polyphosphate synthesis or is used for PHA production but not for growth under aerobic condition [9, 14, 28]. The dPAOs use not only nitrate and

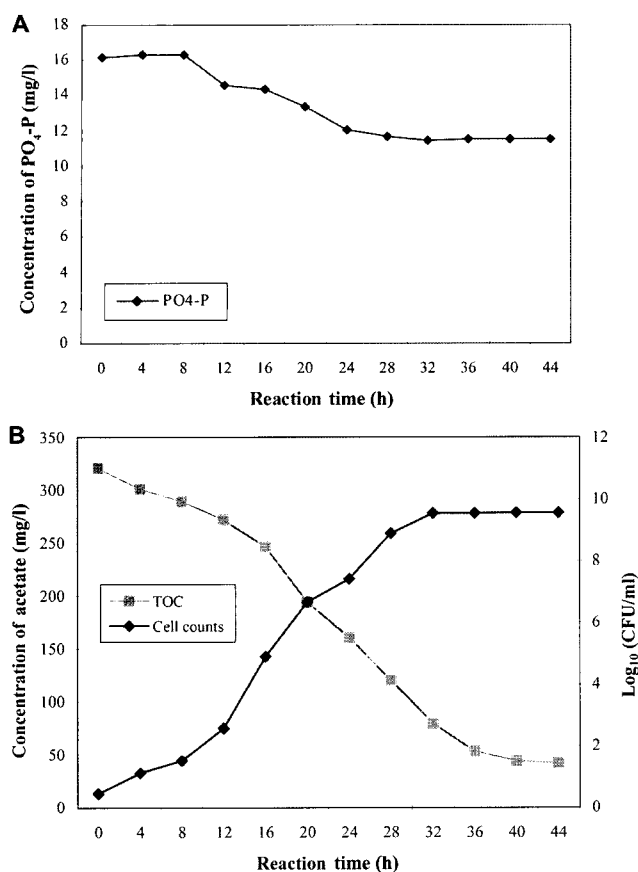


Fig. 8. Polyphosphate uptake (A), and growth rate and acetate concentration (B) of strain YKP-9 under aerobic culture.

nitrite as electron acceptors for polyphosphate accumulation but also accumulate polyphosphate under aerobic condition [18, 19, 27]. Notably, it has been reported that similar metabolism of strain YKP-9 can take place in EBPR [20], where typically in the presence of an external carbon source, phosphate was released in anaerobic conditions and could accumulate polyphosphate under anoxic condition with external carbon sources. Although many metabolisms observed in strain YKP-9 have been demonstrated in EBPR [22], strain YKP-9 is different in that it could not accumulate polyphosphate under oxic-condition and did not consume intracellular PHA for polyphosphate accumulation in anoxic-condition.

In this study, strain YKP-9 is different from the general characteristics of PAOs and dPAOs. The physiological characteristics of strain YKP-9 were: (i) it was able to use an external carbon source for PHA synthesis and to release polyphosphate under anaerobic condition, and (ii) it accumulated polyphosphate on energy provided by an external carbon source under anoxic condition with nitrate, but neither accumulated polyphosphate nor grew in the absence of an external carbon source under anoxic condition. moreover, it did not consume intracellular PHA for polyphosphate accumulation. Instead, it consumed only an external carbon

source for polyphosphate accumulation under anoxic condition. Finally, (iii) it could not accumulate polyphosphate under oxic-condition with external carbon sources. Our results indicate that strain YKP-9 is a new-type denitrifying polyphosphate-accumulating bacterium that accumulates polyphosphate with nitrate and nitrite as the electron acceptors in the presence of an external carbon source. Therefore, the metabolism of this strain may support metabolic models [19, 22] and reactors [5, 18] of biological phosphate removal under a denitrifying environment. The significance of this study is that, contrary to the general assumption among wastewater engineers, biological phosphate and nitrate removal in wastewater may be conducted in only one treatment step without aeration as well as in many steps, as it is currently done.

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