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Polyhydroxyalkanoate production potential of heterotrophic bacteria in activated sludge

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This study was conducted to evaluate the polyhydroxyalkanoate (PHA) production potential of cultivable heterotrophic bacteria in activated sludge by genotypic and phenotypic characterizations. A total of 114 bacterial strains were isolated from four activated sludge samples taken from a lab-scale sequencing batch reactor and three wastewater treatment processes of two municipal wastewater treatment plants. PCR detection of the *phaC* genes encoding class I and II PHA synthase revealed that 15% of the total isolates possessed *phaC* genes, all of which had the closest similarities to known *phaC* genes of α- and β-*Proteobacteria* and *Actinobacteria*. PHA production experiments under aerobic and nitrogen-limited conditions showed that 68% of the total isolates were capable of producing PHA from at least one of the six substrates used (acetate, propionate, lactate, butyrate, glucose and glycerol). Genotypic and phenotypic characterizations revealed that 75% of the activated sludge bacteria had PHA production potential. Our results also indicated that short-chain fatty acids would be the preferable substrates for PHA production by activated sludge bacteria, and that there might be a variety of unidentified *phaC* genes in activated sludge.

[**Key words:** Activated sludge; Heterotrophic bacteria; *phaC* gene; Polyhydroxyalkanoate; Polyhydroxyalkanoate production potential]

Bioplastics have received considerable attention in recent years owing to environmental concerns regarding conventional petroleum-based plastics. Polyhydroxyalkanoates (PHAs), which are polyesters of hydroxyl fatty acids, are a group of bioplastics synthesized by a specific group of bacteria as intracellular carbon and energy storage compounds. To date, approximately 150 different types of PHA monomers have been identified (1). Unlike other bioplastic materials, PHAs can be completely synthesized by microorganisms (2). In addition, PHAs are completely biodegradable, biocompatible and thermoplastic (1,3). Consequently, these compounds are regarded as environmentally-compatible bioplastic materials and have thus attracted attention as promising alternatives to conventional petroleum-based plastics. Due to a wide variety of material properties depending on the monomer composition, PHAs can be used for various purposes. The potential applications of PHAs include packaging (e.g., containers, films), personal hygiene products (e.g., diapers), printing (e.g., toners) and coating (e.g., adhesives) materials, electronic products (e.g., mobile phones), and medical devices (e.g., sutures, repair patches, orthopedic pins, adhesive barriers, stents, nerve guides, bone marrow scaffolds) (4).

PHA production ability has been found in as many as 300 different bacteria (5). Industrial production of PHAs has been accomplished using pure cultures of highly effective PHA producing bacteria, including wild and recombinant organisms, which can accumulate 80%–90% of the cell dry weight (2,5). However, current PHA production with pure strains has technical constraints and economical problems associated with the need for feed and equipment sterilization. In particular, because the feed (PHA substrate) has been estimated to be approximately 40–50% of the total production cost (6), the use of low-cost substrates has been desired. One possible solution to these problems is the use of non-sterile processes employing mixed microbial cultures as the PHA producing agents. PHA production with mixed microbial cultures and renewal raw materials such as agro-industrial wastes or by-products without sterilization can largely reduce the production cost (7,8). However, low PHA accumulation has been a problem associated with PHA production by mixed cultures such as activated sludge. For instance, previous studies have shown that PHA accumulation from acetate by intact activated sludge taken from municipal wastewater treatment plants (WWTPs) was at most approximately 20 wt% with a single batch feeding (9), nearly 30 wt% with continuous feeding (10), and 45 to 67 wt% with fed-batch feeding (11). Conversely, mixed cultures enriched from activated sludge by implementing the feast-famine strategy achieved a very high PHA accumulation of up to 90 wt% from single substrates such as acetate (12) and lactate (13) and nearly 75 wt% from sugar molasses, which are renewable complex feedstocks (14), suggesting the presence of bacteria with a very high PHA accumulation ability in activated sludge and the possibility for practical PHA production using activated sludge-derived mixed

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cultures. To enable methodological development of processes that rationally enhance the PHA accumulation ability of activated sludge, it is essential to thoroughly understand the abundance and taxonomical, genetic and functional diversity of PHA producing bacteria in activated sludge. Nevertheless, only a few studies have investigated the diversity of PHA producing bacteria in intact activated sludge in municipal WWTPs and their PHA production properties at the strain-level (15–17).

This study was conducted to evaluate the PHA production potential of activated sludge bacteria, with a focus on cultivable heterotrophic bacteria. Test strains were isolated from activated sludge samples collected from a lab-scale sequencing batch reactor (SBR) and three wastewater treatment processes of two municipal WWTPs. PHA production potential was evaluated qualitatively through genotypic and phenotypic characterizations, that is PCR detection and sequencing analysis of the *phaC* genes encoding the PHA synthase and PHA production experiments using six different substrates under the aerobic and nitrogen-limited conditions.

MATERIALS AND METHODS

Activated sludge samples and test strains Four different activated sludge samples were collected to isolate test strains used in this study (Table 1). Sample LB was collected from a lab-scale SBR that had been adapted to synthetic wastewater consisting mainly of peptone and glucose. The other samples were collected from two municipal WWTPs (O and K), both of which receive mainly (approximately 90%) domestic wastewater with small amounts of industrial discharge. WWTP O employed a conventional activated sludge (CAS) process, and the collected sample was designated sample OC. WWTP K employed CAS and anaerobic-anoxic-oxic (A2O) processes; thus, samples were collected from both processes and designated samples KC and KA, respectively.

To detect heterotrophic bacteria, serial dilutions of activated sludge samples were plated onto R2A agar (Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 28°C for 7–8 d. For each sample, morphologically distinct colonies (≥1 mm diameter) that appeared on R2A agar were selected, purified, and used as test strains in this study.

Phylogenetic characterization Genomic DNA was extracted from test strains using the Cica Geneus DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan) according to the manufacturer's instructions. Partial sequences of 16S rRNA genes were amplified with the EUB8F (18) and SRV3-2 (19) primer set, which covers the V1 to V3 region. PCR amplification was conducted using the following thermal profile: initial denaturation at 94°C for 1 min; 22 cycles of denaturation at 98°C for 5 s, annealing at 55°C for 5 s and extension at 72°C for 10 s; and final extension at 72°C for 10 min. The PCR products were purified with NucleoSpin Extract II (Macherey–Nagel, Düren, Germany) and sequenced on an Applied Biosystems 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) with primer EUB8F at Macrogen Japan (Tokyo, Japan). The obtained sequence data were compared with sequences in the NCBI database using the BLAST search program (specifically, BLASTN program) (http://www.ncbi.nlm.nih.gov/blast/), and test strains were phylogenetically identified at the genus level based on 16S rRNA gene sequences that had 97% or greater homology to those of bacteria registered in the database.

Detection and sequencing of *phaC* genes The presence of *phaC* genes in test strains was examined by PCR using the CF1 and CR4 primer set (20,21), which was designed specifically to detect *phaC* genes for class I and II PHA synthases. To our knowledge no universal primers to detect *phaC* genes for class III and/or IV PHA synthases were available, albeit there have been specific primers for class III PHA synthase of sulfate-reducing bacteria (22) and haloarchaea (23) and for class IV PHA synthase of *Bacillus* spp. (24). Thus, this study aimed to evaluate the presence of *phaC* genes only for class I and II PHA synthases. PCR was conducted using the following thermal profile: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 98°C for 15 s, annealing at 57°C for 30 s and extension at 72°C for 30 s; and final extension at 72°C for 2 min. The presence of *phaC* genes in test strains was judged by detection of PCR products with a size of around 500 bp. The PCR products were purified and sequenced as described above, excepting the use of both primers CF1 and CR4 for sequencing. The sequences obtained from the sequencing with both primers were combined to yield single sequences. The amino acid sequences deduced from the *phaC* gene sequences were compared with sequences in the NCBI database using the BLAST search program (specifically, TBLASTX program). Furthermore, the deduced amino acid sequences were aligned using CLUSTAL W (25), and a phylogenetic tree was produced by TreeView X (26).

Carbon source utilization experiments All test strains were examined for their ability to utilize six carbon sources (acetate, propionate, lactate, butyrate, glucose, and glycerol) that were used for the PHA production experiments. Colonies of the isolates were seeded onto agar plates (1.7% (w/v) agar) of basal salt medium (BSM: NH_4Cl 181 mg/L, KH_2PO_4 169 mg/L, $MgSO_4 \cdot 7H_2O$ 68.4 mg/L, KCl 26.8 mg/L, trace element solution (27) 1.5 ml/L, pH 7.2) containing one of the aforementioned carbon sources at a final concentration of 324.5 mg-C/L. Carbon source utilization was determined visually by growth after incubation at 28°C for around 7 d.

PHA production experiments Batch PHA production experiments were conducted under aerobic and nitrogen-limiting conditions. All experiments were conducted in duplicate. Test strains were precultivated in R2A broth "DAIGO" (Nihon Pharmaceutical, Tokyo, Japan), after which cells of precultivated strains were harvested by centrifugation (8500 ×*g*, 4°C, 5 min), washed twice with sterilized 0.85% (w/v) NaCl, and inoculated in 50-ml glass vials containing 20 ml of BSM that was free from nitrogen (NH_4Cl) and supplemented with one of the six carbon sources at a final concentration of 973.5 mg-C/L to obtain an optical density of 0.5 at 600 nm. The cultures were then incubated at 28 ± 2°C with rotary shaking at 150 rpm. Aliquots (1 ml) of the cultures were sampled at 0, 24 and 48 h and stored at −20°C until analysis.

PHA analysis A Nile blue A staining and fluorescence measurement method developed by Oshiki et al. (28) was applied to evaluate PHA production by test strains in batch PHA production experiments. The fluorescence intensities obtained using this method have been shown to be correlated with PHA concentrations determined by gas chromatography for activated sludge samples (28). A stock solution of Nile blue A (0.02%w/v) was prepared by dissolving Nile blue A powder (Sigma–Aldrich, St. Louis, MO, USA) into dimethyl sulfoxide (Kanto Chemical, Tokyo, Japan). Next, the culture sample was sonicated using a handy sonicator UR-20P (Tomy Seiko, Tokyo, Japan) with the maximal power for 1.5 min, and an aliquot (100 µl) of the culture sample was mixed with 100 µl of Nile blue A stock solution and incubated at room temperature for 3 min, after which the fluorescence intensity was determined using a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Vantaa, Finland) at excitation and emission wavelengths of 490 and 590 nm, respectively. The ability of test strains to produce PHA was determined by comparing the fluorescence intensity at 24 and 48 h with that at 0 h.

Nucleotide sequence accession numbers The nucleotide sequence data determined in this study were deposited in GenBank/EMBL/DDBJ databases under accession numbers LC018217 to LC018330 for the partial 16S rRNA gene sequences and LC017719 to LC017735 for the partial *phaC* gene sequences.

RESULTS

Phylogenetic characteristics of isolated heterotrophic bacteria A total of 114 colonies (13–51 morphologically distinct colonies per sample) were isolated from four activated sludge samples (Table 2) and used to evaluate the PHA production potential. Phylogenetic analysis based on partial 16S rRNA gene sequences revealed that test strains isolated from four activated sludge samples consisted of 0%–12% α-*Proteobacteria*, 6%–37% β-*Proteobacteria*, 6%–39% γ-*Proteobacteria*, 0%–4% ε-*Proteobacteria*, 0%–15% *Firmicutes*, 0%–27% *Actinobacteria*, 7%–48% *Cytophaga-Flexibacter-Bacteroidetes* (CFB group), and 4%–27% unclassified bacteria (Supplementary Table S1 and Fig. S1).

***PhaC* gene possession** The products of PCR conducted using primers targeting *phaC* genes that encode the class I and II PHA

TABLE 1. Activated sludge samples used in this study.

Sample	Reactor/WWTP	Biological treatment process	MLSS (mg/L)	Heterotrophic bacteria (CFU/g-MLSS)	Sampling date
LB	Laboratory scale reactor	SBR	13090	1.2×10^9	June 1, 2012
OC	Municipal WWTP O	CAS	985	1.0×10^{10}	July 10, 2013
KC	Municipal WWTP K	CAS	1510	8.2×10^9	August 2, 2013
KA		A2O	1213	3.9×10^9	August 2, 2013

A2O, anaerobic-anoxic-oxic process; CAS, conventional activated sludge process; MLSS, mixed liquor suspended solids; SBR, sequencing batch reactor; WWTP, wastewater treatment plant.