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Development and validation of an HPLC-based screening method to acquire polyhydroxyalkanoate synthase mutants with altered substrate specificity

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A rapid and convenient method for the compositional analysis of polyhydroxyalkanoate (PHA) was developed using high-performance liquid chromatography (HPLC) and alkaline sample pretreatment in a 96-well plate format. The reliability of this system was confirmed by the fact that a mutant with a D171G mutation of *Aeromonas caviae* PHA synthase ($PhaC_{Ac}$), which gained higher reactivity toward 3-hydroxyhexanoate (3HHx), was selected from the D171X mutant library. Together with D171G mutant, several single mutants showing high reactivity toward 3HHx were isolated by the HPLC assay. These new mutants and double mutants combined with an N149S mutation were used to synthesize P(3-hydroxybutyrate-*co*-3HHx) in *Ralstonia eutropha* $PHB^{-}4$ from soybean oil as carbon source, achieving higher levels of 3HHx fraction than the wild-type enzyme. Based on these results, the high-throughput screening system will serve as a powerful tool for exploring new and beneficial mutations responsible for regulating copolymer composition of PHA.

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[**Key words:** Polyhydroxyalkanoates; High-throughput screening; High-performance liquid chromatography (HPLC); Alkaline pretreatment; PHA synthase; Substrate specificity]

Polyhydroxyalkanoates (PHAs) are biological polyesters produced by a wide variety of microorganisms as an intracellular storage material for carbon and energy. PHAs have attracted industrial attention for use as biodegradable and biocompatible thermoplastics (1,2). PHAs are synthesized by PHA synthases (PhaCs) which catalyze the polymerization reaction of 3-hydroxyalkanoates (3HAs) as monomer substrates. Therefore, the substrate specificity of PhaC significantly influences the monomer composition of synthesized PHA.

Poly(3-hydroxybutyrate) [P(3HB)] is the most common PHA synthesized by bacteria in nature. P(3HB) has high rigidity but is brittle with low elasticity. Therefore, flexible 3HB-based copolymers such as P(3HB-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)], P(3HB-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], P(3HB-*co*-3-hydroxy-4-methylvalerate) [P(3HB-*co*-3H4MV)], and P(3HB-*co*-medium-chain-length-3-hydroxyalkanoate) [P(3HB-*co*-mcl-3HA)] are recognized as more suitable polymers for practical use (3–5). These monomer structures are shown in Fig. 1.

Aeromonas caviae is capable of synthesizing P(3HB-*co*-3HHx) random copolymer from vegetable oils as the carbon source (6), because this bacterium possesses the PHA synthase ($PhaC_{Ac}$) that has the unique ability to polymerize 3HB and 3HHx units. P(3HB-*co*-3HHx) is highly desired by industry as a bio-based plastic, but this bacterium has poor ability to produce and accumulate it (less than about 30 wt.% of the cells). On the other hand, *Ralstonia eutropha* is a PHA over-producer (greater than 80 wt.% of the cells), while the type of PHA synthesized by this strain is limited to P(3HB) with vegetable oils as the carbon source. Thus, the higher production of P(3HB-*co*-3HHx) was achieved from vegetable oils in recombinant *R. eutropha* $PHB^{-}4$ transformed with a vector-borne $PhaC_{Ac}$ gene (7,8). The resultant host-vector system, however, suffered from weak incorporation of the 3HHx unit into P(3HB-*co*-3HHx), limiting the 3HHx fraction to 3–4 mol% in cultivation on soybean oil. This phenomenon was mainly ascribed to the substrate specificity of $PhaC_{Ac}$ (7). We applied directed evolution to create mutant enzymes that gain higher reactivity toward 3HHx. In the initial stage, two beneficial single mutants, N149S (asparagine 149 → serine) and D171G (aspartic acid 171 → glycine), were obtained (9). Subsequent mutation studies with $PhaC_{Ac}$ allowed us to regulate the 3HHx fraction from 0 to 5.2 mol% (10,11). The highest 3HHx fraction (5.2 mol%) was obtained using the $PhaC_{Ac}$ double mutant with N149S and D171G mutations (NSDG mutant) (10).

To extend the regulation range of the 3HHx fraction, $PhaC_{Ac}$ mutants with further increased reactivity toward 3HHx were required. For this, a much more efficient high-throughput screening system was necessary. Kichise et al. developed an initial assay

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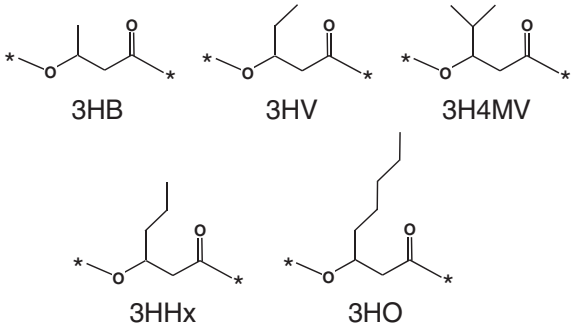

FIG. 1. Structure of monomer units in PHA synthesized in this study. 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3H4MV, 3-hydroxy-4-metylvalerate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate.

method to measure cellular P(3HB) content using high-performance liquid chromatography (HPLC) (12). To prepare samples for this method, P(3HB)-accumulating cells are treated with sulfuric acid at 100°C to convert P(3HB) to crotonic acid (*trans*-2-butenoic acid). Subsequently, the treated samples are subjected to HPLC with an ultraviolet (UV) detector to measure absorption at 210 nm due to unsaturated crotonic acid bonds (13). This method is very convenient to measure P(3HB) concentration; however, it is not applicable to other PHAs because PHA monomers longer than 3HB cannot be converted to the corresponding unsaturated fatty acids. In a previous study (12), $PhaC_{Ac}$ mutants showing higher reactivity toward 3HHx were obtained as a result of screening for high-polymerization activity mutants based on P(3HB) accumulation levels in host *Escherichia coli*. Thus, establishing a sample preparation method applicable to longer PHA monomers including 3HHx would allow direct screening for substrate-specificity-altered synthases based on PHA copolymer composition using HPLC, improving screening efficiency. On the other hand, gas chromatography (GC) analysis is widely used to determine PHA composition regardless of 3HA carbon chain length. It requires derivatization process of polymer samples, which is difficult to simplify for a high-throughput assay, prior to GC analysis.

In this study, a new HPLC-based screening method capable of analyzing PHA copolymer composition was developed by applying alkaline (sodium hydroxide) pretreatment instead of acid pretreatment to sample preparation for HPLC analysis. In addition, a high-throughput protocol was established by introducing a 96-well plate format for cultivation of the PHA producing host and for sample preparation. The new method was used to isolate $PhaC_{Ac}$ mutants with high reactivity toward 3HHx from a D171 random point mutation (D171X) library to determine whether the D171G mutation is the most effective for increasing $PhaC_{Ac}$ reactivity toward 3HHx.

MATERIALS AND METHODS

Bacterial strains and plasmids *E. coli* JM109 was used as the host strain for screening $PhaC_{Ac}$ mutants and for P(3HB-*co*-3HHx) accumulation from dodecanoate, while *R. eutropha* PHB^{-}4 (PHA-negative mutant, DSM541) was used for PHA copolymer production from soybean oil, octanoate, or 4-methylvalerate. Plasmid pBBR1*pha*$PCJ_{Ac}AB_{Re}$ was constructed by introducing a 6.4-kb *Xba*I–*Hin*dIII fragment of pBSEE32phbAB (12) into the same sites of a broad-host-range vector pBBR1MCS-2 (14). The resultant plasmid carries PHA polycistronic genes (accession no. D88825) for $PhaP_{Ac}$ (granule-associated protein), $PhaC_{Ac}$, and $PhaJ_{Ac}$ (*R*-specific enoyl-CoA hydratase) with a promoter derived from *A. caviae* FA440 and the genes for the (*R*)-3HB-CoA monomer supplying enzymes $PhaA_{Re}$ (3-ketothiolase) and $PhaB_{Re}$ (NADPH-dependent acetoacetyl-CoA reductase) from *R. eutropha* H16 (accession no. J04987).

Random point mutagenesis at position 171 of $PhaC_{Ac}$ Random point mutagenesis at position 171 of $PhaC_{Ac}$ (Fig. 2A) was performed using an inverse polymerase chain reaction (PCR) method described by Imai et al. (15). The PCR primers used in this study were designed in inverted tail-to-tail directions to amplify pBBR1*pha*$PCJ_{Ac}AB_{Re}$ with the target sequence for amino acid substitution as follows: for D171X, 5′-CCT GGA GTC CNN NGG CCA GAA CCT G-3′ (the underlined sequence indicates a mutation site, and N represents a random nucleotide) as the sense primer and 5′-GTC AGC TTG AGC AGC TCG GGG TTG G-3′ as the antisense primer. After PCR amplification with the primer set, the amplified linear DNA was phosphorylated and self-ligated using a BKL kit (Takara Bio Inc., Otsu). Subsequently, the self-ligated PCR products were transformed into *E. coli* JM109 to prepare a $PhaC_{Ac}$ mutant library.

HPLC-based screening of $PhaC_{Ac}$ mutants Fig. 2B shows a schematic diagram of the HPLC-based screening system developed in this study. Briefly, it is consisted of site-specific random mutagenesis, preparation of a mutant library, primary assay of P(3HB) accumulation in *E. coli* JM109 using Nile red (9-(diethylamino)-5H-benzo[α]phenoxazin-5-one) dye on an agar plate, liquid cultivation in M9 medium plus dodecanoate (C12) using a 96-well plate for P(3HB-*co*-3HHx) accumulation, alkaline sample pretreatment, HPLC assay, and nucleotide sequence determination. A $PhaC_{Ac}$ D171X mutant library constructed with *E. coli* JM109 was spread on Luria–Bertani (LB) agar plates (Bacto-Tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, and agar 15 g per liter of distilled water) supplemented with 20 gL^{-1} glucose, 0.5 mg/L^{-1} Nile red, and 50 mgL^{-1} kanamycin and cultured at 37°C overnight. The polymerization ability of $PhaC_{Ac}$ mutants was judged based on the intensity of the pinkish pigmentation of the cells caused by Nile red staining (16). Next, single pinkish pigmented colonies were inoculated in M9 medium (0.6 mL) containing 1.0 gL^{-1} sodium dodecanoate, 1.0 gL^{-1} Bacto-yeast extract, 0.4 vol% Brij35, and 50 mgL^{-1} kanamycin in each 1.2-mL well of a 96-deep well culture plate (BM Equipment Co., Ltd., Tokyo). After sealing the plate with an air-permeable film (4titude, Ltd., Surrey, UK), the cells were cultured at 37°C for 72 h in a reciprocal shaker (1035 rpm, Bio Shaker, Taitec Co., Ltd., Saitama). After the grown cells were replicated on LB agar plates, the cultured 96-well plates were centrifuged using a Hitachi R6S swing rotor at 3000 rpm (1500 × *g*) for 10 min, and then the culture supernatants were discarded. The 96-well plates with cell pellets remaining at the bottom of each well were dried at 55°C for 3 days.

For alkaline hydrolysis of the dried cells, 200 μL of 1 N NaOH was added to each well of the 96-well plates using a handheld multichannel pipettor. After heat-sealing with a polypropylene/aluminum film (4titude) using a microplate heat sealer (ABgene Ltd., Surrey, UK), the 96-well plates were heated at 100°C for 3 h on a hot plate. The cell hydrolysates were neutralized with 200 μL of 1 N HCl and then filtrated with 96-well filter plates (0.45 μm pore size polytetrafluoroethylene (PTFE) membrane, Pall Co., NY, USA) by centrifuging at 3000 rpm (1500 × *g*) for 30 min. The filtrates were collected with a new 96-well assay plate, sealed with a cover film to prevent evaporation, and subjected to HPLC analysis.

HPLC analysis was performed using a Shimadzu LC-10Avp system with an auto-sample injector applicable to 96-well plates. The samples were separated on two types of ion-exclusion columns, Aminex HPX-87H (300 mm × 7.8 mm I.D., Bio-Rad, CA, USA) and Fast Acid Analysis (100 mm × 7.8 mm I.D., Bio-Rad), at 60°C using 0.014 N H_2SO_4 with or without 20% CH_3CN as a mobile phase at a flow rate of 0.7 mL/min. The chromatograms were recorded at 210 nm using a UV detector.

Site-specific mutation of $PhaC_{Ac}$ Aspartic acid (D) at position 171 of the $PhaC_{Ac}$ N149S mutant was replaced by alanine (A), leucine (L), or histidine (H) as a second mutation to yield a doubly mutated gene (*pha*C_{Ac} NSDA, NSDL, or NSDH) using a QuickChange Multi Site-directed Mutagenesis Kit (Stratagene Co., CA, USA) or a similar method. The primer was designed as 5′-CTG GAG TCC NNN GGC CAG AAC CTG G-3′. The underlined NNN sequences in the primer were GCC, CTG, and CAC for alanine (A), leucine (L), and histidine (H) replacement, respectively, and were designed based on the codon usage of *R. eutropha*.

Recombination of *R. eutropha* and PHA analysis To express the *pha*C_{Ac} gene in *R. eutropha* PHB^{-}4 under our basal conditions, a 0.6-kb *Pst*I–*Sca*I fragment of pBBR1*pha*$PCJ_{Ac}AB_{Re}$ was introduced into the same pBBREE32d13dPB sites (10,11) to yield pBBREE32d13dPB D171X carrying only the mutated *pha*C_{Ac} gene downstream of the *pha* promoter from *A. caviae*. These plasmids were introduced by transconjugation from *E. coli* S17-1 into *R. eutropha* PHB^{-}4 (17).

The recombinant *R. eutropha* strain PHB^{-}4 was cultivated at 30°C for 72 h on a reciprocal shaker (130 strokes/min) in 500-mL flasks containing 100 mL of nitrogen-limited mineral salt (MS) medium supplemented with a carbon source (20 gL^{-1} soybean oil, 5 gL^{-1} sodium octanoate, or 2.5 gL^{-1} 4-methylvalerate). The composition of the MS medium was as follows (per liter of distilled water): 9 g of $Na_2HPO_4 \cdot 12H_2O$, 1.5 g of KH_2PO_4, 0.5 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution (18). The pH of the medium was adjusted to 7.0. Kanamycin (50 mgL^{-1}) was added to the medium to maintain the expression plasmid. The PHA content in dry cells was determined by GC after methanolysis of the lyophilized cells in the presence of 15% sulfuric acid (18). GC analysis was carried out by using Shimadzu GC-14B system with a non-polar capillary column (InertCap 1, 30 m × 0.25 mm, GL Sciences Inc., Tokyo) and a flame ionization detector.

The polymers accumulated in the cells were extracted with chloroform for 72 h at room temperature and purified via precipitation with methanol. Molecular weight data were obtained by gel permeation chromatography (GPC) at 40°C using a Shimadzu 10A GPC system and a 10 A refractive index detector with Shodex K806M and K802 columns. Chloroform was used as the eluent at a flow rate of 0.8 mL/min, and sample concentrations of 1.0 mg/mL were applied. Polystyrene standards with a low polydispersity were used to make a calibration curve.

RESULTS

Pretreatment by acid or alkaline for HPLC analysis Karr et al. established an HPLC technique for rapid analysis of P(3HB) with

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