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Biosynthesis of polyhydroxyalkanoates containing 2-hydroxy-4-methylvalerate and 2-hydroxy-3-phenylpropionate units from a related or unrelated carbon source

Shoji Mizuno,[1] Yukio Enda,[2] Azusa Saika,[3] Ayaka Hiroe,[4] and Takeharu Tsuge[1,*]

Department of Materials Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan,[1] Akita Industrial Technology Center, 4-11 Sanuki, Araya-machi, Akita 010-1623, Japan,[2] Research Institute for Innovation in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan,[3] and Department of Chemistry for Life Sciences and Agriculture, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan[4]

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The discovery of the lactate-polymerizing enzyme (LPE) enabled the biosynthesis of a polyhydroxyalkanoate (PHA) containing 2-hydroxyalkanoate (2HA). Amino acids are potential precursors of 2HA with various side chain structures if appropriate enzymes are used to convert amino acids to 2HA-coenzyme A (CoA) as the substrate for LPE. In this study, the suitability and utility of (*R*)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase (LdhA) and 2H4MV-CoA transferase (HadA) from *Clostridium difficile* as 2HA-CoA–supplying enzymes were investigated. By expressing LPE, LdhA, and HadA in *Escherichia coli* DH5α, we successfully produced poly(3-hydroxybutyrate-*co*-2HA) [P(3HB-*co*-2HA)] from a related or unrelated carbon source. The 2HA units incorporated into PHA from unrelated carbon sources were primarily 2H4MV and 2-hydroxy-3-phenylpropionate (2H3PhP), which were assumed to be derived from endogenous leucine and phenylalanine, respectively. Furthermore, P(3HB-*co*-22 mol% 2HA) synthesis was demonstrated by means of saccharified sugars, which are an abundant and renewable feedstock for polymer production from hemicellulosic biomass (Japanese cedar) as the carbon source. Our study shows that several types of 2HA units such as 2H4MV and 2H3PhP are endogenous monomers for PHA biosynthesis in *E. coli* expressing LdhA and HadA.

[**Key words:** Polyhydroxyalkanoate; Lactate-polymerizing enzyme; Leucine; Phenylalanine; 2-Hydroxyalkanoate; *Clostridium difficile*]

A polyhydroxyalkanoate (PHA), an aliphatic polyester, is synthesized as a carbon and energy storage source in bacterial cells. PHAs can be produced by bacterial fermentation from renewable biomass such as sugars and plant oils (1,2). Thus, much attention has been directed to the use of PHAs as renewable, biodegradable, and biocompatible thermoplastics. The most general type of PHA is poly[(*R*)-3-hydroxybutyrate] [P(3HB)]; however, P(3HB) is stiff and brittle owing to its high crystallinity. Therefore, via copolymerization of 3HB with other units, such materials can be given more preferable properties of flexibility and ductility through a reduction in the crystallinity (3).

A variety of monomer units for the synthesis of 3HB-based copolymers have been reported, e.g., 3-hydroxyvalerate (3HV) with an ethyl group side chain (4), 3-hydroxyhexanoate (3HHx) with a propyl group (5), 3-hydroxy-4-methylvalerate (3H4MV) with an isopropyl group (6,7), and 3-hydroxy-3-phenylpropionate (3H3PhP) with a phenyl group (8,9). These comonomers, except for 3HV, are known to be excluded from the P(3HB) crystalline phase (7), resulting in an effective decrease in the crystallinity of the copolymers.

Recently, copolymerization of 2-hydroxyalkanoates (2HAs) such as lactate (2-hydroxypropionate, 2HP), glycolate (2-hydroxyacetate), and 2-hydroxybutyrate by PHA synthase (PhaC) has been demonstrated after the discovery of a lactate-polymerizing enzyme (LPE) by Taguchi's group (10–13). LPE is an engineered PhaC that is derived from *Pseudomonas* sp. 61-3 by introduction of several amino acid mutations (e.g., PhaC1$_{Ps}$ STQK). It has been demonstrated that introduction of 2HA units into the polymer chain effectively and stably inhibits crystallization of 3HB units (14). To develop metabolic pathways to supply these 2HA units from sugars, many challenges are being addressed (10–15).

The obligate anaerobe *Clostridium difficile* (currently designated as *Peptoclostridium difficile*) is known to have a unique metabolic pathway for conversion of leucine to 4-methylvalerate (Fig. 1A), where 2-hydroxy-4-methylvaleryl-coenzyme A (2H4MV-CoA) is an intermediate (16,17). 2H4MV-CoA is produced from deaminated leucine under the action of two enzymes: (*R*)-2H4MV dehydrogenase (LdhA) and 2H4MV-CoA transferase (HadA). Because 2H4MV-CoA is expected to be the substrate for LPE, a novel PHA copolymer containing 2H4MV units can be synthesized by employing LPE, LdhA, and HadA.

In this study, by means of LPE and codon-optimized *C. difficile* LdhA and HadA, we attempted to synthesize P(3HB-*co*-2H4MV) in *Escherichia coli* with supplementation with leucine as a 2H4MV precursor or without supplementation with amino acids. It was revealed that 2H4MV-containing PHA was produced by using leucine as a 2H4MV precursor. Additionally, the PHA biosynthesized from sugars as a sole carbon source is composed of not

* Corresponding author. Tel.: +81 45 924 5420; fax: +81 45 924 5426.
E-mail address: tsuge.t.aa@m.titech.ac.jp (T. Tsuge).

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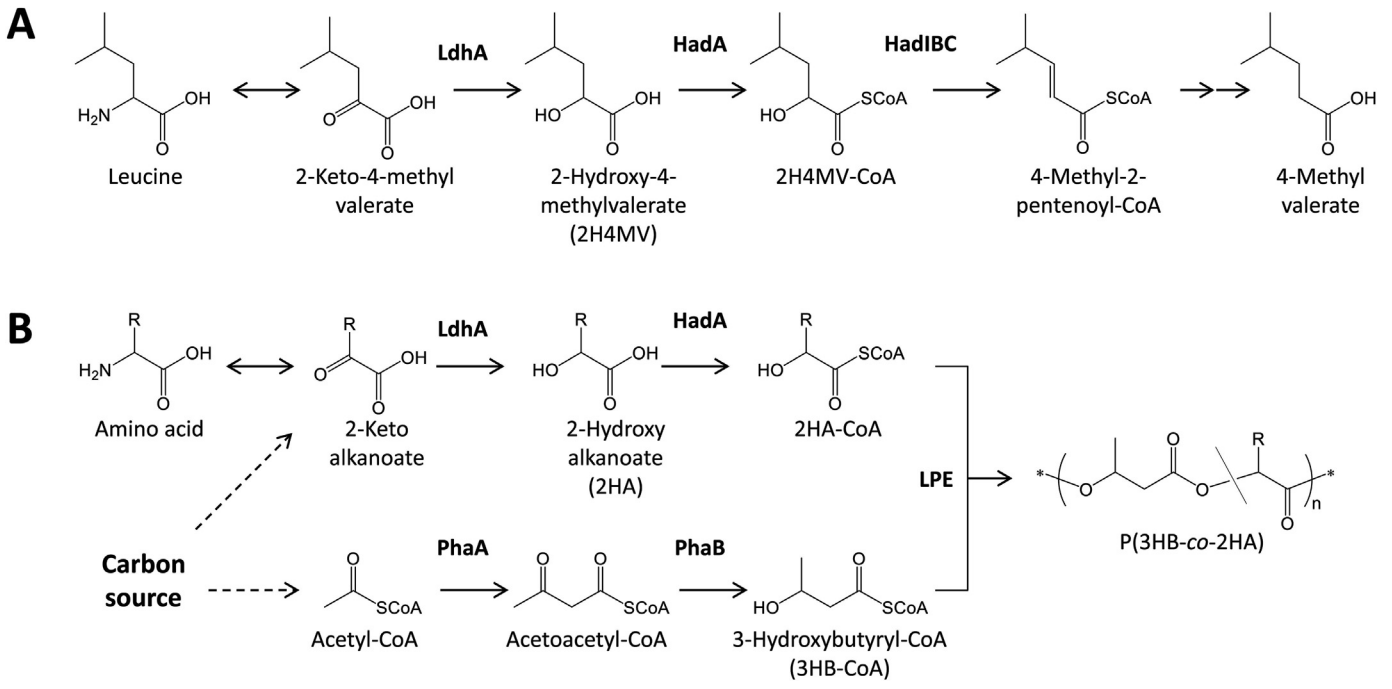

FIG. 1. (A) The leucine degradation pathway in *C. difficile* and (B) the P(3HB-*co*-2HA) biosynthesis pathway constructed in engineered *E. coli*. LdhA, (*R*)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase; HadA, 2H4MV CoA transferase; HadBC, 2H4MV-CoA dehydratase; HadI, activator of HadBC; PhaA, 3-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; LPE, lactate-polymerizing enzyme (PHA synthase, $PhaC1_{Ps}$ STQK).

only 3HB and 2H4MV units but also 2-hydroxy-3-phenylpropionate (2H3PhP) and other minor 2HA units. Furthermore, P(3HB-*co*-2HA) synthesis was performed using saccharified sugars derived from Japanese cedar. This study shows suitability of 2H4MV and 2H3PhP as endogenous monomers for PHA biosynthesis in LdhA- and HadA-expressing *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids *E. coli* strain DH5α (Takara Bio, Ohtsu, Japan) served as the host for PHA production. Plasmid $pTTQldhAhadA_{Cd\_opt}$ was constructed by inserting the *ldhA-hadA*-containing DNA fragment derived from *C. difficile* 630 (16) at the *Pst*I and *Bam*HI sites of the pTTQ19 vector (18). The *ldhA-hadA*-containing DNA fragment was chemically synthesized with optimized codons for expression in *E. coli*. Plasmid $pBBR1''C1_{Ps}(STQK)AB_{Re}$ (9) harbors the following genes: a PHA synthase gene ($phaC1_{Ps}$) with the STQK mutation (S325T and Q481K) (10), a 3-ketothiolase gene ($phaA_{Re}$), and a NADPH-dependent acetoacetyl-CoA reductase gene ($phaB_{Re}$). Genes $phaC1_{Ps}$ and $phaAB_{Re}$ were derived from *Pseudomonas* sp. 61-3 and *Ralstonia eutropha* H16, respectively.

PHA synthesis in *E. coli* Engineered *E. coli* DH5α was cultured in the Luria–Bertani (LB) medium (10 g NaCl, 10 g tryptone, and 5 g yeast extract per liter of distilled water) at 30°C as the seed culture. This culture was inoculated into 100 mL of the M9 medium (17.1 g $Na_2HPO_4 \cdot 12H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mL of 1 M $MgSO_4$, and 0.1 mL of 1 M $CaCl_2$ per liter of distilled water) or LB medium supplemented with a carbon source (glucose, xylose, glycerol, or saccharified sugars), and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in a 500-mL shake flask. In all cases, 50 mg/L carbenicillin and 50 mg/L kanamycin were added to the medium to maintain the plasmid in the cell. The cells were cultivated at 30°C for 72 h and washed with distilled water to remove medium components before lyophilization.

[1-^{13}C] labeled L-leucine (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was added into the M9 medium as an isotope tracer.

Saccharification of Japanese cedar Pulverization of Japanese cedar was performed in a tandem-ring mill to facilitate enzymatic saccharification of the cellulosic feedstock by reducing the size of the particles (to 40 μm) and crystallinity of cellulose (19). Subsequently, the 30 wt% Japanese cedar powder in 0.1 M acetate buffer (pH 5.5) was saccharified by means of a mixture of cellulases (1 wt% Cellic CTec2 and 0.1 wt% HTec2, Novozymes, Denmark) at 50°C for 48 h with stirring (300 rpm). The obtained saccharified suspension was centrifuged for solid–liquid separation, prior to use. The component sugars of the saccharified solution were 114 g/L glucose, 10.8 g/L xylose, 8.4 g/L mannose, 1.2 g/L arabinose, and 0.5 g/L galactose, as determined by high-performance liquid chromatography. The saccharified solution was freeze-dried and then subjected to elemental analysis on a CHNS analyzer (Vario EL Cube, Elementar, Germany).

PHA analysis PHA content and composition were determined by gas chromatography (GC14B, Shimadzu, Kyoto, Japan) with a flame ionization detector and by gas chromatography with mass spectrometry (GC–MS, QC2010, Shimadzu). Approximately 30 mg of the lyophilized cells was methanolyzed in the presence of 15% of sulfuric acid before analysis (20).

PHA was extracted from lyophilized cells with chloroform at room temperature and purified by reprecipitation in methanol. Molecular weight was determined by gel permeation chromatography (10A GPC system, Shimadzu). Approximately 2 mg of the extracted polymer was dissolved in 1 mL of chloroform and analyzed at a column temperature of 40°C.

The structure of PHA was determined by NMR spectroscopy. Purified polymer (50 mg) was dissolved in 1 mL of $CDCl_3$ and subjected to both 400 MHz ^{1}H and 100 MHz ^{13}C NMR analyses. NMR spectra were recorded using a Bruker Biospin AVANCE III spectrometer.

For thermal analysis, PHA films were prepared by solvent casting in Petri dishes. For this purpose, the purified PHA polymer was dissolved in chloroform, and the polymer solution was poured into Petri dishes. The solvent was evaporated in a draft chamber for several days, followed by aging for 4 weeks at room temperature (approximately 25°C) to attain equilibrium crystallinity prior to analysis. To analyze the PHA copolymers by differential scanning calorimetry (DSC), 3 mg of the PHA film was weighed, encapsulated in aluminum pans, and heated under nitrogen by means of a Perkin–Elmer Pyris 1 DSC instrument from −50°C to 200°C at a heating rate of 20°C/min (first heating scan). The melted samples were then maintained at 200°C for 1 min, followed by rapid quenching at −50°C. After that, the samples were heated from −50°C to 200°C at a heating rate of 20°C/min in a nitrogen atmosphere (second heating scan) (9).

RESULTS AND DISCUSSION

Pathway design for 2HA supply Fig. 1B shows the newly designed pathway for P(3HB-*co*-2HA) biosynthesis in engineered *E. coli*. In this pathway, LdhA reduces 2-ketoalkanoate to 2HA. Next, HadA adds CoA to 2HA, yielding 2HA-CoA. Finally, LPE, namely $PhaC1_{Ps}$ STQK, polymerizes the acyl moiety of 2HA-CoA with 3HB-CoA, which was supplied by PhaAB from *R. eutropha* H16, yielding the P(3HB-*co*-2HA) copolymer.

To construct this pathway, the genes encoding LdhA and HadA from *C. difficile* were synthesized with codon optimization and

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