Biosynthesis of Poly(3-Hydroxybutyrate-co-3-Hydroxy-4-Methylvalerate) by Recombinant
Escherichia coli Expressing Leucine Metabolism-Related Enzymes Derived from
Clostridium difficile

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Keywords: polyhydroxyalkanoate, *Clostridium difficile*, leucine metabolism, 3H4MV,
Aeromonas caviae, *Ralstonia eutropha*

Running title: P(3HB-co-3H4MV) synthesis by *E. coli*

26

27 **Abstract**

28 An obligate anaerobic bacterium *Clostridium difficile* has a unique metabolic pathway to
29 convert leucine to 4-methylvalerate, in which 4-methyl-2-pentenoyl-CoA (4M2PE-CoA) is an
30 intermediate of this pathway. 4M2PE-CoA is also able to be converted to
31 3-hydroxy-4-methylvalerate (3H4MV), a branched side-chain monomer unit, for synthesis of
32 polyhydroxyalkanoate (PHA) copolymer. In this study, to synthesize 3H4MV-containing
33 PHA copolymer from leucine, the leucine metabolism-related enzymes (LdhA and HadAIBC)
34 derived from *C. difficile* and PHA biosynthesis enzymes (PhaPCJ_{Ac} and PhaAB_{Re}) derived
35 from *Aeromonas caviae* and *Ralstonia eutropha* were co-expressed in the codon
36 usage-improved *Escherichia coli*. Under microaerobic culture conditions, this *E. coli* was
37 able to synthesize P(3HB-*co*-12.2 mol% 3H4MV) from glucose with the supplementation of
38 1 g/L leucine. This strain also produced P(3HB-*co*-12.6 mol% 3H4MV) using the culture
39 supernatant of leucine overproducer *E. coli* strain NS1391 as the medium for PHA production,
40 achieving 3H4MV copolymer synthesis only from glucose. Furthermore, we tested the
41 feasibility of the 3H4MV copolymer synthesis in *E. coli* strain NS1391 from glucose. The
42 recombinant *E. coli* NS1391 was able to synthesize P(3HB-*co*-3.0 mol% 3H4MV) from
43 glucose without any leucine supplementation. This study demonstrates the potential of the
44 new metabolic pathway for 3H4MV synthesis using leucine metabolism-related enzymes
45 from *C. difficile*.

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Introduction

Polyhydroxyalkanoates (PHAs) are aliphatic polyester that can be synthesized by a wide variety of microorganisms as an intracellular storage material (1). PHAs have attracted industrial attention for use as biodegradable and biocompatible thermoplastics, which can be produced by bacterial fermentation from renewable biomass such as sugars and plant oils. Therefore, PHAs are expected to solve some environmental problems associated with the use of petroleum.

The most commonly found PHA is poly[(*R*)-3-hydroxybutyrate], P(3HB); however, P(3HB) is a brittle and rigid material with low flexibility due to its high crystallinity (2). Therefore, flexible 3HB-based copolymers such as P(3HB-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and P(3HB-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] are recognized as more suitable polymers for practical use. In previous studies, we reported that *Ralstonia eutropha* PHB⁻4 (currently designated *Cupriavidus necator*) harboring PHA synthase 1 gene from *Pseudomonas* sp. 61-3 (*phaCI*_{Ps}) synthesized PHA copolymer containing 0.5 mol% 3-hydroxy-4-methylvalerate (3H4MV), a novel branched monomer, from fructose as a sole carbon source (3, 4). The 3H4MV fraction of P(3HB-*co*-3H4MV) could be increased up to 46 mol% by feeding a structurally related precursor 4-methylvalerate (4MV) (4, 5). Because 4MV is a costly and toxic substrate for cell growth, an alternative precursor is desirable. Our previous study demonstrated that the amino acid leucine, which has the same carbon backbone as 3H4MV, works as a natural precursor for 3H4MV unit (6). Furthermore, the leucine analog resistant mutant of *R. eutropha* strain 1F2 showed increased 3H4MV fraction of 3.1 mol% when 10 g/L leucine was fed. This is the highest value of 3H4MV fraction achieved with leucine as a precursor thus far, but still low to confer elastic property in PHA copolymer. Because the yield of 3H4MV from leucine was very low (7.2 mg/g-leucine), it needs to be further improved.

An obligate anaerobe *Clostridium difficile* has unique metabolic pathway from leucine to 4MV, as shown in Fig. 1 (7-9). In this pathway, leucine is first deaminated to 2-keto-4-methylvalerate by branched-chain amino acid aminotransferase (IlvE) and aromatic amino acid aminotransferase (TyrB), followed by reduction to (R)-2-hydroxy-4-methylvalerate (2H4MV) by 2H4MV dehydrogenase (LdhA). Subsequently, 2H4MV is converted to 2H4MV-CoA by 2H4MV-CoA transferase (HadA), and then dehydrated to 4-methyl-2-pentenoyl-CoA (4M2PE-CoA) by 2H4MV-CoA dehydratase (HadBC) with the help of its activator (HadI). Finally, 4M2PE-CoA is converted to 4MV by two additional enzymatic reactions. Because 4M2PE-CoA is predicted to be an intermediate of 3H4MV biosynthesis pathway from 4MV precursor (4, 5), the enzymes related to 4M2PE-CoA supply were thought to be useful for the enhancement of 3H4MV provision from leucine.

In this study, we attempted to supply 3H4MV unit from leucine in *E. coli* more efficiently than did *R. eutropha*. To this end, *E. coli* recombinants harboring PHA synthesis-related genes from *Aeromonas caviae* and *R. eutropha*, and leucine metabolism-related genes from *C. difficile* were generated and subjected to P(3HB-co-3H4MV) synthesis with the supplementation of leucine as 3H4MV precursor. Furthermore, this pathway was also constructed in leucine-overproducer *E. coli* NS1391 to synthesize P(3HB-co-3H4MV) only from glucose. The present study demonstrates the metabolic engineering for 3H4MV synthesis by employing leucine metabolism-related enzymes from *C. difficile*.

Material and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table S1. *E. coli* strains BL21 codonplus (DE3)-RIL and BL21 (DE3) were used as the host for protein expression. *E. coli* strains JM109 and TOP10 were used as the cloning host. Leucine overproducer *E. coli* NS1391 was kindly donated by Ajinomoto Co., Inc and used as a host strain of leucine and PHA production. *C. difficile* 630 genomic DNA (gDNA) was obtained from American Type Culture Collection (ATCC) and used as a template for PCR amplification.

Plasmid construction

Plasmid pTTQldhAhadAIBC_{Cd} (Fig. S1) was constructed using GENEART Seamless Cloning and Assembly Kit purchased from Invitrogen (Carlsbad, CA, USA). Cloning and gene assembly techniques were performed according to standard procedures provided by the manufacturer. Briefly, the *ldhA*, *hadA-hadI*, *hadB-hadC* fragments with their native ribosomal binding sites were amplified from *C. difficile* 630 gDNA using primer sets listed in Table S1. The three PCR amplified fragments sharing 15 base-pair end-terminal homology were purified by Gel/PCR Extraction Kit (Nippon Genetics Co, Ltd., Tokyo) and cloned into *Sma*I-digested linear plasmid pUC19 to yield pUCldhAhadAIBC_{Cd}. A 5.4-kb *Sal*I-*Sac*I fragment of pUCldhAhadAIBC_{Cd} was introduced into the same sites of pTTQ19, to yield pTTQldhAhadAIBC_{Cd}.

Plasmid pBBR1phaP(D4N)CJ_{Ac}AB_{Re} NSDG (Fig. S1) carries the following genes: PHA granules-associated protein gene (*phaP*_{Ac}) with D4N mutation [Asp⁴ (GAC) → Asn (AAC)], PHA synthase gene (*phaC*_{Ac}) with NSDG mutation [Asn¹⁴⁹ (AAC) → Ser (TCT) and Asp¹⁷¹ (GAC) → Gly (GGC)], (*R*)-specific enoyl-CoA hydratase (*phaJ*_{Ac}), 3-ketothiolase gene (*phaA*_{Re}), NADPH-dependent acetoacetyl-CoA reductase gene (*phaB*_{Re}). *phaPC*_{JAc} and *phaAB*_{Re} genes were derived from *A. caviae* FA440 (10) and *R. eutropha* H16 (11), respectively. These five genes were located in this order downstream of *lac* and *A. caviae* *pha*

promoters. The plasmid was constructed as follows: a 1.2-kb *KpnI-SgfI* fragment of pBBR1phaP(D4N)CJAB D171L (sample no. 15-D8), the mutant plasmid unexpectedly obtained during the course of PhaC_{Ac} mutation study (12), was introduced into the same sites of pBBREE32d13dPB NSDG (13, 14), to yield pBBREE32d13dPB D4N_NSDG. Next, a 1.6-kb *KpnI-ScaI* fragment of pBBREE32d13dPB D4N-NSDG was introduced into the same sites of pBBR1phaPCJAB WT, to yield pBBR1phaP(D4N)CJ_{Ac}AB_{Re} NSDG.

Gene expression and enzyme activity assay

E. coli BL21 codonplus (DE3)-RIL was transformed by pTTQldhAhadAIBC_{Cd} and cultured in 100 mL LB medium (10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter of distilled water) with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and 50 μ g/mL carbenicillin using 500 mL shake flask (130 strokes/min) at 30 °C for 24 h. The recombinant cells were resuspended with a suitable volume of buffer. In order to obtain cell-free extract, the resuspended cells were disrupted by sonication for 30 sec $\times$ 3 times (20 kHz, 60W), and centrifuged at 12,000 $\times$ g, 4 °C for 25 min to remove the cell debris. The cell-free extract was used for enzyme activity assay as follows (8, 9):

2H4MV dehydrogenase (LdhA) activity was measured in cuvettes of 1.0-ml total volume containing 50 mM Tris-HCl, pH 8.0, 0.2 mM NADH, and 1 mM 2-keto-4-methylvalerate at 30 °C. After addition of the enzyme, the absorbance decrease of NADH was monitored at 340 nm (ϵ_{340} = 6,300 M⁻¹ cm⁻¹).

2H4MV-CoA transferase (HadA) activity was measured in cuvettes of 1.0-ml total volume containing 50 mM KPi (pH 7.0), 0.2 mM 2H4MV-CoA, 1 mM 4M2PE at 30 °C, using the difference of the molar absorbance coefficient ($\Delta\epsilon_{260}$ = 6,000 M⁻¹ cm⁻¹) between 2H4MV-CoA (ϵ_{260} = 16,000 M⁻¹ cm⁻¹) and 4M2PE-CoA (ϵ_{260} = 22,000 M⁻¹ cm⁻¹).

2H4MV-CoA dehydratase (HadBC and HadI) activity was measured in cuvettes of

1.0-ml total volume containing 50 mM Tris-HCl (pH 8.0), 0.2 mM 2H4MV-CoA, 5 mM MgCl₂, 0.4 mM ATP, 0.1 mM dithionate, 5 mM dithiothreitol, 1 μM bovine serum albumin. After addition of the enzyme, the absorbance increase of 4M2PE-CoA was monitored at 290 nm ($\Delta\epsilon_{290} = 2,200 \text{ M}^{-1} \text{ cm}^{-1}$) at 30 °C.

2H4MV-CoA used for the above enzyme assays was synthesized from lithium salts of CoA (Wako Pure Chemical Industries, Ltd., Osaka) and L-leucic acid (Tokyo Chemical Industry Co., Ltd., Tokyo), based on mixed-anhydride methods (15, 16). One unit is defined as the amount of enzyme required to convert 1 μmol of substrate per minute in a cell-free extract.

To confirm the protein expression, cell-free extract was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 10% gel according to standard procedures. Protein concentration was determined using a Quant-iT Protein Assay Kit (Invitrogen, CA, USA) according to the manufacturer’s instructions.

PHA synthesis in *E. coli*

Recombinant *E. coli* BL21 codonplus (DE3)-RIL and NS1391 were grown in 20 mL LB medium at 37 °C for 16 h as a seed culture. The seed culture was inoculated into a 500 mL apple-shaped shake flask (Sakaguchi flask) with 100 mL (130 strokes/min; aerobic condition) or 250 mL (90 stroke/min; microaerobic condition) M9 medium (17.1 g of Na₂HPO₄·12H₂O, 3 g of KH₂PO₄, 0.5 g of NaCl and 1 g of NH₄Cl, 2 mL of 1 M MgSO₄ and 0.1 mL of 1 M CaCl₂ per liter of distilled water) supplemented with 20 g/L glucose, 0-3 g/L leucine, 0.1 mM IPTG, and 0.5 mL/L of modified trace element solution (0.218 g of CoCl₂·6H₂O, 9.7 g of FeCl₃, 7.8 g of CaCl₂, 0.118 g of NiCl₂·6H₂O, 0.105 g of CrCl₃·6H₂O, 0.156 g of CuSO₄·5H₂O and 16.9 g of ZnSO₄·7H₂O per liter of 0.1 N HCl). The cells were cultivated at 30 °C for 72 h and washed with distilled water to remove medium components

before being lyophilized.

3H4MV production from glucose via leucine was conducted by using two *E. coli* strains, leucine overproducer strain NS1391 (non-PHA producer) and PHA producer strain BL21 codonplus (DE3)-RIL expressing PhaPCJAB with LdhA/HadAIBC. The culture procedures were as follows: First, to produce leucine, *E. coli* NS1391 was cultured in a 2 L shake flask with 1 L M9 medium containing 20 g/L glucose for 24 h at 30 °C (100 strokes/min). The 250 mL culture supernatant of *E. coli* NS1391 was transferred into three new 500 mL shake flasks and supplemented with 5 g/L glucose, 0.1 mM IPTG, 0.5 mL/L modified trace element, and antibiotics. *E. coli* BL21 codonplus (DE3)-RIL expressing PhaPCJAB with LdhA/HadAIBC was inoculated into the leucine-containing supernatant and cultivated for 72 h at 30 °C (90 strokes/min).

Leucine concentration assay

The amino acids present in the culture supernatant were labeled by phenylisothiocyanate (PITC) (Tokyo Chemical Industry) in order to detect by high-performance liquid chromatography (HPLC) at absorbance 254 nm. The 200 µL culture supernatants of recombinant *E. coli* were frozen and lyophilized in glass vials. Dried samples were washed with 40 µL ethanol mixture (ethanol: water: triethylamine = 2: 2: 1) and the ethanol mixture was removed by evaporation. Subsequently, 100 µL of derivatization reagent (ethanol: water: triethylamine: PITC = 7: 1: 1: 1) was added into the sample vial, and incubated at room temperature for 20 min. After that, the derivatization reagent was removed by evaporation, and then dissolved in 2 mL eluate A (see below). The derivatized samples were filtered by 0.45 µm cellulose acetate filter (Merck Millipore, Billerica, MA, USA) prior to HPLC analysis.

The derivatized samples were analyzed by HPLC using a Shimadzu 10A LC-VP

system and a SPD-10A UV-vis detector (Shimadzu, Kyoto). Samples, 50 μ L, were injected into Shodex silica C18M 4E column (250 $\times$ 4.6 mm, Showa Denko, Tokyo) at 45 $^{\circ}$ C equilibrated with eluate A (100 mM ammonium acetate buffer containing 5 % acetonitrile, pH 6.5). The derivatized samples were eluted at 1 mL/min of flow rate by a gradient of eluate B (60% acetonitrile) as follows: 0-30 min, linear gradient of 0-50% B; 30-35 min, linear gradient of 50-100% B; 35-40 min, 100% B; 40-45 min, linear gradient of 100-0% B; 45-50 min 0% B. Effluents were detected by absorption at 254 nm due to their aromaticity. L-Leucine (Kanto Chemical Co., Inc., Tokyo) was used to plot the calibration curve.

Determination of oxygen transfer coefficient (k_{La})

To elucidate oxygen supply levels in 100 (aerobic condition) and 250 (microaerobic condition) mL of medium volumes in 500 mL shake-flask (Sakaguchi flask), the volumetric oxygen transfer coefficient (k_{La}) was measured by a sulfite oxidation method (17) with suitable modifications. Shake-flasks were filled with Na₂SO₃ solution (0.2 N Na₂SO₃ and 0.1 mM CuSO₄), and were shaken at 30 $^{\circ}$ C and 90 or 130 strokes/min. A portion of Na₂SO₃ solution was taken at appropriate time intervals for iodometry. This method is based on the oxidation reaction of Na₂SO₃ ($\text{Na}_2\text{SO}_3 + 1/2 \text{O}_2 \rightarrow \text{Na}_2\text{SO}_4$) whereby, residual Na₂SO₃ after shaking for a definite period of time is measured by titration. The amount of oxygen absorbed by the Na₂SO₃ solution is calculated from the concentration of residual Na₂SO₃ and therefore, the oxygen-supplying ability of each cultivation condition can thus be evaluated.

PHA analysis

PHA contents and composition were determined by gas chromatography (GC14B, Shimadzu) with flame ionization detector and gas chromatography-mass spectrometry (GCMS-QC 2010, Shimadzu). Approximately 30 mg lyophilized cells were methanolized in

the presence of 15 % sulfuric acid before analysis (18).

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

The structure of 3H4MV was determined by NMR spectroscopy. Purified copolymer (50 mg) was dissolved in 1 mL of CDCl₃ and subjected to both 500 MHz ¹H and 125 MHz ¹³C NMR analyses. NMR spectra were recorded using a JEOL LA500 spectrometer. Tetramethylsilane [(CH₃)₄Si] was used as an internal chemical shift standard.

Results

Expression of *C. difficile* enzymes in *E. coli*

The leucine metabolism-related genes of *C. difficile* were expressed from the plasmid pTTQldhAhadAIBC_{Cd} by IPTG induction (0.1 mM) in *E. coli* BL21 (DE3) and BL21 codonplus (DE3)-RIL. The gene products were analyzed by SDS-PAGE as shown in Fig. 2. In the case of BL21 codonplus (DE3)-RIL, the protein bands appeared at 37 kDa for LdhA, 43 kDa for HadA, 30 kDa for HadI, 42 kDa for HadB and 47 kDa for HadC (Fig. 2, Lane 7, 8). However, in BL21 (DE3), there were no detectable bands corresponding to each of the protein (Fig. 2, Lane 3, 4). This is because *C. difficile* has AT-rich genome and the codon usage of these genes was not optimized for expression in *E. coli* BL21 (DE3). On the other hand, *E. coli* BL21 codonplus (DE3)-RIL was transformed by the plasmid carrying rare codon tRNA genes (*argU*, *ileY* and *leuW*); therefore, this strain was able to translate recombinant enzymes from *C. difficile*. Of several IPTG concentrations tested (0.1, 0.5 and

1.0 mM), 0.1 mM IPTG was preferred for protein expression in *E. coli* BL21 codonplus (DE3)-RIL.

Activity assay of *C. difficile* enzymes

E. coli BL21 codonplus (DE3)-RIL harboring pTTQldhAhadAIBC_{Cd} was cultivated in LB medium with 0.1 mM IPTG at 30 °C for 24 h under aerobic (130 strokes/min) and non-PHA accumulation condition. The cells were then lysed by sonication. Using this cell-free extract, the specific activities of each enzyme from *C. difficile* were determined as listed in Table S2. The 2H4MV dehydrogenase (LdhA) activity was 1.59±0.07 U/mg, which is much higher than the strain harboring empty vector pTTQ19 (0.07±0.02 U/mg). The activity of 2H4MV CoA transferase (HadA, 0.20±0.01 U/mg) was also higher than that of control strain (0.01±0.01 U/mg). However, the activity of 2H4MV-CoA dehydratase (HadIBC complex consisting of HadBC heterodimer and HadI homodimer) was not detectable in the cell-free extract. Because the activator HadI has [4Fe-4S] cluster, HadIBC complex is thought to be extremely sensitive to oxygen (8). Thus, microaerophilic growth condition should be considered for functional expression of HadIBC.

PHA synthesis by recombinant *E. coli* BL21 codonplus (DE3)-RIL

Because the leucine metabolism-related enzymes from *C. difficile* was unable to be expressed in the common *E. coli* (Fig. 2), *E. coli* BL21 codonplus (DE3)-RIL was employed to construct 3H4MV biosynthesis pathway. From the result of enzyme activity assay (Table S2), HadIBC did not show any activity under aerobic condition, thus, PHA synthesis was carried out under microaerobic condition together with aerobic condition. The engineered PHA synthase, PhaC_{Ac} NSDG, was employed in this study because this synthase showed high affinity towards 3H4MV monomer (12). Additionally, PHA granule associated protein

(PhaP_{Ac}) with D4N mutation was co-expressed. This D4N mutation was unexpectedly discovered during the course of PhaC_{Ac} mutagenesis study (12), and it was found to enhance P(3HB) accumulation levels in *E. coli* from 9 wt% (no mutation) to 48 wt% (D4N mutation) when glucose was fed as the sole carbon source. The precise role of D4N mutation in the enhancement of PHA accumulation is now under investigation in our laboratory.

E. coli BL21 codonplus (DE3)-RIL expressing PhaPCJAB with or without LdhA/HadAIBC was cultured with supplementation of 0-3 g/L leucine under both aerobic and microaerobic conditions, where the k_{La} values were 204 and 49 h⁻¹, respectively, under the model conditions. The cultivation result is shown in Table 1. When PhaPCJAB was expressed alone, 3H4MV was not detected in any of the cultivation conditions. This observation suggests that *E. coli* could not supply 3H4MV monomer from glucose and leucine. In contrast, the strain expressing PhaPCJAB and LdhA/HadAIBC was able to synthesize P(3HB-co-3H4MV). Under microaerobic conditions, 3H4MV fraction was increased from 0.7 to 12.2 mol% by increasing the leucine concentration in the culture medium from 0 to 1 g/L. However, further increase of leucine concentration to 3 g/L was not so effective in enhancing 3H4MV fraction (14.0 mol%). Based on these results, the conversion from leucine to 3H4MV was the most effective at 1 g/L leucine, in which 22 mg/L 3H4MV was produced. This yield (22 mg/g-leucine) is still low but substantially improved from that by *R. eutropha* (7.2 mg/g-leucine). Under aerobic conditions, a small amount of 3H4MV fraction was detected only when 3 g/L leucine was supplemented. Although the HadIBC had no activity in *in vitro* assay under aerobic conditions, it may have weak activity *in vivo*.

Figure 3 shows the time course of P(3HB-co-3H4MV) synthesis under microaerobic conditions with 20 g/L glucose and 1 g/L leucine. Rapid cell growth, leucine consumption, and 3H4MV synthesis were observed between 24-48 h. However, the total amount of leucine

consumed over a period of 72 h was only 0.24 g/L. Based on this, it can be calculated that the conversion rate of leucine to 3H4MV was 92 mg-3H4MV/g-leucine.

Characterization of PHA synthesized by *E. coli*

GPC was used to determine the molecular weights of P(3HB-*co*-12.2 mol% 3H4MV) synthesized by recombinant *E. coli* expressing PhaPCJAB with LdhA/HadAIBC under microaerobic condition in the presence of 1 g/L leucine. The number average molecular weight (M_n) and weight average molecular weight (M_w) were 20×10^4 and 90×10^4 , respectively. The polydispersity index (M_w/M_n) was 4.5.

Figure 4 shows GC-MS spectrum of P(3HB-*co*-12.2 mol% 3H4MV), together with P(3HB-*co*-1.2 mol% 3HV-*co*-3.0 mol% 3H4MV) synthesized by *R. eutropha* mutant 1F2 strain harboring *phaC1* from *Pseudomonas* sp. 61-3 in the presence 10 g/L leucine (6). When using *R. eutropha* as the host strain under any culture conditions, 3HV was always detected as a byproduct of 3H4MV metabolism (Fig. 4B). On the other hand, the PHA synthesized by the *E. coli* strain in this study was composed of only 3HB and 3H4MV units (Fig. 4A).

The 500 MHz ^1H NMR spectrum of P(3HB-*co*-12.2 mol% 3H4MV) is shown in Fig. 5A. The signals were assigned as previously described (4). Strong signals at 5.3, 2.5 and 1.3 ppm were assigned to methine of 3HB [b], methylene of 3HB and 3H4MV [a+d], and methyl proton resonances of 3HB unit [c], respectively. In addition, the obvious signals detected from 3H4MV unit were methine from main chain at 5.1 ppm [e], methine from side chain at 1.9 ppm [f], and methyl at 0.9 ppm [g]. The intensity ratio of signals [e], [f] and [g] was 1: 1: 6, which is in good agreement with the ratio estimated from the structure of 3H4MV. Figure 5B shows 125 MHz ^{13}C NMR spectrum. Strong signals at 169.1, 67.6, 40.8 and 19.7 ppm resulted from carbonyl [1], methine [3], methylene [2], and methyl [4] of 3HB unit, respectively. The smaller signals detected at 169.8, 74.7, 36.6, 31.3 and 18.1-17.4 ppm were

assigned to carbonyl [5], methine [7] of main chain, methylene [6], methine [8] of side chain, and methyl [9] of 3H4MV unit. The carbonyl carbon resonances of this 3H4MV copolymer were resolved into four groups of signals, arising from different diad sequences of connecting 3HB and 3H4MV unit. In order to estimate the randomness of this copolymer which is consisted of 3HB and 3H4MV units, the parameter D value defined as $D=(3HB^*-3HB)(3H4MV^*-3H4MV)/(3HB^*-3H4MV)(3H4MV^*-3HB)$ was used (19). From relative intensities of each signal, the D value was estimated as 1.5. If the copolymer is statistically random, the D value is close to 1.0. Thus, the 3H4MV copolymer synthesized in this study was close to random copolymer, unlike the polymers synthesized by *R. eutropha* ($D=2.2-16.6$) in previous study (4).

PHA synthesis by recombinant *E. coli* NS1391

Furthermore, to test the feasibility of the 3H4MV copolymer synthesis from sugar as the sole carbon source, a modified *E. coli* for leucine over-production termed strain NS1391 was employed as new host (Ptitsyn et al., US Patent WO 2008/010565 A2). Since *E. coli* NS1391 is unable to express AT-rich genes just like *E. coli* BL21 (DE3), this strain was transformed with the tRNA coding plasmid that was obtained from *E. coli* BL21 codonplus (DE3)-RIL. The expression of each enzyme from *C. difficile* in the rare-codon-usage-improved *E. coli* NS1391 was analyzed by SDS-PAGE, and the LdhA and HadAIBC were confirmed to be expressed with the addition of 0.1 mM IPTG (data not shown). Next, the *E. coli* NS1391 expressing PhaPCJAB with LdhA/HadAIBC was cultured in M9 medium containing 20 g/L glucose at 30 °C. This recombinant strain synthesized 1.3 wt% P(3HB-co-3H4MV) with 3.0 mol% 3H4MV, under microaerobic culture condition (Table 2). The leucine concentration in the broth after 72 h cultivation was approximately 0.5 g/L leucine.

PHA synthesis using supernatant of *E. coli* NS1391

The culture supernatant of the *E. coli* NS1391 (non-PHA producer), containing 0.2 g/L leucine derived from glucose by 24 h aerobic cultivation, was used as medium for BL21 codonplus (DE3)-RIL expressing PhaPCJAB with LdhA/HadAIBC. This BL21 strain synthesized 18 wt% P(3HB-co-3H4MV) with 12.6 mol% 3H4MV (Table 2), demonstrating the highest 3H4MV fraction using glucose as a raw material.

Discussion

Unlike 3HV monomer, 3H4MV and 3HHx monomer units are excluded from the P(3HB) crystalline lattice (5). Thus, the introduction of these monomer units gives rise to a remarkable decrease in crystallinity, thereby increasing the flexibility of 3HB-based copolymers. Especially, the flexibility is significantly increased by introducing over 10 mol% 3H4MV unit into the copolymer (4, 5). 3HHx unit also has the same effect; however, 3HHx supply from sugars in bacterial cells is still limited to small amount (20). Therefore, we have studied the feasibility for 3H4MV supply from sugars and sugar-derived products.

In a previous study, we reported that branched amino acid can be converted to 3H4MV unit and incorporated into PHA copolymer by *R. eutropha* (6). However, the conversion efficiency of leucine to 3H4MV by *R. eutropha* was very low. In addition, the 3H4MV synthesis pathway is unknown. In order to improve leucine conversion into P(3HB-co-3H4MV) synthesis, we attempted to construct a novel 3H4MV synthesis pathway in *E. coli* by using leucine metabolism-related enzymes from *C. difficile*.

C. difficile is an obligate anaerobic bacterium and it was chosen because of its unique amino acid metabolic pathway. The leucine metabolism-related enzymes of *C. difficile*

are very attractive for 3H4MV synthesis, among which are the HadBC and HadI. HadBC is activated by HadI, probably by transferring an electron to HadBC for the elimination of 2H4MV's hydroxy group. HadI is highly sensitive to oxygen. Therefore, the cultivation of *E. coli* in this study had to be done under limited oxygen supply for functional expression of HadI. Under reduced oxygen supply, HadI was able to function in the supply of 3H4MV although the cell growth was limited, resulting in the low PHA yield. Thus, the oxygen supply was very critical not only for the composition of P(3HB-co-3H4MV) but also for polymer yield in this production system.

E. coli BL21 codonplus (DE3)-RIL expressing PhaPCJAB with LdhA/HadAIBC showed 3-10 times higher PHA content than that without LdhA/HadAIBC (Table 1). The reason for this difference is unclear but seems to involve the protein(s) of LdhA/HadAIBC. Further studies are needed to evaluate the mechanism and consequences of the observed phenomena.

The leucine concentration in broth after 72 h cultivation of *E. coli* NS1391 expressing PhaPCJAB with LdhA/HadAIBC was about 0.5 g/L. As shown in Fig. 3, *E. coli* BL21 codonplus (DE3)-RIL consumed only 0.24 g/L to synthesize P(3HB-co-12.2 mol% 3H4MV), implying that enough amount of leucine was provided by *E. coli* NS1391 for 3H4MV enhancement. However, the maximum 3H4MV fraction was as low as 3.0 mol% under this culture condition. In *E. coli* NS1391, the expression of tyrosine aminotransferase (TyrB) was enhanced for leucine over-production (Ptitsyn et al., US Patent WO 2008/010565 A2). TyrB converts 2-keto-4-methylvalerate to leucine (21, 22); thus, TyrB and LdhA compete for the same substrate 2-keto-4-methylvalerate for each pathway (Fig. 1). Accordingly, the carbon flux towards leucine was increased while the flux towards 3H4MV might be reduced. Apart from leucine production, the recombinant *E. coli* NS1391 accumulated only 10 wt% PHA, which is about one-third of *E. coli* BL21 codonplus

(DE3)-RIL, even under aerobic conditions (Table 2). By employing *E. coli* NS1391, we expected to produce P(3HB-*co*-3H4MV) from glucose as sole carbon source while maintaining 3H4MV fraction at around 10 mol% and without reduction of PHA yield. Unfortunately, the capability of *E. coli* NS1391 to accumulate PHA proved to be not high.

Several studies have reported the synthesis of 3H4MV copolymer using some bacteria by feeding branched fatty acids as 3H4MV precursor (23-26), but often with the concomitant generation of 3HV unit (4, 25). This trend was also observed for *R. eutropha* when leucine was fed as 3H4MV precursor (6). In this study, by constructing a novel 3H4MV synthesis pathway in *E. coli*, binary copolymer consisting of only 3HB and 3H4MV was successfully produced. Besides, the conversion rate from leucine to 3H4MV and the randomness in sequential distribution of the copolymer were substantially improved when compared to the case of *R. eutropha*. The approach developed in this study would be beneficial for compositional regulation of 3H4MV-containing copolymers. However, further study is needed to achieve P(3HB-*co*-3H4MV) production under aerobic culture conditions so as to increase polymer yield.

In summary, we demonstrated the 3H4MV synthesis using leucine metabolism-related enzymes from *C. difficile* under microaerobic culture conditions. The recombinant *E. coli* BL21 codonplus (DE3)-RIL synthesized P(3HB-*co*-3H4MV) with 12.2 mol% 3H4MV when supplemented with 1 g/L leucine as a precursor. In addition, P(3HB-*co*-12.6 mol% 3H4MV) was synthesized by using the culture supernatant of leucine overproducer *E. coli* strain NS1391 as the medium for recombinant *E. coli* BL21 codonplus (DE3)-RIL, achieving 3H4MV copolymer synthesis only from glucose. By employing recombinant *E. coli* NS1391 as a PHA production host, P(3HB-*co*-3H4MV) with 3.0 mol% 3H4MV was also synthesized from glucose. This is the first report demonstrating the synthesis of 3H4MV copolymer using *E. coli* as the host strain by constructing new synthesis

pathway using heterologous enzymes from *C. difficile*.

Acknowledgments

We thank to Y. Nakamura (Tokyo Institute of Technology) for NMR analysis. *E. coli* NS1391 was kindly donated by Ajinomoto Co., Inc (Tokyo, Japan). This work was supported by funding from the Research for Promoting Technological Seeds of Japan Science and Technology Agency (JST) to T. Tsuge. A. Saika is a recipient of a JSPS young scientist fellowship (12J07871).

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Figure captions

Figure 1. Metabolic pathway for P(3HB-*co*-3H4MV) synthesis. LdhA, (*R*)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase; HadA, 2H4MV CoA transferase; HadBC, 2H4MV-CoA dehydratase; HadI, activator of hydratase HadBC; PhaA, 3-ketothiolase; PhaB, NADPH-dependent acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaJ, (*R*)-specific enoyl-CoA hydratase; 3HB, 3-hydroxybutyrate; 3H4MV, 3-hydroxy-4-methylvalerate. Leucine metabolic pathway of *C. difficile* is shown in bold.

Figure 2. SDS-PAGE analysis of cell-free extracts from *E. coli* BL21 (DE3) and BL21 codonplus (DE3)-RIL harboring leucine-metabolism related genes from *C. difficile*. Lane 1 and 2, BL21(DE3)/pTTQ19; 3 and 4, BL21 (DE3)/pTTQldhAhadAIBC_{Cd}; 5 and 6, BL21 codonplus (DE3)-RIL/pTTQ19; 7 and 8, BL21 codonplus (DE3)-RIL/pTTQldhAhadAIBC_{Cd}. Odd and even lane numbers are soluble and insoluble fractions, respectively.

Figure 3. Time-dependent changes of cell growth, PHA concentration, and leucine concentration under microaerobic culture conditions. *E. coli* BL21 codonplus (DE3)-RIL expressing PhaPCJAB and LdhA/HadAIBC was cultured in M9 medium with 20 g/L glucose and 1.0 g/L leucine.

Figure 4. GC-MS analysis (m/z 103 ion chromatogram) of P(3HB-*co*-3H4MV). (A) P(3HB-*co*-12.2 mol% 3H4MV) synthesized by *E. coli* BL21 codonplus (DE3)-RIL expressing PhaPCJAB and LdhA/HadAIBC; (B) P(3HB-*co*-1.2 mol% 3HV-*co*-3.0 mol% 3H4MV) synthesized by *R. eutropha* PHB⁻4 expressing PHA synthase 1 from *Pseudomonas* sp. 61-3 (6).

Figure 5. (A) 500 MHz ¹H NMR spectrum and (B) 125 MHz ¹³C NMR spectrum of

538 P(3HB-*co*-12.2 mol% 3H4MV) synthesized by *E. coli* BL21 codonplus (DE3)-RIL
539 expressing PhaPCJAB and LdhA/HadAIBC.
540

Figure 1

Figure 2

Figure 3

Figure 4

(A) **(B)**

Figure 5

Table 1 PHA production by *E. coli* BL21 codonplus (DE3)-RIL expressing PhaPCJAB and leucine metabolism-related enzymes from *C. difficile* in the presence of 0-3 g/L leucine

Strain	Culture condition	Leucine conc. (g/L)	Dry cell wt (g/L)	PHA content (wt%)	PHA composition (mol%)	
					3HB	3H4MV
BL/PCJAB ^a	Aerobic ^c	0	1.8±0.1	3±1	100	0
		1	1.8±0.1	3±1	100	0
		3	1.8±0.1	4±1	100	0
	Microaerobic ^d	0	0.5±0.1	5±1	100	0
		1	0.5±0.1	6±1	100	0
		3	0.5±0.1	5±1	100	0
BL/PCJAB_Cd ^b	Aerobic	0	2.2±0.1	27±4	100	0
		1	3.8±0.4	41±4	100	0
		3	2.9±0.1	28±1	99.4±0.3	0.6±0.3
	Microaerobic	0	0.6±0.1	17±1	99.3±0.2	0.7±0.2
		1	0.6±0.1	30±6	87.8±0.7	12.2±0.7
		3	0.5±0.1	36±2	86.0±0.7	14.0±0.7
		0.2±0.1 ^e	0.5±0.1	18±2	87.4±1.0	12.6±3.1

Cells were cultured in M9 medium containing glucose (20 g/L) and leucine (0-3 g/L) at 30 °C for 72 h. The results are the average of three independent cultivations.

^a. *E. coli* BL21 codonplus (DE3)-RIL/ pBBR1phaP(D4N)CJ_{Ac}AB_{Re} NSDG.

^b. *E. coli* BL21 codonplus (DE3)-RIL/ pBBR1phaP(D4N)CJ_{Ac}AB_{Re} NSDG + pTTQldhAhadAIBC_{Cd}.

^c. Aerobic condition: 100 mL of culture volume in 500-mL shake flask at 130 strokes/min.

^d. Microaerobic condition: 250 mL culture volume in 500-mL shake flask at 90 strokes/min.

^e. 24 h-cultured supernatant of leucine overproducer *E. coli* NS1391 (non-PHA producer).

Table 2 PHA production from glucose by *E. coli* NS1391 expressing PhaPCJAB and leucine metabolism-related enzymes from *C. difficile*

Culture condition	Dry cell wt (g/L)	PHA content (wt%)	PHA composition (mol%)		Leucine production (g/L) ^c
			3HB	3H4MV	
Aerobic ^a	1.2±0.1	10±2	100	0	1.0±0.1
Microaerobic ^b	0.4±0.1	1.3±0.1	97.0±0.1	3.0±0.1	0.6±0.1

Cells were cultured in M9 medium containing glucose (20 g/L) at 30 °C for 72 h. The results are the average of three independent cultivations.

^a. Aerobic condition: 100 mL of culture volume in 500-mL shake flask at 130 strokes/min.

^b. Microaerobic condition: 250 mL culture volume in 500-mL shake flask at 90 strokes/min.

^c. Leucine concentration in broth at the end of cultivation.