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Biosynthesis of novel lactate-based polymers containing medium-chain-length 3-hydroxyalkanoates by recombinant *Escherichia coli* strains from glucose

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Novel lactate (LA)-based polymers containing medium-chain-length 3-hydroxyalkanoates (MCL-3HA) were produced in *fadR*-deficient *Escherichia coli* strains from glucose as the sole carbon source. The genes encoding LA and 3-hydroxybutyrate (3HB) monomers supplying enzymes [propionyl-CoA transferase (PCT), D-lactate dehydrogenase (D-LDH), β-ketothiolase (PhaA), and NADPH-dependent acetoacetyl-CoA reductase (PhaB)], MCL-3HA monomers supplying enzymes [(*R*)-3-hydroxyacyl-ACP thioesterase (PhaG) and (*R*)-3-hydroxyacyl (3HA)-CoA ligase] via fatty acid biosynthesis pathway, and modified polyhydroxyalkanoate (PHA) synthase [PhaC1(STQK)] of *Pseudomonas* sp. 61-3 were introduced into *E. coli* LS5218. This resulted in the synthesis of a novel LA-based copolymer, P(LA-*co*-3HB-*co*-3HA). ^{1}H-nuclear magnetic resonance (NMR) analysis revealed the composition of P(LA-*co*-3HB-*co*-3HA) to be 19.7 mol% LA (C_3), 74.9 mol% 3HB (C_4), and 5.4 mol% MCL-3HA units of C_8 and C_{10}. Furthermore, the recombinant *E. coli* CAG18497 strain carrying these genes, excluding the *phaAB* genes, accumulated P(92.0% LA-*co*-3HA) with a novel monomer composition containing C_3, C_8, C_{10}, and C_{12}. ^{13}C-NMR analysis showed the existence of LA-3HA sequence in the polymer. The solvent cast film of P(92.0% LA-*co*-3HA) exhibited transparency similar to poly(lactic acid).

[**Key words:** Biodegradable plastic; Polyhydroxyalkanoate; Poly(lactic acid); D-Lactate dehydrogenase; Biopolymer]

Polyhydroxyalkanoates (PHAs) are biodegradable plastics that are synthesized by a wide variety of bacteria as intracellular carbon and energy storage materials (1–3). PHAs can be categorized into three groups according to monomer length. Short-chain-length PHAs (SCL-PHAs) consisting of monomer units of C_3–C_5, medium-chain-length PHAs (MCL-PHAs) consisting of monomer units of C_6–C_{14}, and SCL-MCL-PHAs consisting of both SCL and MCL monomer units (3,4). Poly(3-hydroxybutyrate) [P(3HB)] homopolymer, the most common SCL-PHA, is stiff and brittle. MCL-PHAs are generally amorphous because they possess low crystallinity (5,6). SCL-MCL-PHAs exhibit a wide range of physical properties depending on the molar ratio of SCL to MCL monomer units. For example, P(94% 3HB-*co*-6% 3HA), a random copolymer of 3HB and MCL-3HA, consisting of 3HA units of C_4–C_{12}, synthesized by the recombinant strain of *Pseudomonas* sp. 61-3 was demonstrated to be a flexible material similar to the low-density polyethylene (7). Therefore, the monomer composition exerts considerable influence of PHA qualities.

Despite not being a native PHA-producer, *Escherichia coli* has been used as a host for producing PHA due to its less doubling time and ease of genetic modification. There are numerous studies on the production of SCL-PHA, MCL-PHA, and SCL-MCL-PHA using *E. coli* as a host. Schubert et al. (8) reported a recombinant *E. coli* that synthesized P(3HB) by incorporating a β-ketothiolase gene (*phaA*), an NADPH-dependent acetoacetyl-CoA reductase gene (*phaB*), and a PHA synthase gene (*phaC*) from *Ralstonia eutropha* (Currently designated as *Cupriavidus necator*). In addition, the recombinant *E. coli* synthesized P(3HB-*co*-3HA) with various monomer compositions comprising C_4 to C_{10} using mixtures of glycerol and dodecanoate by coexpression of PhaA, PhaB, and PhaC with (*R*)-specific enoyl-CoA hydratase (PhaJ), which catalyzes the generation of MCL-3HA-CoA from enoyl-CoA, an intermediate of the β-oxidation pathway (9). In recent studies, P(3HB-*co*-3HA)s have been synthesized from glucose as the sole carbon source by coexpression of PhaA, PhaB, and PhaC with (*R*)-3-hydroxyacyl-ACP thioesterase (PhaG) and (*R*)-3-hydroxyacyl (3HA)-CoA ligase, which are the necessary enzymes to incorporate MCL-3HA units into the polymer chain via the fatty acid biosynthesis pathway (10,11). Only two genes, PP0763 of *Pseudomonas putida* KT2440 and PA3924 of *Pseudomonas aeruginosa* PAO, have so far been known that their translational products have (*R*)-3HA-CoA ligase activity *in vivo* (10–12). The P(3HB-*co*-3HA) copolymers possessed mechanical properties that improved the brittleness of the P(3HB) homopolymer. However, the copolymer lacked transparency, which ultimately limited its application.

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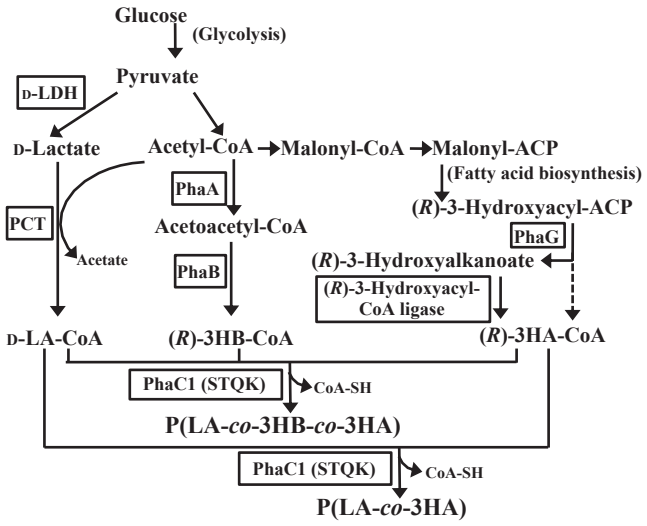

FIG. 1. Metabolic control for the synthesis of P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA) from glucose. D-LDH, D-lactate dehydrogenase; PCT, propionyl-CoA transferase; PhaA, β-ketothiolase; PhaB NADPH-dependent acetoacetyl-CoA reductase; PhaG, (*R*)-3-hydroxyacyl-ACP thioesterase; PhaC1(STQK), engineered PHA synthase of *Pseudomonas* sp. 61-3.

In turn, poly(lactic acid) (PLA), a biodegradable plastic, has attracted considerable attention because it is producible from renewable resources and possess excellent transparency. PLA is normally synthesized by chemical processes using heavy metal catalysts (13). Recently, a new microbial platform for producing LA-based polymer has been established based on a PHA biosynthetic pathway by applying the engineered PHA synthase of *Pseudomonas* sp. 61-3 [PhaC1(STQK)], possessing an acquired LA-polymerizing activity (14). Poly(lactate-*co*-3HB)s [P(LA-*co*-3HB)s], LA-based polymers, have been produced from glucose in a recombinant *E. coli* strain expressing PhaC1(STQK) along with propionyl-CoA transferase (PCT) from *Megasphaera elsdenii* and 3HB monomer-supplying enzymes (PhaA and PhaB) from *R. eutropha*. P(LA-*co*-3HB)s are copolymers containing LA unit and are newly recognized as a member of the PHA family. Moreover, P(LA-*co*-3HB)s exhibit transparency depending on the fraction of LA present (15). Therefore, there have been attempts to enhance LA fractions in the copolymers by changing the host strains and carbon sources, incorporating the engineered enzymes, and controlling the metabolic pathway (16–21). For example, introduction of the exogenous D-lactate dehydrogenase gene (*ldhD*) into *E. coli* strains DH5α, XL1-Blue, and LS5218, together with *phaA*, *phaB*, *pct*, and *phaC1(STQK)* genes led to an enhancement in the LA fraction of P(LA-*co*-3HB)s (21).

PLA exhibits good transparency, and it is a biodegradable polymer with high stiffness. Hence, the biosynthesis of LA-based polymers containing LA, 3HB, and a third monomeric unit in *E. coli* has been established to improve the lack of flexibility of PLA. Shozui et al. (22) have reported that P(LA-*co*-3HB-*co*-3-hydroxyvalerate)s [P(LA-*co*-3HB-*co*-3HV)s], LA-based terpolymers, were produced in the recombinant *E. coli* JW0885 from glucose by feeding propionate as a precursor of 3HV. In addition, the recombinant *E. coli* LS5218 strain synthesized P(LA-*co*-3HB-*co*-3-hydroxyhexanoate)s [P(LA-*co*-3HB-*co*-3HHx)s] via a reverse β-oxidation reaction from glucose and butyrate by coexpression of PhaC1(STQK), PCT, and PhaJ (23). These novel LA-based polymers have been expected to render novel properties to the materials and expand the range of its application. However, there are only a few reports on LA-based polymers with various monomer compositions.

The aim of this study was to synthesize novel LA-based polymers with LA and MCL-3HA units, which could be expected to have transparency and flexibility. We have recently found the (*R*)-3HA-CoA ligase gene (PA3924) from *P. aeruginosa* PAO and succeeded in the biosynthesis of P(3HB-*co*-3HA) copolymer consisting of 3HA units of C_4, C_8, and C_{10} from glucose by the *E. coli* LS5218 strain harboring *phaC1*, *phaA*, *phaB*, *phaG*, and PA3924 genes (11). Based on the results, we constructed metabolic pathways to synthesize P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA) from glucose as the sole carbon source in this study (Fig. 1). D-LA-CoA is obtained from pyruvate by D-lactate dehydrogenase (D-LDH) and PCT (14). On the contrary, (*R*)-3HB-CoA is obtained from acetyl-CoA by PhaA and PhaB (8). (*R*)-3HA-CoA is obtained by PhaG and (*R*)-3HA-CoA ligase via fatty acid biosynthesis pathway (10–12). Here, we report on the biosynthesis and structural analysis of novel LA-based polymers, P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA), synthesized by the recombinant strains of *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this study are listed in Table 1 (11,21,24–27). *E. coli* strains were used as hosts for plasmid construction and biopolymer production. Unless otherwise mentioned, transformation of *E. coli* strains was performed by standard procedures and grown at 37°C in lysogeny broth (LB) medium (28). When needed, ampicillin (100 mg/L), kanamycin (50 mg/L), and tetracycline (12.5 mg/L) were added to the medium.

TABLE 1. Strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Bacterial strain		
Escherichia coli DH5α	*deoR*, *endA1*, *gyrA96*, *hsdR*17 ($r_K^- m_K^+$), *recA1*, *relA1*, *supE44*, *thi-1*, *Δ*(*lacZYA-argFV169*), Φ80*ΔlacZΔ*M15, F^-	Clontech
E. coli LS5218	*fadR*601, *atoC2*(Con)	24
E. coli CAG18497	*fadR*13::Tn10	25
Pseudomonas sp. 61-3	Wild strain	JCM 10015
Lactobacillus acetotolerans HT	Wild strain	26
Plasmid		
T-vector pMD20	*E. coli* cloning vector, Ap^r, *lacPOZ*, SP promoter	Takara, Kusatsu, Japan
pUC118 *Hin*cII/BAP	*E. coli* cloning vector, Ap^r, *lacPOZ*, *lac* promoter	Takara
pBBR1MCS-2	Km^r, broad host range, *lacPOZ*	27
pBBR1MCS-3	Tc^r, broad host range, *lacPOZ*	27
pRTcASc-MCL(Pa)	pBBR1MCS-3 derivative; P_{lac}, PA3924 from *P. aeruginosa* PAO	11
pRTcAA-GMCL(Pa)	pBBR1MCS-3 derivative; P_{lac}, PA3924, $phaG_{Ps}$	This study
pRKmXS-GMCL(Pa)	pBBR1MCS-2 derivative; P_{lac}, PA3924, $phaG_{Ps}$	This study
pTV118NpctC1(STQK)ABdP_{Re}	pTV118N derivative; P_{lac}, *pct*, *phaC1(STQK)*, *phaA*, *phaB*, T_{Re}	21
pTV118NpctC1(STQK)ldhDABdP_{Re}	P_{lac}, *pct*, *phaC1(STQK)*, *ldhD*, *phaA*, *phaB*, T_{Re}	21
pTV118NpctC1(STQK)ldhD	P_{lac}, *pct*, *phaC1(STQK)*, *ldhD*, T_{Re}	This study

Ap^r, ampicillin resistance gene; Km^r, kanamycin resistance gene; Tc^r, tetracycline resistance gene.

TABLE 2. Accumulation of LA-based copolymer by recombinant *Escherichia coli* strains.

Strain	Plasmid	Dry cell weight (g/L)	Polymer content (wt%)	Polymer composition (mol %)					
				LA (C3)	3HB (C4)	3HO (C8)	3HD (C10)	3HDD (C12)	3H5DD (C12′)
DH5α[a]	pTV118NpctC1(STQK)ldhDABdP$_{Re}$	0.70 ± 0.26	9.2 ± 5.2	13.7 ± 3.1	86.3 ± 3.1	0	0	0	0
LS5218[a]	pTV118NpctC1(STQK)ldhDABdP$_{Re}$	1.48 ± 0.12	4.4 ± 0.7	15.7 ± 3.5	84.3 ± 3.5	0	0	0	0
DH5α	pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa)	0.59 ± 0.33	17.0 ± 4.2	9.9 ± 3.5	88.9 ± 3.4	0	1.2 ± 1.0	0	0
LS5218	pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa)	1.14 ± 0.06	2.9 ± 0.3	14.4 ± 1.8	79.7 ± 2.3	2.3 ± 0.9	3.6 ± 1.0	Trace	Trace
CAG18497	pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRKmXS-GMCL(Pa)	1.06 ± 0.12	6.1 ± 3.4	16.6 ± 8.8	80.0 ± 8.2	0.5 ± 0.5	2.9 ± 1.9	Trace	Trace
CAG18497	pTV118NpctC1(STQK)ldhD and pRKmXS-GMCL(Pa)	0.95 ± 0.08	2.9 ± 0.7	70.4 ± 7.0	0	4.2 ± 1.0	25.4 ± 5.9	Trace	Trace

Cells were cultivated at 30°C in a 300-mL conical flask containing 100 mL of LB medium supplemented with 2% (w/v) glucose. LA, D-lactate; 3HB, 3-hydroxybutyrate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3H5DD, 3-hydroxy-5-*cis*-dodecenoate.

[a] Data from Goto et al. (21).

DNA manipulation and plasmid construction All DNA manipulations including isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonuclease, and agarose gel electrophoresis were performed according to standard procedures (28). The *phaG*$_{Ps}$ gene encoding a (*R*)-3-hydroxyacyl-acyl carrier protein (ACP) thioesterase (29) was amplified using PCR from the genomic DNA of *Pseudomonas* sp. 61-3 with the primer pairs phaG-SD(ApaI)-f (5′-GGGCCCGCATACCCGCTTGCCAGGAGT-3′) and phaG-DS(ApaI)-r (5′-GGGCCCTGATCCTTAGGAGCGCGAGGTT-3′) (underlined sequences indicate *Apa*I restriction site). The PCR product of the *phaG*$_{Ps}$ gene was cloned into T-vector pMD20. The 0.8-kb *Apa*I fragment containing the *phaG*$_{Ps}$ gene from the pMD20 derivative was inserted into the *Apa*I restriction site of pRTcASc-MCL(Pa) in the same direction as the (*R*)-3HA-CoA ligase gene to yield pRTcAA-GMCL(Pa). The *phaG*$_{Ps}$ gene and medium-chain-length 3-hydroxyacyl-CoA ligase gene (PA3924) of *P. aeruginosa* PAO were amplified using PCR from pRTcAA-GMCL(Pa) with primers phaG-SD(XhoI)-f (5′-CTCGAGCATACCCGCTTGCCAGGAGT-3′) and MCL-SacI-r(PAO) (5′-GAGCTCGTGTAGGAAAGCCCCGTCAGACGG-3′) (underlined sequences indicate *Xho*I and *Sac*I restriction sites, respectively). The 3.0-kb PCR product was cloned into the pUC118 *Hinc*II/BAP. Then, the 3.0-kb *Xho*I-*Sac*I fragment containing *phaG*$_{Ps}$ and PA3924 genes from the pUC118 derivative was introduced into the *Xho*I and *Sac*I restriction sites of the pBBR1MCS-2 to generate pRKmXS-GMCL(Pa). To remove the *phaA* and *phaB* genes of *R. eutropha* from pTV118NpctC1(STQK)ldhDABdP$_{Re}$ (21), inverse PCR was performed using primers phbB(Re)DS-f1 (5′-GCCTGGTTCAACCAGTCGG-3′) and ldhD-PstI(TAA)-r (5′-AACTGCAGGAATAGAAAATTATGCATCTAA-3′) (underlined sequence indicates *Pst*I restriction site). The amplified DNA fragment was phosphorylated by using T4 Polynucleotide Kinase (Toyobo, Osaka, Japan), followed by self-ligation to yield pTV118NpctC1(STQK)ldhD.

Polymer production For LA-based copolymer production, the recombinant strains of *E. coli* were grown in 3 mL LB medium at 30°C for 15 h. A 1% (v/v) inoculum of the preculture broth was transferred to 300- or 500-mL conical flasks with 100 or 300 mL LB medium, respectively. Cells were cultivated at 30°C in shaking culture (100 strokes/min). Filter-sterilized glucose solution was added to the medium at a concentration of 2% (w/v) as the sole carbon source after 8 h of cultivation. The cultivation was continued for a total of 48 h. Determination of cellular polymer composition and content was performed by gas chromatography (GC) as reported previously (30). The cultivation runs were performed at least in triplicates.

Polymer extraction and analysis The LA-based polymers accumulated in the cells were extracted with chloroform for 48 h at room temperature and purified by reprecipitation with methanol as described previously (11). The molecular mass was measured by gel permeation chromatography (GPC) (Shimadzu 20A GPC system and 10A refractive index detector, Shimadzu, Kyoto, Japan) with Shodex K-806M and K-802 columns. Chloroform was used as eluent at a flow rate of 0.8 mL/min, and a sample concentration of 1.0 mg/mL was applied. Polystyrene standards with a low polydispersity were used to make a calibration curve. The 500 MHz ^{1}H- and the 125 MHz ^{13}C-nuclear magnetic resonance (NMR) spectra of $CDCl_3$ solution of LA-based polymers (5 mg/mL) were obtained as described previously (31,32). The LA-based polymer films were aged at room temperature for at least two weeks to completely evaporate the chloroform solvent and to allow stable crystallization before thermal and mechanical analyses. The thermal data were recorded on differential scanning calorimeter (DSC) using a Perkin–Elmer DSC-8500 equipped with a cooling accessory (PerkinElmer, Waltham, MA, USA) in the temperature range of −50°C to 200°C at a heating rate of 20°C/min under a nitrogen atmosphere. The solvent-cast films were encapsulated in aluminum pans and heated from −50°C to 200°C at 20°C/min (the first scan). The samples were then rapidly quenched at −50°C. They were heated from −50°C to 200°C at 20°C/min (second heating scan). The glass transition temperature (T_g) was defined as the midpoint of the heat capacity change. The melting temperature (T_m) and the enthalpy of fusion (ΔH_m) were determined from endotherm. Dynamical mechanical thermal analysis (DMTA) was performed using a dynamic thermal mechanical analyzer DMA-8000 (PerkinElmer). The temperature scan was from −80°C to 100°C at a constant heating rate of 2°C/min and frequency of dynamic force at 1 Hz, under nitrogen atmosphere. The storage modulus (E') and loss factor (tan δ) of the LA-based polymers were obtained as a function of temperature.

RESULTS

Production of P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA) in recombinant *E. coli* The plasmids pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) were co-introduced into *E. coli* strains DH5α and LS5218, respectively, for metabolic control of the biosynthesis of P(LA-*co*-3HB-*co*-3HA) as illustrated in Fig. 1. The recombinant *E. coli* strains harboring both pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) accumulated P(LA-*co*-3HB-*co*-3HA), whereas the strains harboring pTV118NpctC1(STQK)ldhDABdP$_{Re}$ only accumulated P(LA-*co*-3HB) as reported previously (Table 2) (21). *E. coli* DH5α harboring pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) accumulated 17.0 wt% P(LA-*co*-3HB-*co*-3HA) containing a small amount of MCL-3HA unit of 3HD (C_{10}) (1.2 mol%). On the contrary, *E. coli* LS5218 harboring pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) accumulated 2.9 wt% P(LA-*co*-3HB-*co*-3HA) containing 5.9 mol% of MCL-3HA units of C_8, C_{10}, and C_{12}, resulting in higher MCL-3HA fraction in the polymer than that of the recombinant *E. coli* DH5α. LA-based polymers composed of such a monomer composition have not been reported so far.

Biosynthesis of P(LA-*co*-3HA) from glucose was attempted using the plasmid pTV118NpctC1(STQK)ldhD in which *phaAB* genes were removed from pTV118NpctC1(STQK)ldhDABdP$_{Re}$. However, *E. coli* LS5218 harboring pTV118NpctC1(STQK)ldhD and pRTcAA-GMCL(Pa) could not accumulate the polymer (data not shown). Accordingly, *E. coli* CAG18497, which is a *fadR*-strain unlike LS5218, was selected as a host for synthesis of P(LA-*co*-3HA). As a result, *E. coli* CAG18497 harboring pTV118NpctC1(STQK)ldhD and pRKmXS-GMCL(Pa) accumulated 2.9 wt% P(70.4% LA-*co*-29.6% 3HA) (Table 2). This is a novel LA-based polymer that has not been reported previously. In addition, the recombinant CAG18497 harboring pTV118NpctC1(STQK)ldhD and pRKmXS-GMCL(Pa) accumulated 1.5 ± 0.6 wt% P(74.0% LA-*co*-26.0% 3HA) consisting of 74.0 ± 9.0 mol% LA, 1.3 ± 2.2 mol% 3-hydroxyoctanoate (3HO, C_8) and 24.7 ± 7.3 mol% 3-hydroxydecanoate (3HD, C_{10}) (0.41 g/L dry cell weight) when the recombinant was relatively anaerobically cultivated in a 500-mL conical flask containing 300 mL of medium (data not shown).

NMR analyses of P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA) P(LA-*co*-3HB-*co*-3HA) synthesized in *E. coli* LS5218 harboring pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) was

subjected to 500 MHz ^{1}H-NMR and 125 MHz ^{13}C-NMR analyses. The mole fractions of LA, 3HB, and MCL-3HA units were determined from the intensity ratio of main-chain methylene proton resonance to methyl proton resonance in the ^{1}H-NMR spectra (data not shown). Consequently, the composition of the P(LA-*co*-3HB-*co*-3HA) synthesized by the recombinant LS5218 from glucose was 19.7 mol% LA, 74.9 mol% 3HB and 5.4 mol% 3HA. Fig. 2 shows ^{13}C-NMR spectrum of the P(LA-*co*-3HB-*co*-3HA), together with the chemical shift spectrum of carbonyl resonances. Compared to P(3HB-*co*-3HA) that was reported previously (7), the peaks at around 67.5 and 169 ppm were assigned to the dyad sequences of 3HB*-3HB and 3HB*-3HA + 3HA*-3HB. The other peaks (68–69 ppm and 169–170 ppm), which were also observed for P(LA-*co*-3HB) (16), presumably corresponded to the carbonyl group of 3HB with the adjacent LA unit. However, the resonance indicating the sequence of LA and 3HA could not be confirmed.

The sample of P(74.0% LA-*co*-26.0% 3HA) synthesized by the recombinant CAG18497 harboring pTV118NpctC1(STQK) ldhD and pRKmXS-GMCL(Pa), which was cultivated in a 500-mL conical flask containing 300 mL of medium, was subjected to NMR analyses. The results of ^{1}H-NMR analysis revealed the molar fractions of LA and 3HA units in the polymer to be 92.0 mol% and 8.0 mol%, respectively. These values were not consistent with those from GC analysis of methanolyzed PHA. The difference could be due to the production of MCL-3HA oligomers which could not precipitate with methanol. Fig. 3 shows the ^{13}C-NMR spectrum of P(LA-*co*-3HA) synthesized by the recombinant CAG18497, together with the peaks of C_3, C_8, C_{10}, and C_{12}, and the chemical shift spectrum of carbonyl resonances. The carbonyl carbon resonances (169–170 ppm) of P(LA-*co*-3HA) were resolved into peaks, arising from different sequences connecting LA and 3HA units: LA-3HA*-LA, LA-LA*-3HA, LA-LA*-LA and 3HA-LA*-LA. The peak at around 69.0 ppm, with the top split into two, was assigned to LA*-3HA. These indicate that P(LA-*co*-3HA) synthesized in this study is a copolymer with LA and MCL-3HA repeating units, and not a mixture of PLA and MCL-PHA.

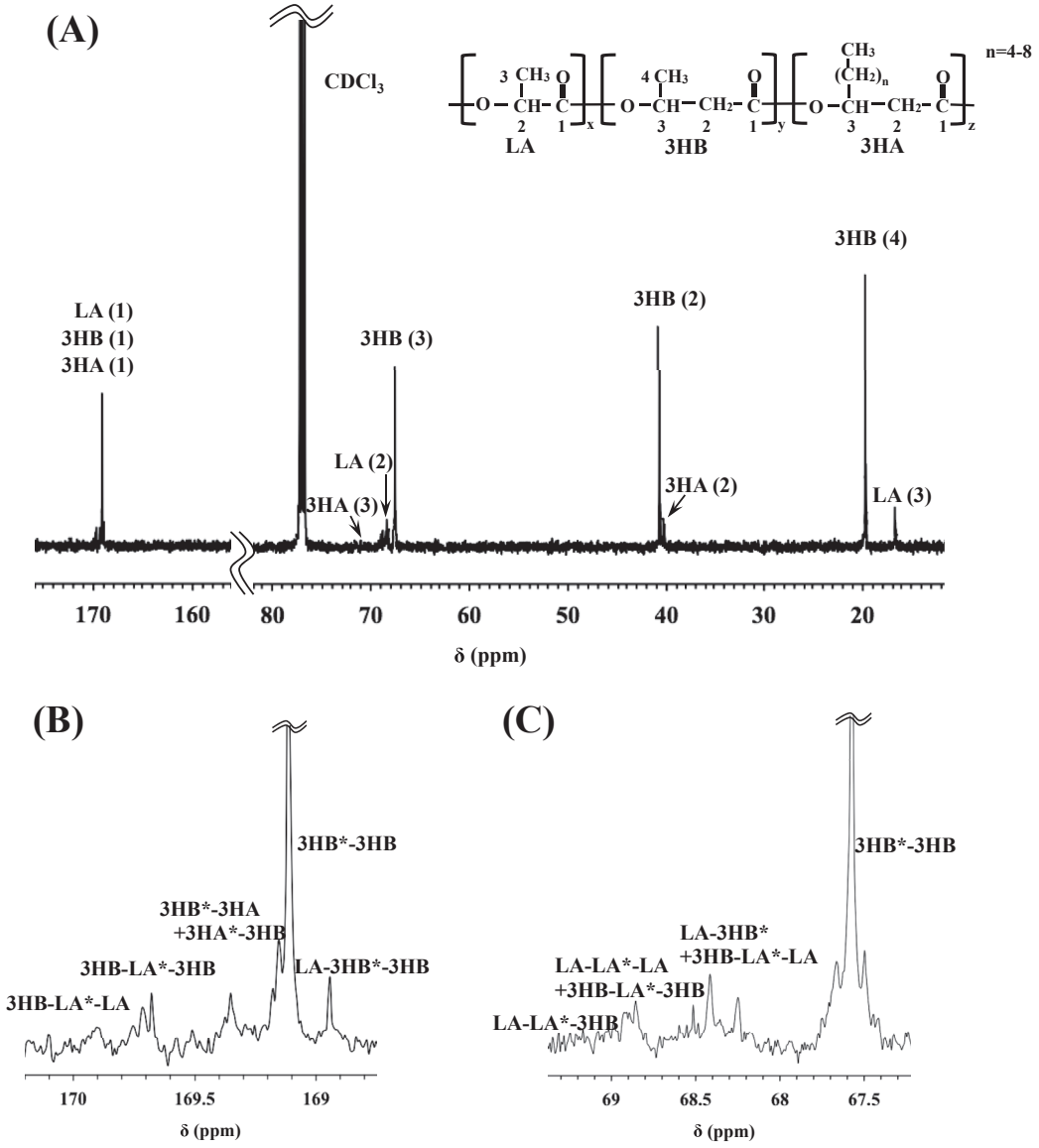

FIG. 2. ^{13}C-NMR spectrum (125 MHz) of the P(19.7% LA-*co*-3HB-*co*-5.4% 3HA) synthesized by the recombinant *E. coli* LS5218 strain harboring pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa). (A) Full ^{13}C-NMR spectrum containing all detected peaks. (B) Expanded 169–170 ppm part of full spectrum. (C) Expanded 67.5–69 ppm part of full spectrum. Carbon atoms in the copolymer are numbered and assigned to peaks in the spectrum. LA, D-lactate; 3HB, 3-hydroxybutyrate; 3HA, MCL-3-hydroxyalkanoate (C_8–C_{12}).

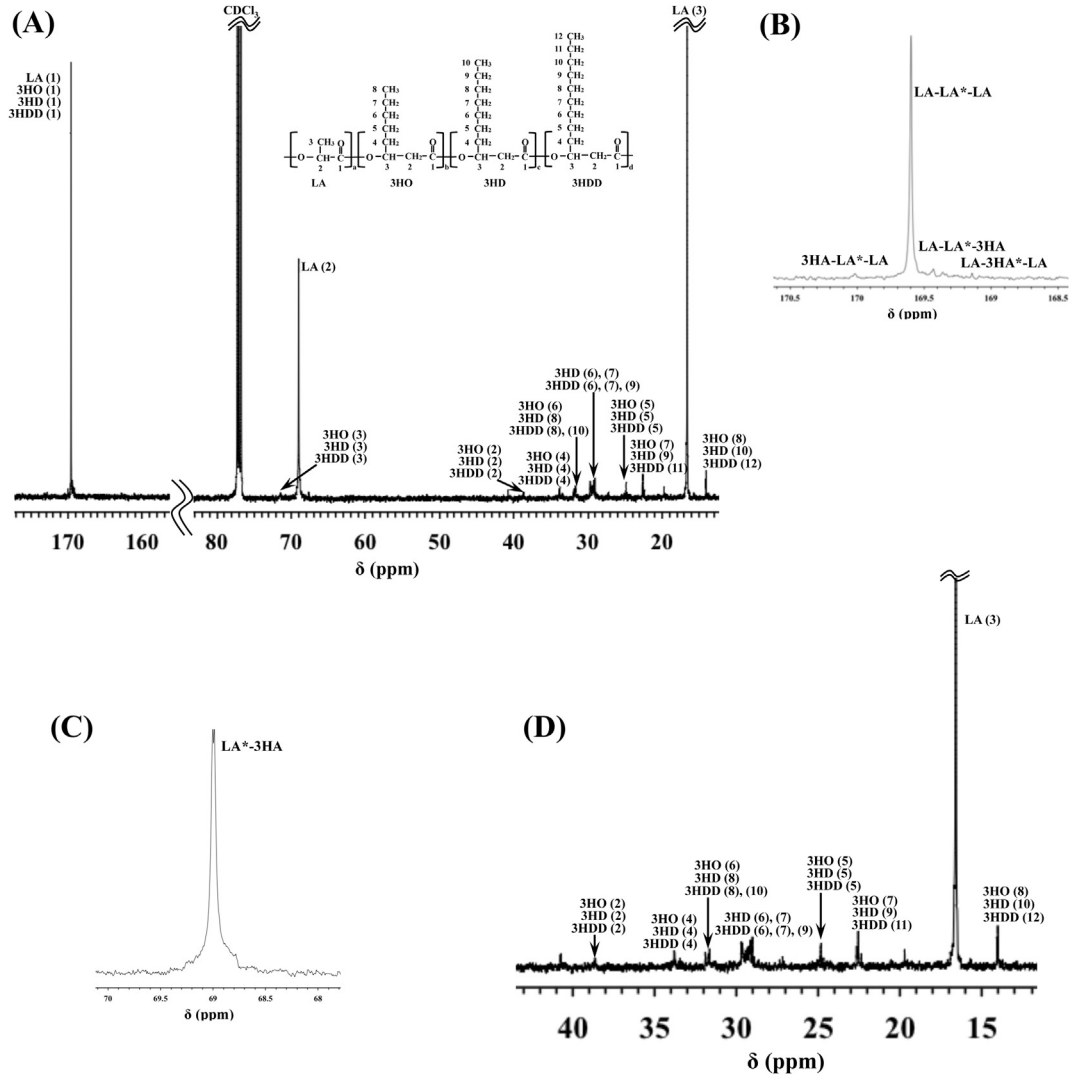

FIG. 3. ^{13}C-NMR spectrum (125 MHz) of the P(92% LA-*co*-8% 3HA) synthesized by the recombinant *E. coli* CAG18497 strain harboring pTV118NpctC1(STQK)ldhD and pRKmXS-GMCL(Pa). (A) Full ^{13}C-NMR spectrum containing all detected peaks. (B) Expanded 169.5–170.5 ppm part of full spectrum. (C) Expanded 68–70 ppm part of full spectrum. (D) Expanded 15–40 ppm part of full spectrum. Carbon atoms in the copolymer are numbered and assigned to peaks in the spectrum. LA, D-lactate; 3HB, 3-hydroxybutyrate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3HA, MCL-3-hydroxyalkanoate (C_8–C_{12}).

Molecular weight and thermal properties of LA-based copolymers Table 3 shows the molecular weights and the thermal properties of P(LA-*co*-3HB-*co*-3HA) and P(LA-*co*-3HA) obtained in this study (7,11,15,31,33). The number-average molecular weight (M_n) and the polydispersity (M_w/M_n) of P(19.7% LA-*co*-3HB-*co*-5.4% 3HA) synthesized by *E. coli* LS5218 harboring

TABLE 3. Molecular weight and thermal properties of polyester.

Sample	Molecular weight			Thermal properties		
	M_w ($\times 10^4$)	M_n ($\times 10^4$)	M_w/M_n	T_m (℃)	T_g (℃)	ΔH_m (J/g)
PLA (4042D, NatureWorks, Minnetonka, MN, USA)[a]	7.7			150	52	1.3
P(29% LA-*co*-3HB)[b]	9	4	2.2	141, 158	−8, 25	0.8, 3.8
P(19.7% LA-*co*-3HB-*co*-5.4% 3HA)[c]	31.7	5.3	5.9	124, 147	6.7	19.6
P(92.0% LA-*co*-8.0% 3HA)[c]	2.7	1.4	2.0	157	36	6.4
P(3HB-*co*-5.4% 3HA)[d]	46.6	23.3	2.0	161	4.6	34.5
P(3HB-*co*-6% 3HA)[e]	139	60.5	2.3	133, 146	−8	39
P(3HB)[f]	117	65.0	1.8	178	4	91

PLA, L/D ratios from 24:1 to 30:1; LA, D-lactic acid; 3HB, 3-hydroxybutyrate (C_4); 3HA, 3-hydroxyalkanoate (C_6–C_{12}); M_w, weight-average molecular weight; M_n, number-average molecular weight; T_m, melting temperature; T_g, grass-transition temperature; ΔH_m, enthalpy of fusion.

[a] Kamthai and Magaraphan (33).
[b] Yamada et al. (15).
[c] This study.
[d] Hokamura et al. (11).
[e] Matsusaki et al. (7).
[f] Abe et al. (31).

pTV118NpctC1(STQK)ldhDABdP$_{Re}$ and pRTcAA-GMCL(Pa) were 5.3×10^4 and 5.9, respectively. The M_n of the P(LA-*co*-3HB-*co*-3HV)s containing 8.7–27.4 mol% LA and 0.2–7.2 mol% 3HV were $2.5–4.2 \times 10^4$ when 10–100 mg/L sodium propionate was added (M_w/M_n: 2.8–4.8) (22). The M_n (5.3×10^4) of P(19.7% LA-*co*-3HB-*co*-5.4% 3HA) obtained in this study was almost the same as that of the P(LA-*co*-3HB-*co*-3HV)s reported by Shozui et al. (22).

The M_n and the M_w/M_n of the P(92.0% LA-*co*-8.0% 3HA) synthesized by *E. coli* CAG18497 harboring pTV118NpctC1(STQK)ldhD and pRKmXS-GMCL(Pa) were 1.4×10^4 and 2.0, respectively. Yamada et al. (15) have reported that the molecular weight ($7–9 \times 10^4$) of P(LA-*co*-3HB) decreased as LA fraction (29–47 mol%) of the polymer increased. Indeed, the M_n of P(92.0% LA-*co*-8.0% 3HA) with high LA fraction was lower than that of P(29% LA-*co*-3HB) (Table 3).

The melting temperatures (T_m) of P(LA-*co*-3HB-*co*-3HA) obtained in this study had two peaks (124°C and 147°C) by DSC thermograms, which were similar to those of P(29% LA-*co*-3HB) and P(3HB-*co*-6% 3HA) random copolymers (7,15). The glass transition temperature (T_g) and enthalpy of fusion (ΔH_m) of P(LA-*co*-3HB-*co*-3HA) were 6.7°C and 19.6 J/g, respectively, indicating in a higher T_g and lower ΔH_m than those of P(3HB-*co*-3HA) reported previously (7,11).

In contrast, the T_m of P(LA-*co*-3HA) obtained in this study was 157°C, which was almost the same as PLA (150°C). The T_g and the ΔH_m of P(LA-*co*-3HA) were 36°C and 6.4 J/g, respectively. The ΔH_m of P(LA-*co*-3HA) was even lower than P(LA-*co*-3HB-*co*-3HA), and the T_g was lower than PLA (52°C). In DMTA, tan δ peak temperature of P(LA-*co*-3HA), which is commonly known as the T_g, was approximately 58°C, which was almost the same as PLA (60°C) (33). The storage modulus (E') of P(LA-*co*-3HA) is approximately 2.3 GPa at room temperature. E' of PLA is generally 2.0–3.0 GPa at room temperature (33,34). Moreover, elastic modulus of P(LA-*co*-3HA) was maintained at approximately 2.0 GPa even at the temperatures above the glass transition point in the range untill 100°C.

P(92.0% LA-*co*-8.0% 3HA) film are shown in Fig. 4. The P(LA-*co*-3HA) film showed transparency similar to PLA.

DISCUSSION

LA-based polymers have been synthesized by bioprocess and exhibit transparency depending on their LA fraction (14,15,35). Attempts have been undertaken to biosynthesize novel LA-based polymers containing LA, 3HB, and a third monomeric unit such as 3HV and 3HHx to render new properties to materials (22,23). In this study, we attempted to biosynthesize novel LA-based polymers containing MCL-3HA units because P(94% 3HB-*co*-6% 3HA), consisting MCL-3HA units, has been demonstrated as a flexible material (7). In a previous study, P(3HB-*co*-3HA)s were accumulated in the *fadR*-deficient *E. coli* LS5218 strain from glucose as the sole carbon source via the fatty acid biosynthesis pathway by PhaG and (*R*)-3HA-CoA ligase (10,11). In addition, we have reported on various *E. coli* strains that synthesized P(LA-*co*-3HB)s with higher LA fraction through introduction of the exogenous *ldhD* gene (21). Therefore, *E. coli* LS5218 introducing *ldhD*, *phaG*, and (*R*)-3HA-CoA ligase (PA3924) genes together with the P(LA-*co*-3HB)-biosynthetic genes was used for biosynthesis of P(LA-*co*-3HB-*co*-3HA). As a result, the recombinant LS5218 produced P(LA-*co*-3HB-*co*-3HA) consisting of LA, 3HB, and MCL-3HA ($C_8–C_{12}$) units from glucose (Table 2). In particular, the MCL-3HA fraction in P(LA-*co*-3HB-*co*-3HA) was increased to 5.9 mol%, and LA fraction (14.4 mol%) was also higher than that of the recombinant DH5α. This suggested that *E. coli* LS5218 was preferred as a host for the synthesis of P(LA-*co*-3HB-*co*-3HA).

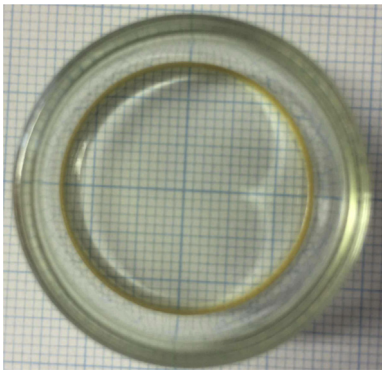

FIG. 4. Solvent-cast film of P(92.0% LA-*co*-8.0% 3HA).

Generally, the T_m, T_g, and ΔH_m of P(3HB-*co*-3HA) copolymers are known to decrease as 3HA fraction increases, compared to the P(3HB) homopolymer. Similarly, the T_m and ΔH_m of P(LA-*co*-3HB-*co*-3HA) were lower than those of P(3HB) (Table 3). Two T_m peaks of P(LA-*co*-3HB-*co*-3HA) were observed and ^{13}C-NMR analysis could not show the signal of the LA-3HA sequence in the copolymer, probably due to the low fractions of LA and 3HA. Although two T_m peaks are often observed in random copolymers (7,15), the possibility of a blend polymer of P(LA-*co*-3HB) and P(3HB-*co*-3HA) could not be excluded.

Thereafter, biosynthesis of P(LA-*co*-3HA) consisting of LA and MCL-3HA units was attempted. *E. coli* LS5218 strain harboring *pct*, *phaC1(STQK)*, *ldhD*, *phaG* and PA3924 genes accumulated no polymer. Whereas, *E. coli* CAG18497 harboring the same genes accumulated P(LA-*co*-3HA) consisting of LA and MCL-3HA ($C_8–C_{12}$) (Table 2). The strain LS5218, *fadR*601 and *atoC2*(Con) mutant, was developed by chemical mutagenesis treatment, whereas the strain CAG18497 (*fadR*13::Tn10) was developed by the site-specific insertion of Tn10 into *fadR* (24,25). Thus, the difference was expected to affect the expression levels of the genes involved in fatty acid metabolism system. Actually, the P(3HB) accumulation levels which were produced via enoyl-CoA hydratase-mediated P(3HB) synthesis from glucose by the recombinant strains of LS5218 and CAG18497, were different between two *fadR*-strains (36).

^{13}C-NMR analysis of P(LA-*co*-3HA) revealed the LA-3HA sequence, although the sequence was not detected in the analysis of P(LA-*co*-3HB-*co*-3HA). The molecular weight of P(92.0% LA-*co*-3HA) was lower than that of P(19.7% LA-*co*-3HB-*co*-5.4% 3HA) and P(29% LA-*co*-3HB). Incorporation of LA units into the polymer chain led to a reduction in molecular weight of the polymer as described previously (15,17,18). This phenomenon has been reported to explain that the synthesis of high molecular weight polymers is prevented due to low affinity to D-LA-CoA as a substrate for PhaC1(STQK) and low mobility of LA-based polymer (37). It has been concluded that molecular dynamics is a determining factor of the productivity and molecular weight of LA-based PHA.

The solvent cast film of P(92.0% LA-*co*-3HA) demonstrated transparency as expected. The T_m, T_g and ΔH_m of P(92.0% LA-*co*-3HA) were 157°C, 36°C and 6.4 J/g, respectively. The storage modulus of the P(LA-*co*-3HA) film was approximately 2.3 GPa at room temperature. Unfortunately, P(LA-*co*-3HA) did not have flexibility as expected. In other words, the properties of P(92.0% LA-*co*-3HA) were almost similar to those of PLA. Poor flexibility of the polymer would be due to the very high LA fraction in the polymer chain and its low molecular weight.

In conclusion, we succeeded in producing novel LA-based polymers with monomer compositions consisting of C_3 to C_{12} from glucose as the sole carbon source. Production of P(LA-*co*-3HB-*co*-3HA) was achieved by supplying the 3HA-CoA via the fatty acid biosynthesis pathway to the P(LA-*co*-3HB) synthesis pathway. Furthermore, P(LA-*co*-3HA) was synthesized by removing 3HB-CoA supply route from the P(LA-*co*-3HB-*co*-3HA) synthesis pathway.

The *fadR*-deficient *E. coli* strains were preferred as hosts for novel LA-based polymers containing MCL-3HA units, especially, *E. coli* CAG18497 was preferred as a host for P(LA-*co*-3HA). P(LA-*co*-3HA) with 92% LA fraction demonstrated transparency and other properties similar to PLA. We are now attempting to expand the range of monomer composition of P(LA-*co*-3HA) to render transparency and various properties to the materials.

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