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Microbial Platform for Tailor-made Production of a Biodegradable Polylactide Modifier: Ultrahigh-Molecular-Weight Lactate-Based Polyester LAHB

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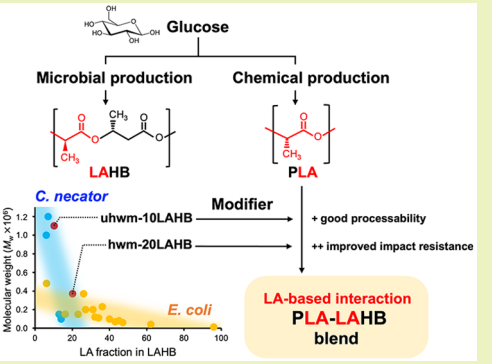
ABSTRACT: Polylactide (PLA) is a biobased synthetic polyester with diverse commercial applications. Nevertheless, PLA has been deemed unfavorable owing to its constrained processability, impact resistance, and biodegradability. Therefore, this study aimed at engineering novel biodegradable modifiers based on a highly chiral enantiopure D-lactate (D-LA)-based polyester called poly[D-LA-*co*-(*R*)-3-hydroxybutyrate (3HB)] (LAHB) to improve the physical properties of PLA. High-molecular-weight (hmw) LAHB was synthesized from the chemoautotrophic *Cupriavidus necator* in substantial quantities. The tailor-made overproduction of LAHB was achieved by using a minimal medium containing glucose and retaining the intrinsic synthetic pathway for the 3HB homopolymer in *C. necator*, which produced the highest yields, reaching up to 27 g/L/48 h. The molecular weight of LAHB substantially elevated up to 1.1 × 10^6 g/mol and was termed ultrahigh-molecular-weight (uhmw) LAHB. The LA faction in LAHB was modulated via a synergistic optimized combination of the lactate dehydrogenase and propionyl-coenzyme A transferase variants as well as by effective shut-off of the D-LA escape route. Combination of PLA and the two selected biodegradable uhmw-/hmw-LAHBs as demand-oriented biodegradable modifiers allowed improved processability and impact resistance of PLA while retaining transparency. These benefits of LAHB are distinguishable from those of conventional biobased modifiers, including 3HB-based polymers.



KEYWORDS: *Cuprividus necator, polylactic acid, polyester, polyhydroxyalkanoate, LAHB, PLA, engineering biology, synthetic biology*

INTRODUCTION

Current global concerns regarding greenhouse gas emissions and solid waste management have driven the search for sustainable replacements for petroleum-based plastics, such as biobased polylactide (PLA), which is a 100% renewable biosourced polyester that can replace fossil-fuel-based products in numerous applications. The mechanical performance of PLA is comparable to that of commodity thermoplastics, such as polystyrene and poly(ethylene terephthalate). PLA is biocompatible with biomedical applications. Nevertheless, despite this benefit, the commercial applicability of PLA is restrained owing to its brittleness, lack of elongation properties, and limited biodegradability.[1] Consequently, extensive efforts have been invested to enhance the mechanical properties of PLA by blending it with low-molecular-weight plasticizers.[2−6]

In contrast, polyhydroxyalkanoate (PHA) is a naturally occurring polyester that is biodegradable in various environments including the ocean. Over 160 members of the PHA family have been identified, including various combinations resulting from copolymerization.[7−9] The monomeric variation of PHA allows optimization of its physical properties through design of controlled random and sequence-regulated copolymers.[10,11] For example, poly[3-hydroxybutyrate (3HB)-*co*-3-hydroxyhexanoate (3HHx)] (PHBH) has been industrially produced and tailor-made with 6 mol % 3HHx for hard-type and 11 mol % 3HHx for soft-type PHBH.[12] The customizable composition of highly crystalline (3HB) and elastomeric (3HHx) units effectuates the acquisition of a broader processing window, enhanced thermal stability, and efficacious mechanical performance. Additionally, it offers excellent biodegradability and facilitates the efficient utilization of renewable feedstocks.[13,14] PHBH is commercially manufactured by KANEKA Co. Ltd. under the trade name KANEKA Biodegradable Polymer GreenPlanet, and is currently used for various applications, including the production of straws, shopping bags, cutlery, and single-use coffee capsules. The annual production capacity of GreenPlanet reached 20,000

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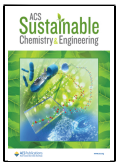

tons in 2024, and the demand is expected to grow further as an alternative to conventional fossil fuel-based commodity plastic.[12] The industrialization of KANEKA PHBH has inspired further investigation of PHA on a global scale.

In this study, we aimed to manipulate the copolymerization of the D-lactate (D-LA)-based polyester [poly(D-LA-*co*-3HB)], known as LAHB.[10,11,15] LAHB exhibits promising material properties compared with chemically synthesized PLA and can be distinguished from PHBH owing to differences in excellent transparency and thermal and mechanical properties. The D-LA unit of LAHB confers controllable flexibility and transparency, which is an advantageous polymeric property, compared with the rigidity of the PLA homopolymer.[16] Furthermore, incorporation of D-LA monomeric units into the 3HB polymeric backbone enhances the biodegradability of LAHB,[17] whereas PLA is only biodegradable under specific conditions. As the D-LA unit of LAHB is a key factor affecting PLA material properties and would serve as an interactive connecter with PLA when blending these two polymers, we focused on the D-LA unit of LAHB to create demand-oriented LAHB modifiers. This is a game-changing technology from studying LAHB itself to studying LAHB as a modifier. As this study required higher molecular weight synthesis and high yield LAHB production for in-depth investigation of the value-added properties of LAHB and its modifier, herein, we attempted to establish a microbial plastic factory for tailor-made LAHB production.

In 2008, we initiated a project for producing LAHB in an *Escherichia coli* platform using a novel LA-polymerizing enzyme (LPE) developed from class II PHA synthase derived from *Pseudomonas* sp. 61−3.[15] The first LPE had Ser325Thr and Gln481Lys substitutions and was designated as $PhaC1_{Ps}$(ST/QK). Although *E. coli* is often used as a standard platform to produce LAHBs with different LA fractions and numerous studies have successfully established LAHB tailor-production systems in *E. coli*,[18−26] we encountered limited production levels and molecular weight of LAHB in *E. coli*.

Herein, we propose strategic approaches to achieve polymeric, performance-oriented LAHB production using an engineered chemoautotrophic *Cupriavidus necator* platform based on the strategies employed in the GreenPlanet supply chain. We used the *C. necator* strain, which exhibits high intrinsic 3HB-CoA-supply capacity, for LAHB overproduction rather than the previously used mutant strain with reduced 3HB-CoA-supply ability to facilitate incorporation of the LA monomer unit into the 3HB polymeric backbone.[27−30] As for PHBH production, the remarkable intrinsic 3HB-supply ability of *C. necator* assumes a key strategy for the LAHB overproduction strategy. Notably, this strategy is very effective for overcoming the limitation in the production performance and molecular weight of LAHB performed by the previous study.[27−30] Consequently, we achieved the greatest production of LAHB (27 g/L/48 h) through enzymatic reinforcement of the LA-CoA-supply ability followed by overexpression of the LPE gene and shut-off of the D-LA escape route. As hypothesized, these strategies allowed effective synthesis of ultrahigh-molecular-weight (uhmw-) LAHB, thus providing promising biodegradable modifiers to improve PLA impact resistance and processability. Taken together with the superior ability of LAHB that serves as the PLA-biodegradable modifier,[31] LAHB would be positioned as an advantageous multiple-functioned PLA modifier over conventional biobased candidates.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Flask Culture Conditions. The bacterial strains used in this study are summarized in Table S1. All *E. coli* strains were cultured in a Luria−Bertani (LB) medium. *E. coli* strains JM109 and S17-1 were used for plasmid construction and as donors for conjugation with recipient cells of *C. necator*, respectively. For LAHB polymer production, *C. necator* strains were cultured in 500 mL shaking flasks containing 100 mL modified basal mineral (MBM) medium supplemented with 20 g/L fructose, D-LA, and glucose as carbon sources, and incubated at 30 °C for 72 h.[13] The MBM medium constituted 0.578% w/v $Na_2PO_4{\cdot}12H_2O$, 0.101% w/v KH_2PO_4, 0.437% w/v $(NH_4)_2SO_4$, 0.15% w/v $MgSO_4{\cdot}7H_2O$, and 0.75% v/v trace element solution (0.1 M HCl solution with 1.6% w/v $FeCl_2{\cdot}6H_2O$, 1% w/v $CaCl_2{\cdot}2H_2O$, 0.02% w/v $CoCl_2{\cdot}6H_2O$, 0.016% w/v $CuSO_4{\cdot}5H_2O$, and 0.012% w/v $NiCl_2{\cdot}6H_2O$).

Plasmid Construction for Gene Expression and Transformation of *C. necator*. The plasmids used to construct gene-modified *C. necator* strains are listed in Supplementary Table S2. LA-polymerizing enzyme (LPE),[15] D-lactate dehydrogenase (LDH), and propionyl-CoA transferase (PCT) genes were cloned for expression under the control of trc, lacUV5, REP, or lacN17 promoters followed by the potential ribosome binding site of phaC1.[13] The sequence of trc, lacUV5, REP, and lacN17 promoters is listed in Supporting Information, Figure S1. This fragment was digested with *Mun*I and *Spe*I and cloned into the same site of the pCUP3 expression vector. These plasmids were used to transform *C. necator* by electroporation, as previously described.[32] The DNA sequences of the LDHs and PCTs are listed in Figure S1. *C. necator* gene deletion and insertion were conducted by homologous recombination using pNS2X-sacB[32] derivatives listed in Supplementary Figure S2. Suicide vector pNS2X-sacB was introduced in *C. necator* by conjugation of S17-1. The selected LDH and PCT genes were introduced into the genome by replacing the *phaJ*4a (H16_A1070) and *phaJ*4b (H16_B0397) open reading frames.

Overproduction of LAHB Using Jar Fermentation. A fed-batch fermentation was performed in a 5 L jar fermenter (B.E. Marubishi, Co., Ltd.; Bioneer-Neo) containing 1.8 L of MBM medium. Glucose was used as the carbon source and NH_4OH was used for pH regulation. The initial glucose concentration was 20 g/L. Once glucose was consumed to 10 g/L, the glucose concentration was maintained at 10 g/L. Fermentation was maintained for 48 h at 30 °C, 500 rpm, 1.8 vvm, and pH 6.8.

Analysis of Dry Cell Weight and LAHB Production. Following fermentation, cells were collected by centrifugation, washed, and lyophilized, and the dry cell weight was determined. The dried cells were subsequently methanolized using 15% sulfuric acid in methanol, as previously described,[15] and the cellular contents and LAHB monomer composition were determined using gas chromatography. The culture supernatant was subjected to high-pressure liquid chromatography to evaluate the LA concentration.

RNA Sequencing Analysis. The strain S1 cells were cultured at 30 °C in 100 mL of nitrogen-limited mineral salt (MB) medium on a reciprocal shaker at 125 strokes/min. A filter-sterilized solution of sodium D-LA and fructose was added to the medium at a final concentration of 20 g/L. The cells in 100 mL culture broth were harvested by centrifugation (4,000 *g*, 10 min, 4 °C) at 22 and 32 h, and total RNA was isolated from the cell pellet using RNeasy Mini Kits (Qiagen, Hilden, Germany). Samples were subsequently purified by phenol-chloroform extraction and ethanol precipitation and dissolved in RNase-free water. The quality and yield of the extracted RNA were confirmed using a Bioanalyzer D1000 instrument (Agilent Technologies, Santa Clara, CA, USA). RNA-seq template libraries were constructed with approximately 10 μg of enriched mRNA using Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus 96 Samples (Illumina, San Diego, CA, USA) according to the manufacturer's instruction. Deep sequencing was performed using a NextSeq 500 sequencer (Illumina). The raw reads were mapped onto genome sequences of *C. necator* H16 (accession No. GCA_000009285). Reads per kilobase of exon per million (RPKM)

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values for each coding DNA sequence was calculated as a quantitative gene expression index. Data were generated from three independent experiments. All data are provided in the Supplementary additional file (PDF).

Gel Permeation Chromatography. The molecular weights of LAHB were determined by using gel permeation chromatography (GPC; EcoSEC Elite; HLC-8420GPC; Tosoh; Japan) with two TSKgel Super HM-H and two Super H-RC columns connected in series. Chloroform was used as the solvent (flow rate; 1 mL/min). Polystyrene standards were used for calibration.

Monomer Sequence Analysis Using ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H NMR analyses of the copolymers were performed by using a 600 MHz NMR spectrometer (Avance III, Bruker) with deuterated chloroform as the solvent. Measurements were conducted at room temperature with an accumulation of eight scans. Tetramethylsilane was used as an internal standard at 0 ppm. The typical ^{1}H NMR spectrum of an LAHB copolymer is shown in Figure S3, indicating assignments of each proton in the chemical structure. The monomer ratio of LA and 3HB were determined from the area ratio of methine protons (H_b and H_c). The LA monomer content (C_{LA}) was calculated as follows:

$$C_{LA}(\text{mol}\%) = \frac{\text{area}_{H_b}}{\text{area}_{H_b} + \text{area}_{H_c}} \times 100$$

The calculated C_{LA} values are summarized in Table 2.

Monomer sequence distributions were further characterized by analyzing the triad ratios. LAHB copolymers can contain three types of 3HB-centered triads, including 3HB-3HB-3HB, LA-3HB-3HB (or 3HB-3HB-LA), and LA-3HB-LA. In the methyl-proton region of 3HB (H_d), a doublet peak at 1.25 and 1.29 ppm can be assigned to 3HB-3HB-3HB, while the peaks between 1.29 and 1.39 ppm are assigned to LA-3HB-3HB, 3HB-3HB-LA, and LA-3HB-LA. The 3HB-3HB-3HB triad content was calculated as follows:

$$C_{\text{obs}(3\text{HB}-3\text{HB}-3\text{HB})}(\%) = \frac{\text{area}_{1.25-1.29\text{ppm}}}{\text{area}_{1.25-1.39\text{ppm}}} \times 100$$

Supposing the monomer sequence is completely random, the probability of the 3HB-3HB-3HB triad may be calculated as follows:

$$C_{\text{random}(3\text{HB}-3\text{HB}-3\text{HB})}(\%) = C_{3\text{HB}}{}^2 = (1 - C_{\text{LA}})^2$$

The observed and calculated values of the 3HB-3HB-3HB triad content (Table 2) correspond well with each other, indicating that the monomer sequences of the obtained LAHB copolymers are highly random.

Biochemical Oxygen Demand (BOD) Measurement. The biodegradability of LAHBs in ocean water was evaluated by using BOD measurements. All tests were performed under aerobic conditions in a temperature-controlled BOD reactor (Oxi-Top IDS, WTW GmbH, Weilheim, Germany) at 30 °C with stirring. Powdered samples (average particle diameter, 500 μm; weight, 25 mg) of uhmw-10LAHB and hmw-20LAHB were incubated in 250 mL of ocean water in a 500 mL BOD reactor. Ocean water was collected from Takasago port (Hyogo, Japan) and used as the inoculate by adding 0.5 g/L NH_4Cl and 0.1 g/L K_2HPO_4 as specified in ASTM D-7081. Polylactide (PLA 2003D) and cellulose (Supelco, cellulose microcrystalline, Merck, USA) were used as references and incubated under the same conditions in accordance with ASTM D-6691. The internal pressure of the vessel containing ocean water and polymer was monitored, and the oxygen consumption by microorganisms during LAHB biodegradation was calculated. The BOD biodegradability of the polymer sample was calculated by subtracting the BOD of the control blank (BODb) from the BOD of the test sample (BODt) and dividing the value (BODt − BODb) by the theoretical oxygen demand (ThOD) of the test sample, as previously described.[33] Two LAHB samples, uhmw-10LAHB and hmw-20LAHB, were subjected to a BOD test in three independent experiments.

Compounding PLA and LAHB. We used PLA (PLA 2003D, D-lactate content 4.3%[34]) manufactured by NatureWorks LLC (Nebraska, USA). The average molecular weight was determined as 1.8×10^5 g/mol using GPC. PLA and LAHB were combined in a plastic container with manual shaking to obtain a homogeneous mixture. The mixture was dried in an oven at 50 °C for 12 h and extruded using a corotating twin screw extruder TECHNOVEL ULT nano 05 (Techinovel Co., Ltd., Osaka, Japan) with a screw with a 1.5 cm diameter and a 13.33 L/D ratio. Screw rotation speed was set at 80 rpm. Barrel and die temperatures were set at 190 °C. The strand extruded from the nozzle (φ = 2.5 mm) was cooled under atmospheric conditions, and a pelletizer was used to obtain PLA-LAHB blend pellets.

Sheet Sagging Test. The sheet sagging test was conducted to measure the melt strength to simulate processability.[35] The PLA-LAHB blend pellets were heat-pressed using a SICK C4000 hydraulic press (Shinto Metal Industries, Ltd., Osaka, Japan) with 1.0 MPa at 180 °C for 10 min to obtain a 0.4 mm thick sheet. Square 20 × 20 cm sheets were placed horizontally on 20 × 20 cm square metal frames with a width of 1 cm, held in position by a second metal frame and placed in an oven at 140 °C. Sagging length was recorded every 30 s.

Impact Test. The PLA-LAHB pellets were heat-pressed using a hydraulic vacuum press (MOTO IMC-11FD (Imoto machinery Co., Ltd. Kyoto, Japan) with 1.4 MPa at 170 °C to obtain 4 mm-thick sheets. The sheets were cut into 80 × 10 mm samples and subjected to an un-notched Charpy impact test at 23 °C using an impact tester equipped with a 7.5 J pendulum hammer, in accordance with JIS K7110. Test results were calculated as the average of five tested samples.

Elongational Viscosity. The uniaxial elongational viscosity was measured using a rotational rheometer equipped with a universal extensional fixture (Anton Paar MCR102, CTD450 TD ready, UXF12/CTD) at 150 °C. The strain rate was established at 0.1 s^{-1}. Rectangular specimens (10 mm wide, 20 mm long, and 0.3 mm thick) for measurement were portioned from the sheets that had been used for the sagging test.

RESULTS

Synthetic Pathway for Incorporating an LA Monomer into the 3HB Polymeric Backbone in *C. necator*. One-pot fermentative LAHB production can be used with microorganisms other than *E. coli*. Wild-type *C. necator* does not produce LA, and strain KNK-005 dZ126, which does not express the three genes encoding PHB depolymerases (PhaZ1, PhaZ2, PhaZ6), was previously used for industrial production of high-molecular-weight (hmw) PHBH.[36] We therefore chose LDH and PCT as beneficial LA-CoA monomer-supplying enzymes. The results are summarized in Figure 1. First, exogenous LDH was evaluated by analyzing the LA fraction of the LAHB produced by strain S1 harboring genes encoding PCT and LPE, using fructose as the sole carbon source. We focused on three candidate genes that encode LDH, including LDH_{Lm} derived from *Leuconostoc mesenteroides*, LDH_{Ec} derived from *E. coli*, and LDH_{Ldb} derived from *Lactobacillus delbruekii* subsp. *bulgaricus*. Introduction of the pCUP3 vector[32] expressing the three LDH candidate genes into strain S1 identified LDH_{Lm} as the best enzyme for enhancing the incorporation of LA into LAHB (Figure 1b). However, the incorporation of the LA-monomer into the 3HB-based polymeric backbone was challenging because of the high supply of 3HB-CoA. We therefore explored PCT variants to regulate the 3HB-CoA-supply ability and identified three strains with different 3HB-CoA-supply abilities, defined as S0dAB (weak), S0dA (middle), and S0 (strong) (Figure 1c). Genome-mining was used to identify new beneficial PCTs in *C. necator* by providing D-LA as the sole carbon source. In addition, the recombinant strain S0dAB (weak) with a weakened 3HB-CoA-supply ability was used to develop

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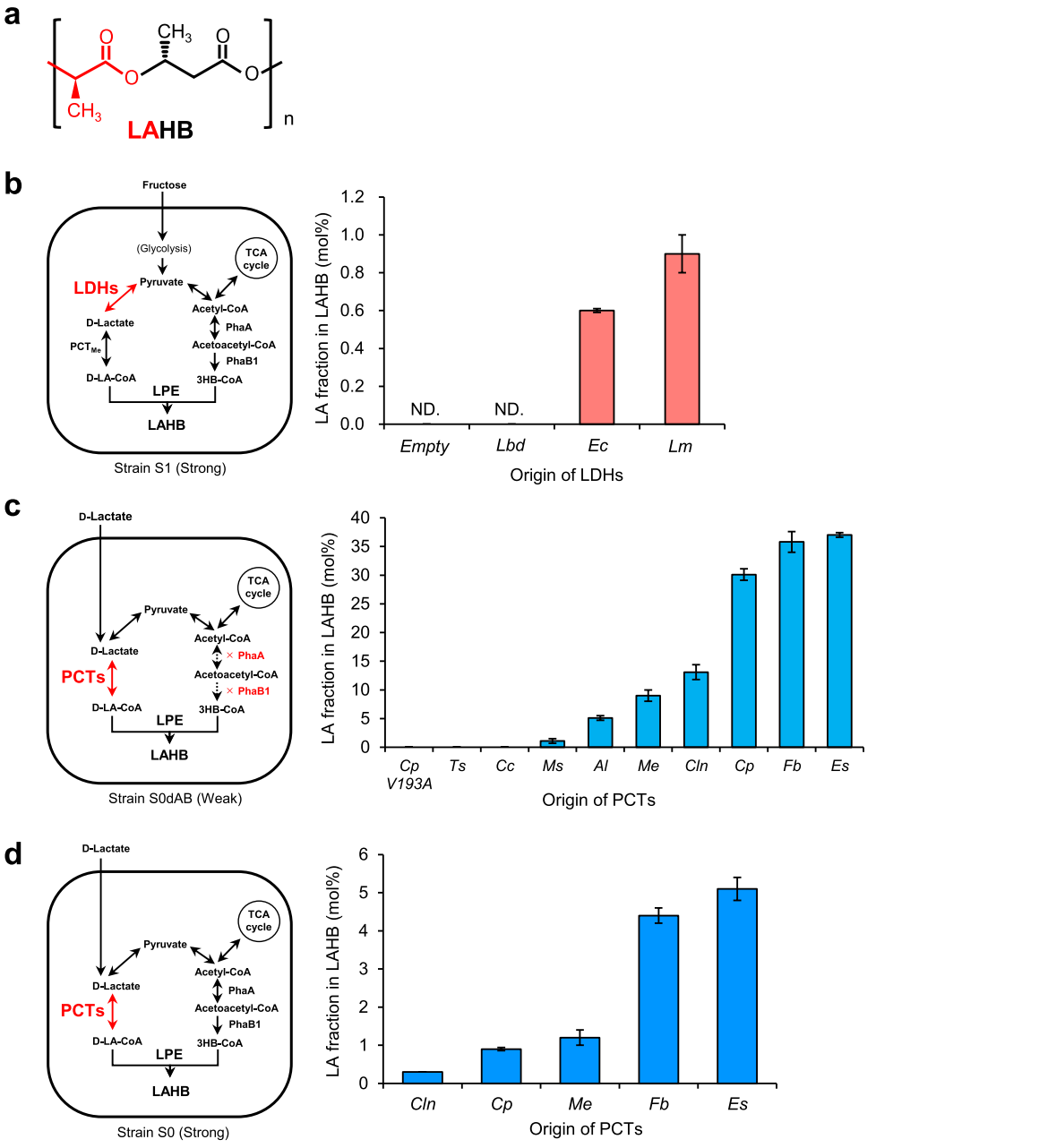

Figure 1. Screening of lactate dehydrogenases (LDHs) and propionyl-CoA transferases (PCTs) for lactate (LA)-enriched copolymer synthesis in *C. necator* by controlling the intrinsic capacity for 3HB-CoA monomer supply. (a) Chemical structure of poly[D-LA-*co*-(*R*)-3-hydroxybutyrate (3HB)] (LAHB). (b) Best LDH_{Lm} was selected based on its ability to incorporate the LA unit into LAHB in the recombinant *C. necator* that carries the genes encoding PCT_{Cp} and LPE, $PhaC1_{Ps}$(ST/QK). (c) Best PCT_{Es} was selected by investigating the LA fraction of LAHB produced by strain S0dAB (weak) cultivated in a minimal salt medium containing 20 g/L of D-LA. (d) Best PCT_{Es} was selected by investigating the LA fraction of LAHB produced by strain S0 (strong) as well as S0dAB (weak) (Table S1).

PCT-positive candidates that were more efficient than the original strain S0 with strong 3HB-CoA-supply ability. The capacity to incorporate LA was evaluated by introducing the pCUP3 vector expressing seven PCT candidates, including PCT_{Me}, PCT_{Cp}, and its mutant (PCT_{Cp} (V193A)) previously used in *E. coli* (Figure 1c).[15,37] The results indicated the successful acquisition of seven PCTs, and the top five candidates were subjected again to strain S0. Comparison of the LA fraction in LAHB indicated that PCT_{Es} derived from *Epulopiscium* sp. is the most effective for enhancing LA incorporation (Figure 1d). Based on these results, we developed the LAHB-producing strain S3 using genes encoding LDH_{Lm} and PCT_{Es} that conferred the greatest LA fraction in LAHB in *C. necator* (Table S1).

Tailor-made Production of LAHBs with Varying LA Fractions by Controlling the 3HB-CoA Supply Pathway. To change the carbon source to glucose, which can be obtained from various renewable feedstocks, we needed to modify *C. necator* H16 as this strain cannot intrinsically assimilate glucose. We modified strain S3 by introducing a point mutation in the *N*-acetylglucosamine transporter NagE and deleting the gene encoding the regulator NagR (Figure 2a),[38] and the resultant strain was named GS3 (strong). With glucose as the carbon source, strain GS3 produced 1.4 mol %

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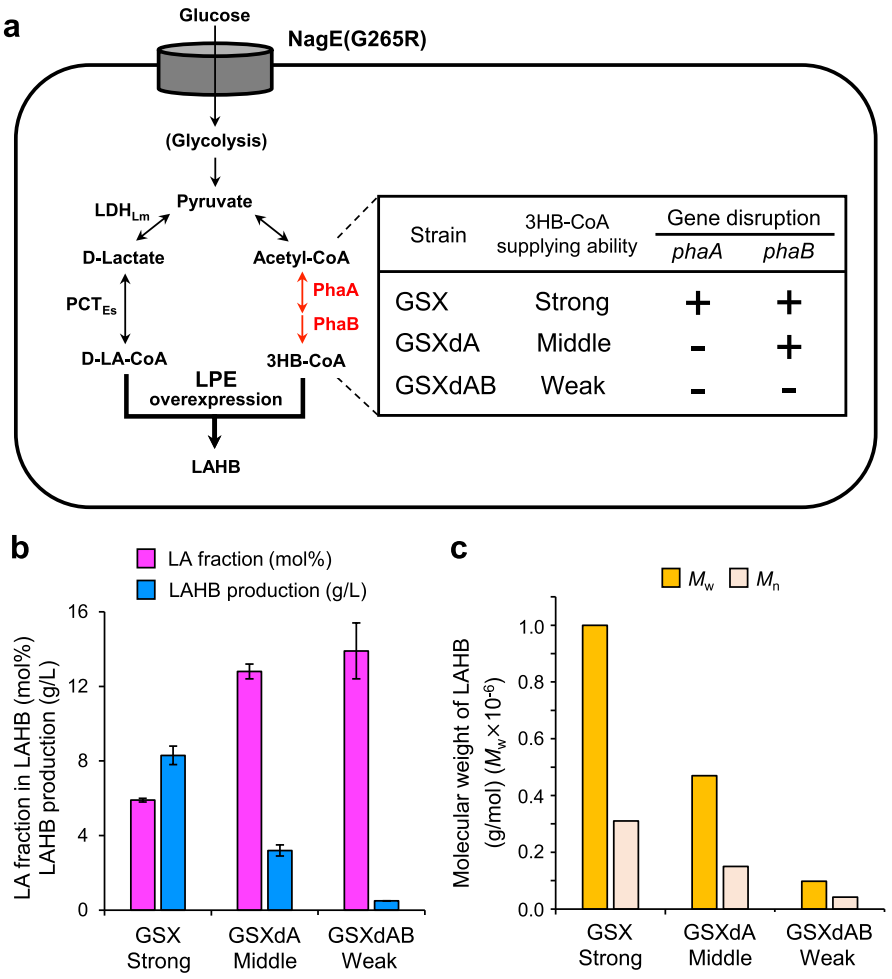

Figure 2. Overproduction of LA-enriched LAHB by controlling the intrinsic 3HB-CoA supply ability. (a) LAHB production was manipulated by modifying the intrinsic 3HB-CoA supply pathway catalyzed by PhaA and PhaB1 in *C. necator*. The + or - indicates the presence or disruption of the target gene. (b) LAHB production profiles of strains GSX (strong), GSXdA (middle), and GSXdAB (weak) after 72 h of cultivation indicating the amount of LAHB produced and the LA mol %. Data are presented as the mean ± standard deviation of three independent experiments. (c) Weight-average molecular weight (M_w) and number-average molecular weight (M_n) of LAHB produced by strains GSX (strong), GSXdA (middle), and GSXdAB (weak). The 3HB-CoA supply ability (strong, middle, weak) was modulated by combinated disruption of phaA and/or phaB1.

LA-containing copolymer with a cellular content of 75 wt % (productivity 4.7 g/L). In addition, overexpression of the LPE gene under the control of the strong promoter lacUV5, the resultant strain was named GSX (strong), increased the production of LA-enriched LAHB (up to 5.9 mol %) and enhanced LAHB content (up to 88 wt %), as well as LAHB production (up to 8.3 g/L, 1.8-fold) (Figure 2b). The average molecular weight (M_w) of the extracted LAHB from GSX (strong) reached 1.0 × 10^6 g/mol, which is the highest reported value (Table 1). These results indicate that LPE overexpression improves all of the performance parameters of LAHB production.

To further enhance the LA fraction in LAHB, the metabolic flux of the 3HB-supply pathway was intentionally weakened by disrupting the 3HB-CoA supply pathway genes (*phaA* and *phaB1*; strain GSXdAB) as previously reported.[27−30] It should be noted that the three strains with different 3HB-CoA-supply abilities, GSXdAB (weak), GSXdA (middle), and GSX (strong), should exhibit varying LA-incorporation capacities. Accordingly, strain GSXdAB (weak) produced LA-enriched LAHB (up to 13.9 mol %), although the cellular content and LAHB production level were drastically decreased (23 wt % and 0.5 g/L, respectively) (Figure 2b), which corresponds to previous reports.[27−30] Previous studies also reported such an inverse relationship between the LA fraction and M_w of LAHB in *E. coli*,[9] and the disruption of the *phaB1* may negatively impact PHBH production in *C. necator*.[39] Our results indicated that the M_w of LAHB produced by strain GSXdAB (weak) decreased to one-tenth (9.8 × 10^4 g/mol) of that of GSX (strong) (Figure 2c), which suggests that the intrinsic 3HB-supply pathway is required for high yield and high M_w of the polymer. Strain GSXdA (middle) successfully produced LA-enriched LAHB (12.8 mol %) while retaining cellular content and LAHB production level (65 wt % and 3.2 g/L, respectively), as well as high M_w (4.7 × 10^5 g/mol) (Figure 2b,c).

Overproduction of uhmw-LAHB by Disrupting the D-LA Escape Route. The low LA fraction of LAHB implied the presence of an D-LA escape route that limits the conversion of D-LA to D-LA-CoA in strain S1. We therefore assessed the D-LA escape route using RNA sequencing (RNA-seq) to analyze the expression levels of the transcriptome related to the glycolysis pathway in *C. necator* when D-LA is provided as the sole carbon source (Figure S4a). We identified a gene cluster of glycolate dehydrogenases (GDHs; *glcD1*, *glcE*, and *glcF*) paralog to D-LDH (*dld*) that converts D-LA to pyruvate

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Table 1. Summary of Yield and Molecular Weight of LAHB Produced by Engineered *C. necator* Strains[c]

C. necator strain	3HB-CoA supplying ability[a]	Gene disruption ± (presence/disruption)		LAHB production				Average molecular weight[b]			ref.
		phaA	*phaB1*	Dry-cell weight (g/L)	Cellular contents (wt %)	LAHB (g/L)	LA fraction (mol %)	M_w	M_n	M_w/M_n	
GSXdAB	weak	−	−	2.2 ± 0.1	23 ± 1	0.5 ± 0.0	13.9 ± 1.5	9.8×10^4	4.2×10^4	2.3	this study
GSXdA	middle	−	+	4.9 ± 0.3	65 ± 3	3.2 ± 0.3	12.8 ± 0.4	4.7×10^5	1.5×10^5	3.1	this study
GSX	strong	+	+	9.5 ± 0.3	88 ± 2	8.3 ± 0.5	5.9 ± 0.1	1.0×10^6	3.1×10^5	3.2	this study
GS3d147	strong	+	+	4.3 ± 0.2	69 ± 2	3.0 ± 0.2	6.9 ± 0.2	1.2×10^6	5.0×10^5	2.4	this study
NCIMB11599	weak	−	−				4.5	2×10^4	1×10^4		27
437−540/pKM212-EcLDHsacC	weak	−	−	1.53	35.8	0.55	7.3				29
437−540/pKM212-EcLDHsacC	weak	−	−	0.37	19.5	0.07	21.5	4×10^4	2×10^4		28
437−540/pKM212-EcLDH	weak	−	−	0.42	33.9	0.14	37				30

[a]The 3HB-CoA supply ability was modulated by combinated disruption of *phaA* and/or *phaB1*. [b]Molecular weight was determined by gel permeation chromatography (GPC). [c]Yield and molecular weight of LAHB were compared with those of a previous study.[27−30]

Table 2. Primary Structures and Properties of Two Selected LAHBs Overproduced by Jar Fermentation[e]

Polymer	Jar fermenter-based LAHB production				Monomer sequence[a]		Molecular weight[b]		DSC[c]	Tensile test[d]
	Strain	Dry-cell weight (g/L)	Cellular contents (wt %)	LAHB (g/L)	LA fraction (mol %)	$C_{obs(3HB\text{-}3HB\text{-}3HB)}$ ($C_{random(3HB\text{-}3HB\text{-}3HB)}$)	M_w	M_w/M_n	T_g (°C)	Tensile modulus (GPa)
uhmw-10LAHB	*C. necator* GS3d147	45	60	27	10.2	80.9 (80.6)	1.1×10^6	2.2	4.6	1.7 ± 0.1
hmw-20LAHB	*C. necator* GSXdA	47	50	24	20.1	64.3 (63.8)	3.7×10^5	3.6	10.2	0.6 ± 0.0
PLA	-	-	-	-	100	N.A.	1.8×10^5	1.7	60.0	3.3 ± 0.2

[a]LA fraction and LA-monomer randomness were analyzed using NMR. Raw values are presented in the upper row and the theoretical values for truly random copolymers are presented in parentheses. [b]Molecular weight was determined using gel permeation chromatography (GPC). [c]Glass-transition temperature was determined using temperature-modulated differential scanning calorimetry (DSC). [d]Tensile modules were measured using a universal test machine attached with a 5 kN load cell at 22 ± 1 °C. Dumbbell-shaped tensile test specimens were prepared by die-cutting from the films. Five specimens were tested for each composition. [e]The production performance, primary structures, and thermal and physical properties of the two new LAHBs with increased molecular weights are compared with those of commercially available PLA.

(Figure S4b). These results suggested that the genes encoding D-LDH and GDH are related to D-LA escape routes in *C. necator*. Based on these results, we disrupted the gene cluster including *dld*, *glcD1*, *glcE*, and *glcF* (single-gene disruption mutants: GS3d1, GS3d4, GS3d6, and GS3d7) related to the LA escape route in strain GS3 (strong), which increased the LA fraction in LAHB to 1.4−5.3 mol % (Figure 3a,b). The molar fraction of LA in the produced polymer can therefore be modulated by controlling intracellular D-LA. In addition, a wide-range LA fraction LAHB series was successfully produced through various gene disruptions, including double mutants GS3d14, GS3d16, and GS3d17; triple mutants GS3d146 and GS3d147; and quadruple mutant GS3d1467 (Figure 3b). As shown in Figure 3b, triple mutant GS3d147 (strong) exhibited the greatest LA fraction (up to 6.9 mol %) with the highest cellular content (up to 69 wt %) as well as LAHB production (up to 3.0 g/L/72h).

Furthermore, we achieved the highest reported M_w of LAHB (1.2×10^6 g/L),[40] termed uhmw-LAHB (Table 1). This may be attributed to the absence of LA in strains GS3d17 and GS3d147, which produced LA-enriched LAHB in high quantities (Figure S5). These results suggest that effective PCT_{Es} facilitates reuptake of D-LA, leading to the efficient conversion of D-LA to D-LA-CoA and subsequent incorporation of the LA unit into the polymeric backbone.

Jar Fermentation Overproduction of LAHBs Using Engineered *C. necator* Strains. Table 2 summarizes the results of jar fermentation of LAHB production from the low-cost medium containing glucose. Strains GS3d147 (strong) and GSXdA (middle) were selected for overproduction of uhmw-LAHB and LA-enriched LAHB, respectively. Jar fermentation of strain GS3d147 achieved the highest LAHB productivity (27 g/L/48 h; cellular content 60 wt %; 10.2 mol % LA fraction). Furthermore, strain GS3d147 (strong) reproducibly produced uhmw (M_w 1.1×10^6 g/mol) LAHB, termed uhmw-10LAHB. Strain GSXdA (middle) yielded LA-enriched LAHB with the highest LA fraction (20.1 mol %) with a high-molecular-weight (hmw) (M_w 3.7×10^5 g/mol), termed hmw-20LAHB. Nuclear magnetic resonance (NMR) analysis was used to investigate the monomer sequence

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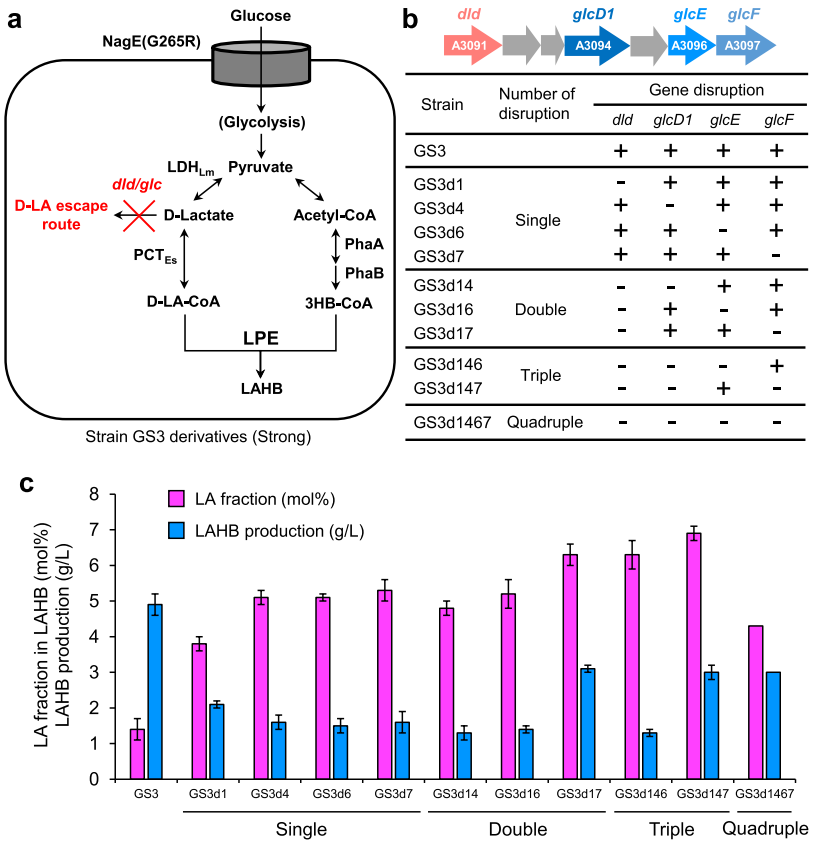

Strain	Number of disruption	Gene disruption			
		dld	*glcD1*	*glcE*	*glcF*
GS3		+	+	+	+
GS3d1	Single	-	+	+	+
GS3d4		+	-	+	+
GS3d6		+	+	-	+
GS3d7		+	+	+	-
GS3d14	Double	-	-	+	+
GS3d16		-	+	-	+
GS3d17		-	+	+	-
GS3d146	Triple	-	-	-	+
GS3d147		-	-	+	-
GS3d1467	Quadruple	-	-	-	-

Figure 3. Multiple gene disruption within the *dld*/*glc* gene cluster was required for effective LAHB production. (a,b) Metabolic designs of GS3-derived strains. The red arrow indicates the target for confirming a possible D-LA escape route. The + or − indicate the presence or disruption of the target gene. (c) Production of LAHB (blue) and its LA fraction (pink) by strains harboring various gene disruptions cultured in a minimal salt medium containing 20 g/L glucose for 72 h.

distributions of uhmw-10LAHB and hmw-20LAHB by analyzing the triad ratios and comparing these to the theoretical triad ratios for truly random copolymers (Table 2). The 3HB methyl proton region of the 3HB-3HB-3HB triad was assigned to 1.25−1.29 ppm based on our previous study.[15] The observed 3HB triad ratios ($C_{obs(3HB\text{-}3HB\text{-}3HB)}$) deviated from the theoretical values for truly random copolymers ($C_{random(3HB\text{-}3HB\text{-}3HB)}$) by less than 0.5%, indicating that the microbially produced uhmw-10LAHB and hmw-20LAHB have highly random monomer sequences. The uhmw-10LAHB and hmw-20LAHB were therefore chosen as suitable demand-oriented modifiers.

Biodegradability of hmw-20LAHB and uhmw-10LAHB Overproduced by Engineered *C. necator*. The biodegradability of hmw-20LAHB and uhmw-10LAHB was evaluated based on BOD measurements using ocean water samples from Takasago, Japan (Figure 4). Both uhmw-10LAHB and hmw-20LAHB exhibited biodegradation during the 7 day test period at 30 °C, suggesting that LAHB may be biodegradable in the natural ocean environment.

Acquired Impact Resistance of PLA by Blending with LAHB as a Modifier. The applicability of PLA is limited owing to its inherent brittleness. Multiple studies have been conducted to improve the impact resistance of PLA through polymer blending using biodegradable elastomers and polymers.[2] However, the poor compatibility between biodegradable polymers and PLA warranted additional efforts, such as use of compatibilizer, chemical modification, or reactive processing.[3−6,41] LAHB copolymers have low T_g (Table 2) and the moduli of LAHB are much lower than that of PLA. Additionally, the existence of the LA comonomer in LAHB is expected to facilitate compatibility with PLA *via* LA-based interaction. Thus, LAHB as soft additive was expected to improve PLA brittleness without the need for additional components by a simple melt extrusion process. We blended PLA with different ratios of the higher LA-content hmw-20LAHB ranging from 0.5 to 10 wt % and conducted unnotched Charpy impact strength tests (Figure 5a,b). A maximum value of 24.5 kJ/m^2 was achieved at 3 wt % hmw-20LAHB, which is 1.5 times higher than that of unmodified PLA (Figure 5c). The fracture surface of the LAHB-modified PLA specimen after the impact test showed obvious whitening in a wide area (Figure 5d), which is characteristic of toughened materials.[42,43] In contrast, the fracture surface of PLA was smooth, which is typical for a brittle material. The morphology of the polymer blend was evaluated by SEM observation of the fractured surfaces. Fine spherical structures were found only in the PLA-LAHB blends (Figure 5e), which can be attributed to the dispersed phase of LAHB. The number of spherical structures measuring less than 1 μm, which were hypothesized to contribute to the enhancement of impact strength, was maximized with the blend containing 3 wt % of hmw-20LAHB. Although the spherical dispersed phases were also observed in the blends at the higher LAHB concentrations (6 and 10 wt %), the average size of spherical structures became larger, and coalescence occasionally occurred between them. These morphological modifications reportedly effectuated the maximum improvement in impact strength at 3 wt % of LAHB.

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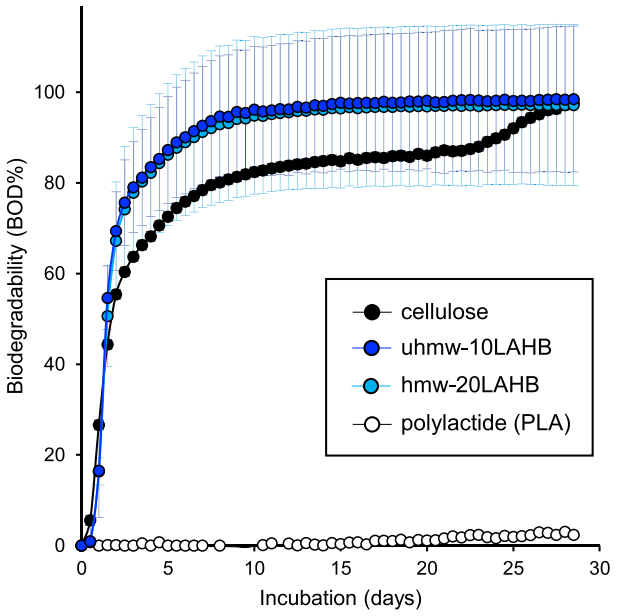

Figure 4. Ocean biodegradability of uhmw-10LAHB and hmw-20LAHB produced by engineered strains of *C. necator*. The curves illustrate biodegradation of uhmw-10LAHB (blue circles) and hmw-20LAHB (cyan circles) as well as that of cellulose (black circles) as a positive control and polylactide (PLA; white circles) as a negative control. Biochemical oxygen demand (BOD) measurements were conducted at 30 °C using ocean water samples. The error bars are presented as the standard deviations of three independent experiments.

Impact of LAHB on PLA Processability. In addition to poor biodegradability and brittleness, PLA has limited processability. Industrial manufacturing and polymer processing, such as vacuum molding, inflation molding, and fiber spinning, require high melt tension, which can be maintained by using a high M_w polymer.[37] The M_w of commercially available PLA is in the order of 10^5, and synthesizing PLA with higher M_w is difficult.[35] The melt tension of PLA is therefore not satisfactory for application of the abovementioned processing methods.[44−47] Our uhmw-10LAHB (Table 2) with a M_w of greater than 1.0×10^6 was therefore expected to improve the melt tension of PLA. Combining 15 wt % uhmw-10LAHB with PLA yielded highly transparent PLA-LAHB-blend sheets (Figure 6a). Sagging tests,[48] which simulate vacuum molding, indicated that sagging of the PLA-LAHB-blend sheets was delayed by more than 40% compared with the pure PLA sheet (Figure 6b,c). The elongational viscosities of the PLA-LAHB 15 wt % blend were higher than those of PLA (Figure 6d). Especially in the large elongational strain region, the viscosity was greater by more than five times the original value. These results indicate that LAHB is an effective melt tension modifier for PLA.

DISCUSSION

Most studies of LAHB investigated the properties of LAHB alone.[18−26] This study aimed to develop an LAHB-based modifier that can improve the material properties, including processability and impact resistance, of PLA. In-depth investigation and development of LAHB-based modifiers require overproduction of hmw-LAHBs. Specifically, the synthesis of uhmw-LAHB was expected to realize an improved PLA processability. We used systematic synthetic biology to

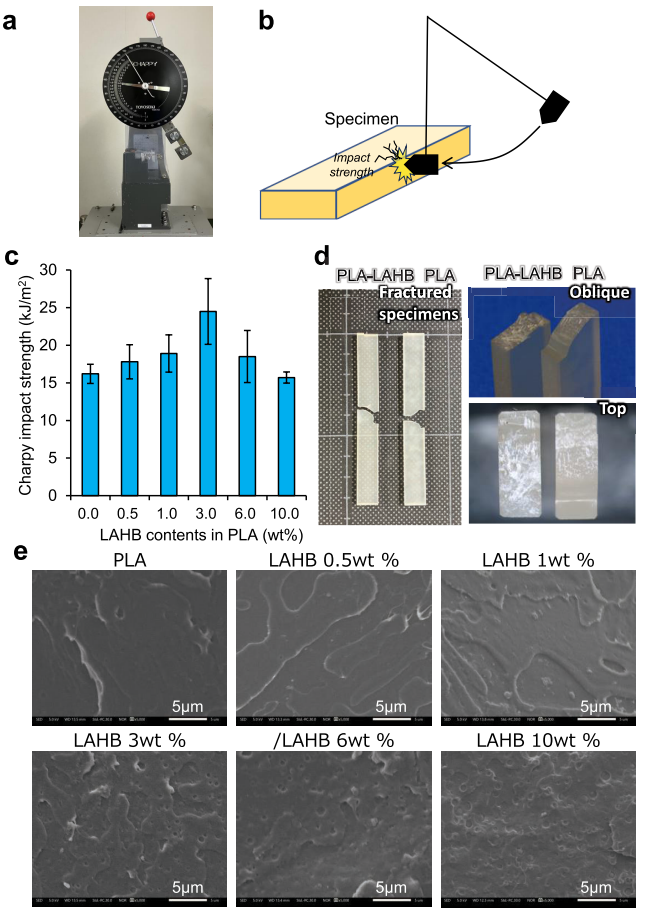

Figure 5. Blending effect of LAHB on the impact resistance of PLA. (a) Image of the Charpy impact tester. (b) Schematic drawing of a specimen hit by the pendulum hammer to measure the Charpy impact energy. (c) Charpy impact strength of the PLA-LAHB polymer blends with varied LAHB ratios ranging from 0.5 to 10 wt %. (d) Photos of the specimens after impact. (e) Scanning electron microscopy images of the fracture surfaces of the specimens.

successfully establish a microbial plastic factory for tailor-made production of uhmw-LAHB by utilizing the 3HB-CoA-supply ability of *C. necator* strain GS3d147 (strong). Equivalent production could not be achieved using *E. coli*. Furthermore, improved impact resistance requires enriched LA units for LA-based interaction with PLA, and strain GSXdA (middle) produced hmw-20LAHB with an effectively enriched LA fraction (Graphical abstract).

Our production strategy using engineered *C. necator* strains GSXdA (middle) and GS3d147 (strong) allowed tailor-made overproduction of LAHB-based modifiers hmw-20LAHB and uhmw-10LAHB that improve the material properties of PLA. The synergistic effects of the newly selected LDH_{Lm} and PCT_{Es} enhanced incorporation of LA units into LAHB. Furthermore, RNA-seq identified a glycolate-degradation related pathway and shut-off of this D-LA escape route through triple gene-deletion of *dld*, *glcD1*, and *glcF* drastically increased both the yield and LA fraction of LAHB. We therefore successfully converted *C. necator* from a non-LA producer to an LA producer. *C. necator* strain GS3d147 (strong) produced uhmw-10LAHB with a M_w higher than that of LAHBs produced by *C. necator* strains with a reduced 3HB-CoA-supply ability. The strong 3HB-CoA supply pathway intrinsic to *C. necator* is therefore a crucial driving force for higher productivity, as well

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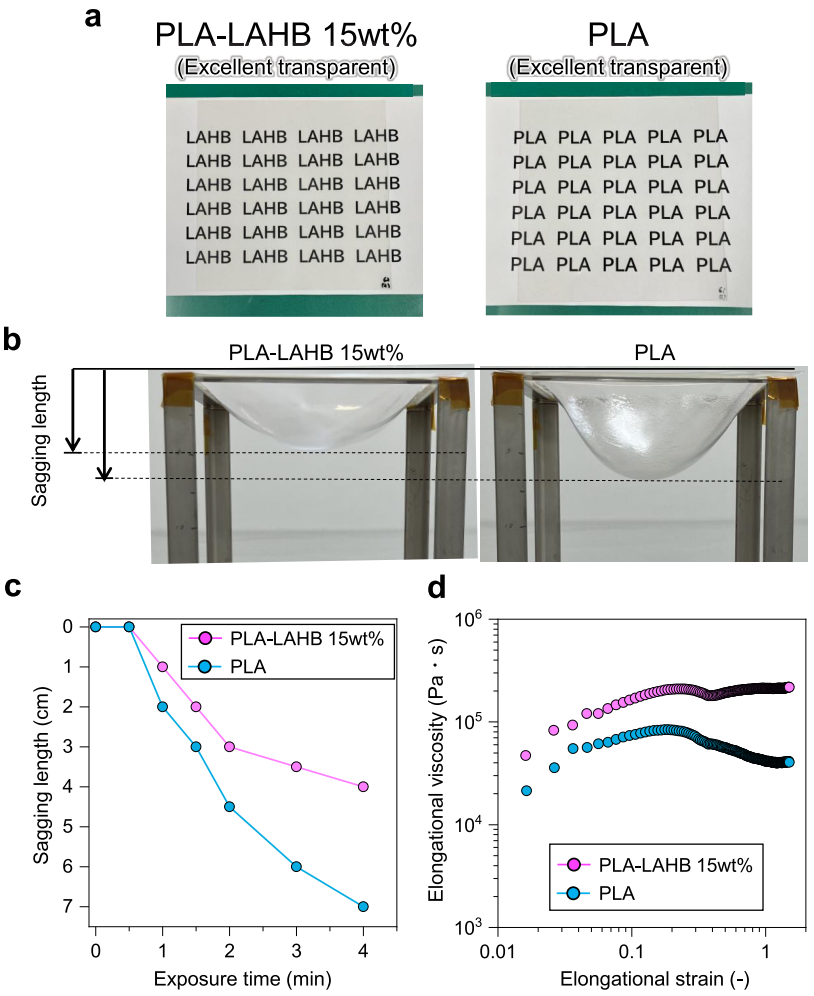

Figure 6. Blending effect of LAHB on PLA processability. (a) Photos of PLA-LAHB 15 wt % and PLA sheets indicating excellent transparency. (b) Photos of PLA and PLA-LAHB 15 wt % sheets after the sagging test. (c) Plot of sagging length versus exposure time at 140 °C. (d) Elongational viscosities of PLA and PLA-LAHB 15 wt %.

as higher M_w of LAHB. However, the class II PHA synthase-derived enzyme, LPE, tends to synthesize lower M_w PHAs,[35,44] suggesting that the M_w of biosynthesized PHA is determined not only by PHA synthase activity but also by monomer-supply ability. The engineered strains of *C. necator* may therefore serve as versatile platforms for the production of various LAHBs for use as biobased modifiers capable of improving the properties of PLA. This demonstrates the potential to contribute to the growth of the global market for biobased PLA. Recent studies have also been conducted on polymer blends of PLA with biobased polymers, including PHB, PHBH, and PBAT, used for plasticizers and compatibilizers.[5,6] Notably, in contrast to these conventional biobased polymeric modifiers, LAHB is highly dispersed into PLA with a domain size on the order of less than 1 μm estimated in SEM observation (Figure 5e), which is also consistent with the high transparency of the polymer blends (Figure 6a). This could be achieved via LA-based interaction between LAHB and PLA. The aforementioned benefits of LAHB are distinguishable from those of conventional biobased modifiers.

LAHB itself exhibits excellent biodegradability in various environmental samples, including soil and rivers.[10,49,50] Using BOD analyses, we found that both uhmw- and hmw-LAHBs were fully biodegraded in ocean water samples, even at extremely low microorganism concentrations, suggesting that these LAHB-based modifiers can potentially confer biodegradability to PLA. As additional promising evidence, we recently discovered that LAHB can facilitate the biodegradability of PLA by blending.[31]

CONCLUSIONS

Biotechnology strives to create value-added products and develop efficient production processes, and simultaneous improvements are required to realize a circular bioeconomy. The present study successfully achieved tailor-made microbial bioproduction of uhmw-LAHB utilizing renewable biomass resources and established its efficiency as a biodegradable LAHB-based modifier for PLA. The findings demonstrated the potential of tailor-made LAHBs to serve as demand-oriented modifiers for improving the properties of PLA while retaining transparency. Our findings provide the foundation for sequential design of optimized, targeted LAHBs for PLA modification. Our sustainable bioengineering approach for improving the properties of PLA may therefore contribute to the reduction of CO_2 emission.

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■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c07662.

Table S1: List of strains used in this study; Table S2: List of plasmids used in this study; Figure S1: DNA sequences of LDHs and PCTs; Figure S2: DNA sequences of gene deletion vector pNS2X-sacB derivatives; Figure S3: ^{1}H NMR spectra of a uhmw-10LAHB and b hmw-20LAHB; Figure S4: Gene identification of the D-lactate escape route based on transcriptome analysis; Figure S5: Extracellular pH and lactate and acetate concentrations in the culture medium of the strain GS3-derivatives in Figure 5; Additional file: Comparative RNA-seq analysis of *C. necator* S1 (PDF)

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Author Contributions

[▽]S.K. and S.F. contributed equally. Se.T. designed and supervised the study; S.K., S.F., Y.I., T.K., Y.T., and Sh.T. conducted the experiments; S.K., S.F., Y.I., T.K., Y.T., Sh.T., S.S., and Se.T. analyzed the results; Se.T., S.K., S.F., T.K., and Y.I. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BOD, biochemical oxygen demand; DSC, differential scanning calorimetry; GPC, gel permeation chromatography; LA, lactate; LAHB, P(D-lactate-*co*-(*R*)-3-hydroxybutyrate); LPE, lactate-polymerizing enzyme; MBM, modified basal mineral; NMR, nuclear magnetic resonance; PCT, propioyl-CoA transferase; PHBH, P[(3-(*R*)-hydroxybutyrate (3HB)-*co*-(*R*)-3-hydroxyhexanoate (3HHx))]; ThOD, theoretical oxygen demand

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