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Versatile aliphatic polyester biosynthesis system for producing random and block copolymers composed of 2-, 3-, 4-, 5-, and 6-hydroxyalkanoates using the sequence-regulating polyhydroxyalkanoate synthase PhaC_{AR}

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Abstract

Background: Polyhydroxyalkanoates (PHAs) are microbial polyesters synthesized by PHA synthases. Naturally occurring PHA copolymers possess a random monomer sequence. The development of PhaC_{AR}, a unique sequence-regulating PHA synthase, has enabled the spontaneous biosynthesis of PHA block copolymers. PhaC_{AR} synthesizes both a block copolymer poly(2-hydroxybutyrate)-*b*-poly(3-hydroxybutyrate) [P(2HB)-*b*-P(3HB)], and a random copolymer, poly(3HB-*co*-3-hydroxyhexanoate), indicating that the combination of monomers determines the monomer sequence. Therefore, in this study, we explored the substrate scope of PhaC_{AR} and the monomer sequences of the resulting copolymers to identify the determinants of the monomer sequence. PhaC_{AR} is a class I PHA synthase that is thought to incorporate long-main-chain hydroxyalkanoates (LMC HAs, > C₃ in the main [backbone] chain). Thus, the LMC monomers, 4-hydroxy-2-methylbutyrate (4H2MB), 5-hydroxyvalerate (5HV), and 6-hydroxyhexanoate (6HHx), as well as 2HB, 3HB, and 3-hydroxypropionate (3HP) were tested.

Results: Recombinant *Escherichia coli* harboring PhaC_{AR}, CoA transferase and CoA ligase genes was used for PHA production. The medium contained the monomer precursors, 2HB, 3HB, 3HP, 4H2MB, 5HV, and 6HHx, either individually or in combination. As a result, homopolymers were obtained only for 3HB and 3HP. Moreover, 3HB and 3HP were randomly copolymerized by PhaC_{AR}. 3HB-based binary copolymers P(3HB-*co*-LMC HA)s containing up to 2.9 mol% 4H2MB, 4.8 mol% 5HV, or 1.8 mol% 6HHx were produced. Differential scanning calorimetry analysis of the copolymers indicated that P(3HB-*co*-LMC HA)s had a random sequence. In contrast, combining 3HP and 2HB induced the synthesis of P(3HP)-*b*-P(2HB). Similarly, P(2HB) segment-containing block copolymers P(3HB-*co*-LMC HA)-*b*-P(2HB)s

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were synthesized. Binary copolymers of LMC HAs and 2HB were not obtained, indicating that the 3HB or 3HP unit is essential to the polymer synthesis.

Conclusion: PhaC_{AR} possesses a wide substrate scope towards 2-, 3-, 4-, 5-, and 6-hydroxyalkanoates. 3HB or 3HP units are essential for polymer synthesis using PhaC_{AR}. The presence of a 2HB monomer is key to synthesizing block copolymers, such as P(3HP)-*b*-P(2HB) and P(3HB-*co*-LMC HA)-*b*-P(2HB)s. The copolymers that did not contain 2HB units had a random sequence. This study's results provide insights into the mechanism of sequence regulation by PhaC_{AR} and pave the way for designing PHA block copolymers.

Keywords: PHA synthase, Block copolymer, 2-Hydroxybutyrate, 4-Hydroxy-2-methylbutanoate, 5-Hydroxypentanoate, δ -Valerolactone, ϵ -Caprolactone, Sequence regulation, Biodegradable plastic

Background

Aliphatic polyesters comprising hydroxycarboxylates, such as microbial polyhydroxyalkanoate (PHA), chemically synthesized polylactic acid (PLA), and petrol-based polycaprolactone (PCL), are attracting considerable research interest due to their applicative physical properties and degradability in natural environments [1, 2], compost or both [3, 4]. PCL and PLA are typically synthesized via ring-opening polymerization (ROP) of corresponding lactones using inorganic, organic, or enzymatic catalysts [5–7]. In contrast, PHAs are produced in microbial cells from hydroxycyl-coenzyme A (HA-CoA)s via successive transesterification by PHA synthase activity [8]. PHA biosynthesis systems have several advantages over ROP. PHAs are produced via a one-step fermentation process from different feedstock [9, 10]. PHA synthases possess strict enantio-specificity and synthesize isotactic polymers [11]. For certain types of polymers, PHA synthases synthesize polymers with a relatively high molecular weight [12] compared to those synthesized by ROP. Thus, PHA synthase plays a critical role in PHA biosynthesis. In addition, PHAs are well biodegraded in various environments, partly because natural polymers induce the expression of degrading enzymes by microorganisms [13].

Naturally occurring PHAs comprise (*R*)-3-hydroxyalkanoates (3HAs) with 4–12 carbons [11]. Among these, poly(3-hydroxybutyrate) [P(3HB)] is the most abundant PHA in nature and is produced by many microbes. Although the brittleness of P(3HB) has limited its practical use, recent studies have developed several applications by blending P(3HB) with other polymers, such as soy protein fibers [14], chitosans [15], and PLAs [16]. Another effective strategy to reduce brittleness is random copolymerization, which induces increased ductility of the polymer. A random copolymer, P(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx) or PHBHx], has been extensively studied [17] and commercially manufactured and implemented as a commodity plastic [18]. Natural PHA copolymers possess a random

sequence [19] presumably because the monomer supply and polymerization proceed simultaneously.

The limitation of the structural variety of PHA has been recognized as a drawback in practical applications of the material. Therefore, exploring PHA synthases with a wide substrate scope—in other words, low substrate specificity—has been an important research target [20, 21]. In addition, enzyme engineering is an effective approach for further expanding its substrate scope [22]. In particular, the discovery of 2-hydroxyalkanoate (2HA)-incorporating PHA synthase significantly expanded the structural variety of PHAs. A pairwise mutant of class II PHA synthase from *Pseudomonas* sp. 61–3 (PhaC1_{ps}STQK) was the first enzyme that incorporated 2HAs into the polymer [23] (for details of PHA synthase classification, see [8, 24, 25]). PhaC1_{ps}STQK incorporates lactate (LA, 2-hydroxypropionate) [23], glycolate (GL, 2-hydroxyacetate) [26], 2-hydroxybutyrate (2HB) [27, 28], and amino acid-derived 2HAs [29]. A random copolymer of LA and medium-chain-length (MCL) 3HA-CoAs (C_{6–12}) was also synthesized using the same enzyme [30]. Strict enantio-specificity of PHA synthase has enabled the synthesis of highly isotactic polymers from inexpensive racemic precursors, which is an advantage over ROP. This has also enabled the characterization of the mechanical properties of P[(*R*)-2HB] [27].

The monomer sequence of copolymer is an effective factor that influences the physical properties of polymer materials. Block copolymers exhibit useful and characteristic properties, and many studies on chemical block copolymer synthesis exist [31]. Block copolymers comprising immiscible segments spontaneously form phase-separated structure on the nanoscale, known as microphase separation, that is the principle of their physical properties. On the other hand, random copolymerization is effective to reduce the crystallinity of the polymers, which typically softens the materials. As mentioned above, PHA copolymers generally possess a random sequence. Therefore, several attempts have been made to synthesize PHA block copolymers by manipulating monomer supplies during cultivation [32]. However,

the difference in time required to synthesize one polymer chain and cultivation complicates this strategy [33].

We previously reported that PhaC_{AR}—a chimeric class I PHA synthase comprising PhaCs from *Aeromonas caviae* and *Ralstonia eutropha* (formally *Cupriavidus necator*) [34]—has a unique sequence-regulating capacity and spontaneously synthesizes block copolymers from a mixture of substrates [33]. In addition, PhaC_{AR} can efficiently incorporate 2HB and GL units into polymers. Using this enzyme, PHA block copolymers, P(2HB)-*b*-(3HB) [33], P(GL-*ran*-3HB)-*b*-P(3HB) [35], and P(GL-*co*-3HB-*co*-3HHx)-*b*-P(3HB-*co*-3HHx) [36] were synthesized. P(2HB)-*b*-(3HB) exhibits elastomer-like properties that characterize block copolymers [37]. These results also demonstrate that PhaC_{AR} synthesizes block and random copolymers, depending on the monomer combination. Nevertheless, the regularity of monomer sequence formation by PhaC_{AR}, i.e., which monomers trigger random or block sequences, remains unsolved.

As aforementioned, PhaC_{AR} is a class I 2HA-incorporating PHA synthase. Class I PHA synthases can recognize unusual long-main-chain hydroxyalkanoates (LMC HAs, $\geq C_4$ in the main-chain) monomers, such as 4-hydroxybutyrate (4HB) and 5-hydroxyvalerate (5HV). 4HB-containing PHAs have been extensively studied and used in biomedical applications (for details, see review [38]). The inefficiency of P(4HB) synthesis by ROP makes the biosynthetic process attractive [39]. P[3HB-*co*-3-hydroxypropionate (3HP)-*co*-5HV] was produced using *R. eutropha*, which possesses the class I PHA synthase PhaC_{Re} [40]. Little is known about 6HHx-containing PHAs. P(3HB-*co*-4HB-*co*-6HHx) was synthesized by *Methylocystis parvus*, which has uncharacterized PHA synthase [41]. The 3HP and 4HB units in these polymers were generated via β -oxidation of 5HV and 6HHx, respectively. Meanwhile, a binary copolymer, P(3HB-*co*-6HHx), was chemically synthesized by ROP of (*R*)- β -butyrolactone (92% ee) and ϵ -caprolactone [42]. The copolymer had a lower glass transition temperature (T_g), which is approaching -67 °C depending on its 6HHx fraction, than homopolymeric P(3HB) (4 °C). Similarly, P(3HB-*co*-5HV) was synthesized by ROP of (*R*)- β -butyrolactone and δ -valerolactone [43].

3HP possesses the same main-chain structure as 3HB and shares structural characteristics with the LMC monomers regarding the terminal hydroxy group. 3HP units are incorporated by some class I enzymes [44]. P(3HP) and its copolymers have not been found in nature, but they can be synthesized by natural class I PHA synthases. Glycerol [45], β -alanine [46], and exogenously supplemented 3HP are used as precursors of 3HP units. The use of nontoxic starting substances is a benefit of P(3HP) biosynthesis compared to ROP of β -propiolactone [47]

with a carcinogenic effect [48]. P(3HP) has high tensile strength and stretchable properties, and it is degraded by PHA depolymerase activity [44, 49].

These studies on class I PHA synthases combined with our previous results of PhaC_{AR} suggest that PhaC_{AR} potentially possesses a wide substrate scope toward 3HB and 2HB as well as 3HP and LMC monomers. In addition, the monomer sequence of the obtained polymers is imperative. Here we attempted to synthesize homopolymers and copolymers containing 3HB, 3HP, 2HB, and LMC monomers and analyzed their monomer sequences. 4-Hydroxy-2-methylbutyrate (4H2MB) was used as a 4HB analogue. The experiments also aimed at identifying the determinants of the monomer sequence of copolymers synthesized using PhaC_{AR}. Consequently, we demonstrated that PhaC_{AR} provides a versatile system for producing various random and block aliphatic copolyesters.

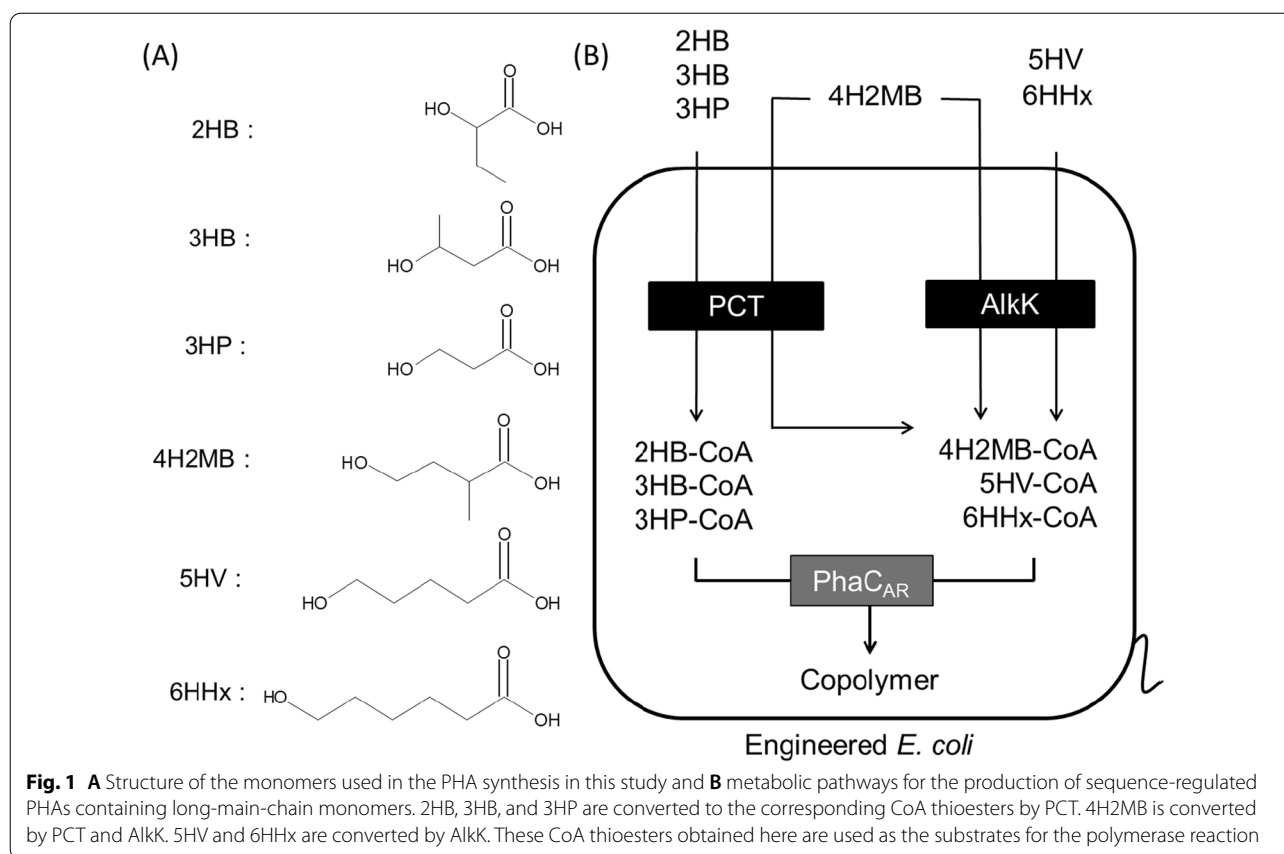
Results

Preparation of LMC HA monomer precursors

Monomer precursors, 4H2MB, 5HV, and 6HHx (Fig. 1A), were prepared by hydrolyzing their corresponding lactones. To avoid unintended ROP, the lactones were hydrolyzed in a mild condition as described in the Methods section. After hydrolysis, the reaction mixtures contained no polymerized products based on diffusion ordered spectroscopy (DOSY) nuclear magnetic resonance (NMR) analysis (Additional file 1: Fig. S1A–C).

3HB-based binary copolymer synthesis

The synthesis of 3HB-based binary copolymers containing 3HP, 4H2MB, 5HV, and 6HHx as secondary monomer units was attempted. It is known that propionyl-CoA transferase (PCT) from *Methanospaera elsdenii* can convert short-chain-length (SCL, $\leq C_5$) 2HAs and 3HAs including 2HB and 3HB to 2HA/3HA-CoAs using acetyl-CoA as the CoA donor [27]. CoA ligase AlkK catalyzes the condensation of MCL 3HA and CoA using ATP [50] [51]. In this study, these enzymes were used to supply 3HP-CoA and LMC HA-CoAs from the corresponding precursors. The polymer production in the presence (Entry 5–8) and absence (Entry 1–4) of AlkK was compared to estimate the substrate specificity of AlkK toward these substrates. Consequently, the copolymer production was observed under all conditions (Table 1, Entry 1–8). The 4H2MB fraction with AlkK exceeded that without AlkK; 5HV and 6HHx units were incorporated with AlkK but not with PCT alone. These results indicate that AlkK is effective for supplying the LMC monomers, and PCT has no activity toward 5HV and 6HHx. In addition, PhaC_{AR} was found to polymerize 3HP-CoA and



LMC-HA-CoAs as substrates. Figure 1B shows the proposed pathway based on the results.

The polymer samples (**Entry 5–8**) were subjected to gas chromatography (GC) analysis to further verify the incorporation of 3HP and LMC HA units. From Additional file 1: Fig. S2A–N, the methanolysis products of the polymers were identical to those from monomer standards. The detected components were consistent with the NMR results. The results indicate that 3HP and LMC HA units were incorporated into the copolymers.

Next, homopolymer production of 2HB, 3HB, 3HP, and LMC HAs was attempted. Polymer production was observed only for 3HB and 3HP (Table 1, **Entry 9–14**).

3HP-based binary copolymer synthesis

Because PhaC_{AR} synthesized P(3HP), we subsequently attempted 3HP-based binary copolymer production containing LMC HAs as the secondary monomer units. However, no LMC HAs were incorporated and P(3HP) was obtained under these conditions (Table 2, **Entry 15–17**). Similarly, no polymer was obtained in the combination of 2HB and LMC HAs (Table 2, **entry 20–22**). In contrast, synergizing 3HP and 2HB induced copolymer production. Overall, it was found that 3HB or 3HP

is essential for polymerization using PhaC_{AR} among the tested monomers, and that LMC HAs were incorporated only in the presence of 3HB.

Synthesis of 2HB-containing terpolymers

2HB-containing ternary copolymer production was conducted using 3HB, 3HP, and LMC HAs in combination. Consequently, ternary copolymers were produced under all conditions (Table 3). The 3HP fraction in P(2HB-co-3HP-co-3HB) significantly exceeded those of 4H2MB, 5HV, and 6HHx fractions in their corresponding copolymers, indicating the preference of PhaC_{AR} for 3HB and 3HP monomers.

Sequence analysis of 3HB-based binary copolymers

The thermal properties of P(3HB-co-LMC HAs) were analyzed by differential scanning calorimetry (DSC) (Table 4) to determine their monomer sequences. The melting point of P(3HB) homopolymer exceed those of P(3HB-co-4H2MB), P(3HB-co-5HV), and P(3HB-co-6HHx), indicating that 4H2MB, 5HV, and 6HHx were randomly introduced into the polymer chains, and they reduced the crystallinity of P(3HB). The 3HB and 3HP units were biasedly copolymerized by ¹³C NMR analysis

Table 1 Production of 3HB-based copolymers and various homopolymers with and without AlkK

Monomer supplying genes ^a	Entry	Precursor concentration (g L ⁻¹) ^b	Cell dry weight (g L ⁻¹)	Polymer production (g L ⁻¹)	Monomer composition (mol%)						M _w (× 10 ⁵)	M _n (× 10 ⁵)	M _w /M _n	
					2HB	3HB	3HP	4H2MB	5HV	6HHx				
<i>pct</i>	1	3HB 2.5	3HP 1.0	3.67 ± 0.07	0.49 ± 0.10	-	51.4	48.6	-	-	-	6.3	2.8	2.3
<i>pct</i>	2	3HB 2.5	4H2MB 1.0	3.68 ± 0.06	0.48 ± 0.03	-	97.9	-	2.1	-	-	2.9	0.8	3.6
<i>pct</i>	3	3HB 2.5	5HV 1.0	3.17 ± 0.05	0.32 ± 0.02	-	100	-	-	-	-	2.0	0.9	2.3
<i>pct</i>	4	3HB 2.5	6HHx 1.0	3.10 ± 0.04	0.33 ± 0.03	-	100	-	-	-	-	1.7	0.7	2.5
<i>pct, alkK</i>	5	3HB 2.5	3HP 1.0	3.70 ± 0.13	0.78 ± 0.13	-	55.5	44.5	-	-	-	4.6	2.3	2.0
<i>pct, alkK</i>	6	3HB 2.5	4H2MB 1.0	3.32 ± 0.21	0.66 ± 0.03	-	97.1	-	2.9	-	-	1.9	0.9	2.1
<i>pct, alkK</i>	7	3HB 2.5	5HV 1.0	2.81 ± 0.38	0.42 ± 0.06	-	95.3	-	-	4.7	-	1.1	0.5	2.2
<i>pct, alkK</i>	8	3HB 2.5	6HHx 1.0	2.85 ± 0.08	0.34 ± 0.02	-	98.2	-	-	-	1.8	0.8	0.4	2.1
<i>pct, alkK</i>	9	-	2HB 2.5	2.61 ± 0.10	ND ^c	-	-	-	-	-	-	-	-	-
<i>pct, alkK</i>	10	-	3HB 2.5	3.61 ± 0.07	0.44 ± 0.05	-	100	-	-	-	-	2.0	1.1	1.8
<i>pct, alkK</i>	11	-	3HP 1.0	3.03 ± 0.08	0.25 ± 0.02	-	-	100	-	-	-	3.2	1.7	1.8
<i>pct, alkK</i>	12	-	4H2MB 1.0	3.37 ± 0.63	ND	-	-	-	-	-	-	-	-	-
<i>pct, alkK</i>	13	-	5HV 1.0	2.62 ± 0.10	ND	-	-	-	-	-	-	-	-	-
<i>pct, alkK</i>	14	-	6HHx 1.0	2.44 ± 0.09	ND	-	-	-	-	-	-	-	-	-

^a pBSP_{Reg}phaC_{Alk}pct and pBSP_{Reg}phaC_{Alk}pctalkk were used respectively. ^bThe concentrations of precursors are given as the sodium salts. ^cND, not detected

Table 2 Production of 3HP- and 2HB-based polymers

Monomer supplying genes	Entry	Precursor concentration (g L ⁻¹) ^a	Cell dry weight (g L ⁻¹)	Polymer production (g L ⁻¹)	Monomer composition (mol%)						M _w (× 10 ⁵)	M _n (× 10 ⁵)	M _w /M _n
					2HB	3HB	3HP	4H2MB	5HV	6HHx			
<i>pct, alkk</i>	15	3HP 1.0	4H2MB 1.0	3.53 ± 0.23	0.18 ± 0.05	-	-	100	-	-	2.2	1.0	2.3
<i>pct, alkk</i>	16	3HP 1.0	5HV 1.0	3.20 ± 0.07	0.19 ± 0.04	-	-	100	-	-	3.4	1.4	2.4
<i>pct, alkk</i>	17	3HP 1.0	6HHx 1.0	3.06 ± 0.06	0.19 ± 0.01	-	-	100	-	-	2.7	1.2	1.6
<i>pct, alkk</i>	18	2HB 2.5	3HB 2.5	3.18 ± 0.05	0.40 ± 0.04	32.5	67.5	-	-	-	2.8	0.9	3.0
<i>pct, alkk</i>	19	2HB 2.5	3HP 1.0	2.61 ± 0.13	0.16 ± 0.02	4.2	-	95.8	-	-	1.9	1.2	1.6
<i>pct, alkk</i>	20	2HB 2.5	4H2MB 1.0	2.46 ± 0.24	ND	-	-	-	-	-	-	-	-
<i>pct, alkk</i>	21	2HB 2.5	5HV 1.0	2.31 ± 0.20	ND	-	-	-	-	-	-	-	-
<i>pct, alkk</i>	22	2HB 2.5	6HHx 1.0	2.47 ± 0.03	ND	-	-	-	-	-	-	-	-

^a The concentrations of precursors are given as the sodium salts

Table 3 Biosynthesis of 2HB-containing ternary copolymers

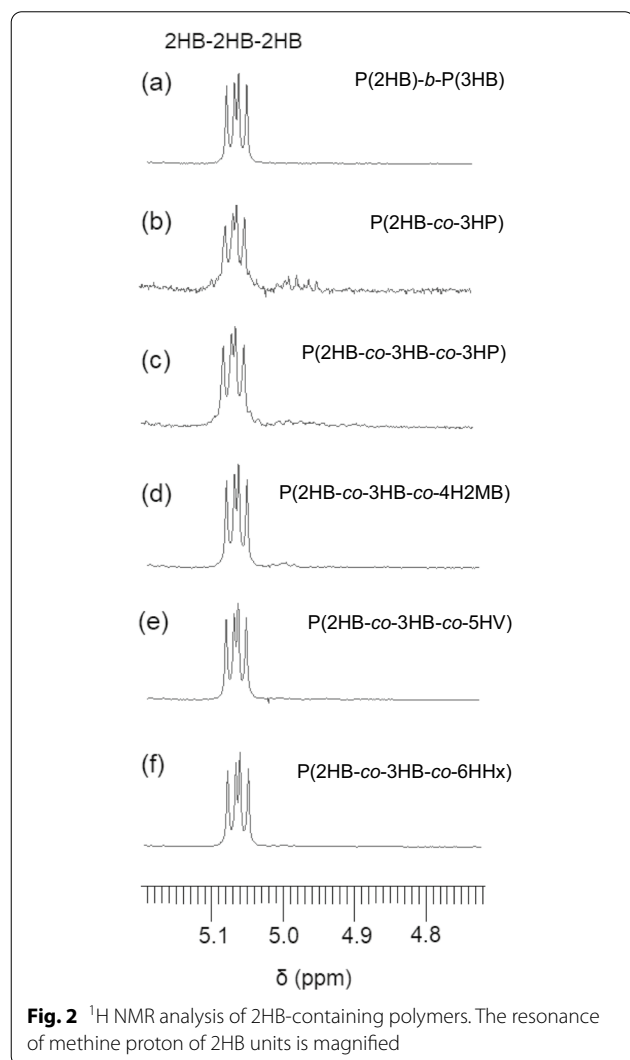
Monomer supplying genes	Entry	Precursor concentration (g L ⁻¹) ^a	Cell dry weight (g L ⁻¹)	Polymer production (g L ⁻¹)	Monomer composition (mol%)						M _w (× 10 ⁵)	M _n (× 10 ⁵)	M _w /M _n
					2HB	3HB	3HP	4H2MB	5HV	6HHx			
<i>pct, alkK</i>	23	2HB 2.5 3HP 1.0 3HB 2.5	3.10 ± 0.03	0.26 ± 0.03	4.4	57.2	38.3	-	-	-	3.7	1.2	3.2
<i>pct, alkK</i>	24	2HB 2.5 4H2MB 1.0 3HB 2.5	3.53 ± 0.16	0.39 ± 0.03	26.8	71	-	2.2	-	-	2.3	1.0	2.4
<i>pct, alkK</i>	25	2HB 2.5 5HV 1.0 3HB 2.5	3.15 ± 0.07	0.45 ± 0.03	31.6	65.3	-	-	3.1	-	1.6	0.7	2.4
<i>pct, alkK</i>	26	2HB 2.5 6HHx 1.0 3HB 2.5	2.98 ± 0.06	0.33 ± 0.01	25.3	73.6	-	-	-	1.1	1.1	0.5	1.9

^a The concentrations of precursors are shown as the sodium salts

Table 4 DSC analysis of homopolymers and copolymers synthesized by PhaC_{AR}

Sample	Synthesis conditions ^a	T _g (°C)	T _m (°C)	ΔH (J/g)
P(3HP)	Entry 11	− 15.0	71.9	62.3
P(3HB)	Entry 10	2.4	169.0	65.9
P(55.5 mol% 3HB-co-3HP)	Entry 1	− 15.5, 0.8	47.9, 161.1	7.7, 10.0
P(97.1 mol% 3HB-co-4H2MB)	Entry 6	1.1	155.9	55.5
P(95.2 mol% 3HB-co-5HV)	Entry 7	− 2.1	159.2	49.4
P(98.3 mol% 3HB-co-6HHx)	Entry 8	0.0	162.4	59.0
P(28.9 mol% 2HB-co-3HB)	Entry 18	3.0	163.4	28.8
P(4.8 mol% 2HB-co-3HP)	Entry 19	− 14.6	73.2	55.1
P(4.4 mol% 2HB-co-3HB-co-3HP 55.1 mol%)	Entry 23	− 11.8, − 1.3	50.3	2.3
P(26.8 mol% 2HB-co-3HB-co-4H2MB 2.2 mol%)	Entry 24	2.7	159.3	35.9
P(32.4 mol% 2HB-co-3HB-co-5HV 3.8 mol%)	Entry 25	2.0	163.7	30.3
P(26.5 mol% 2HB-co-3HB-co-6HHx 1.1 mol%)	Entry 26	1.7	159.4	27.6

^a The polymers were synthesized using conditions in Tables 1, 2 and 4. Combining different batches caused the slight difference in the monomer composition. Thermograms are shown in Additional file 1: Fig. S5AB



(Additional file 1: Fig. S3), as the dyads of 3HB-3HB and 3HP-3HP were more abundant than those of 3HB-3HP and 3HP-3HB. In addition, the copolymer exhibited two melting points. These results suggest that P(3HB-co-3HP) synthesized by PhaC_{AR} is a random copolymer, which possesses 3HB-rich and 3HP-rich heterogeneous copolymer fractions.

Sequence analysis of 2HB-containing copolymers

The monomer sequence of the 2HB-containing segments is estimated based on triad sequence of the 2HB units, which is determined using ¹H NMR [37]. Figure 2 shows the magnified ¹H NMR resonance ascribed to the methine proton of 2HB units (full spectra are shown in Additional file 1: Fig S4). All polymers exhibited the same resonance corresponding to the 2HB-2HB*-2HB triad. The results suggest that the 2HB-containing copolymers have a P(2HB) homopolymer segment. DSC analysis did not detect a melting peak of P(2HB) crystals in 2HB-containing copolymers (Table 4), probably due to the low 2HB fraction and slow crystallization of P(2HB).

NMR analysis clearly distinguishes random and block copolymers, but it detects no difference between a block copolymer and homopolymer blend. Therefore, solvent fractionation of P(3HP-co-2HB) was conducted to verify whether there are covalent linkages between P(3HP) and P(2HB) segments in the polymer. Diethylether dissolves P(2HB) but not P(3HP). Thus, the polymer samples were fractionated into diethylether-soluble and insoluble fractions (Table 5). This procedure completely separates a blend of two homopolymers P(2HB) and P(3HP) (Fig. 3). In contrast, for P(2HB-co-3HP), both signals of 2HB and 3HP were observed in both the diethylether-soluble and insoluble fractions. These results strongly indicate

Table 5 Solvent fractionation of P(2HB-co-3HP) synthesized using PhaC_{AR}

Polymers	Monomer composition (mol%)		Recovery (mol%)
	3HP	2HB	
Blend of P(3HP) and P(2HB)	65	35	100
Soluble fraction	94	6	46
Insoluble fraction	0	100	53
Original Copolymer	56	44	100
Soluble fraction	64	36	39
Insoluble fraction	39	61	52

a covalent linkage between the P(2HB) and P(3HP) segments, thereby indicating that the copolymer of 3HP and 2HB synthesized using PhaC_{AR} is a block copolymer P(3HP)-*b*-P(2HB).

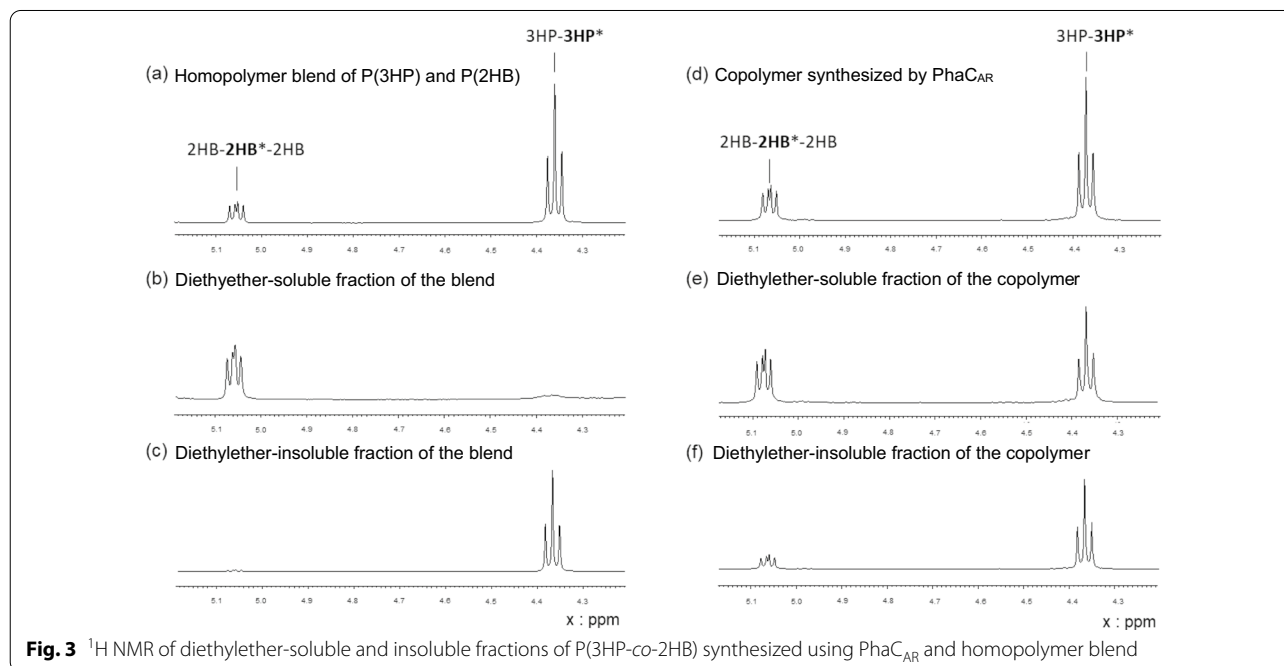
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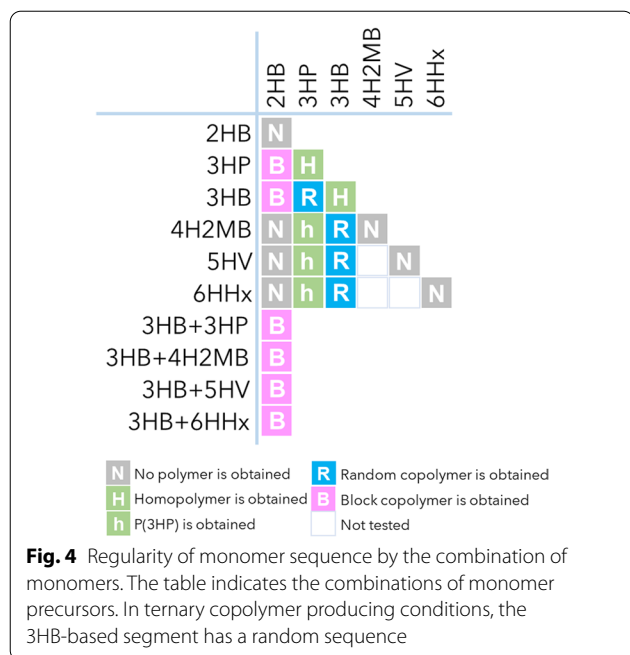
In this study, we described copolymer synthesis containing 3HP, 4H2MB, 5HV, and 6HHx units that possess a terminal hydroxy group using PhaC_{AR} (Fig. 4). Together with the natural monomer unit 3HB, the four unusual monomers were successfully incorporated into their respective polymers. To our best knowledge, only one study has reported the biosynthesis of 6HHx-containing PHA [41]. For the incorporation of 2HA units, PhaC_{AR} is a unique class I PHA synthase that efficiently polymerizes 2HB-CoA. For other class I enzymes, PhaC_{Re}

reportedly incorporates a small amount of 2HB units in vitro [52]. Some engineered class II enzymes, such as PhaC1_{PS}STQK, efficiently incorporate 2HB units, but they have not been reported to incorporate 3HP and LMC HAs. Therefore, PhaC_{AR} possesses the widest substrate scope for the length of the main-chain among the previously characterized PhaCs.

The LMC HA units were randomly incorporated into the P(3HB) backbone by PhaC_{AR}. Random copolymerization is an effective method for reducing the crystallinity and glass transition temperature of polymers (Table 4), which contribute to improving impact resistance [42]. Similar effects can be obtained by introducing the MCL 3HA units [53, 54]. The difference between the LCM HA and MCL 3HA units is their enzymatic degradability. The presence of 4HB, 5HV, and 6HHx in PHAs enhances PHA degradability by lipases [55, 56], which could influence biodegradability in the environment. In fact, in compositing conditions, PCL [e.g. P(6HHx)] is degraded more rapidly than MCL PHA [57]. In addition, PCL and P(3HB) are marine degradable [58]. These results suggest that LCM HA units are potentially used to modulate the biodegradability of PHA and expand the type of its degrading enzymes.

A characteristic of PhaC_{AR} is its limited homopolymer-synthesizing capacity despite its wide substrate scope. Only P(3HB) and P(3HP) were obtained under single precursor supplemented conditions (Table 1). The limited capacity of homopolymer synthesis is presumably due to a potential barrier for initiating polymerization. Notably, some PHA synthases exhibit a slow reaction





rate at the initial reaction stage, termed a lag phase [59]. Polymerization is accelerated in the presence of preferred substrates, which eliminates the lag phase. This study's results indicate that 3HB-CoA and 3HP-CoA can proceed the initiation step of the polymerization while LMC HA-CoAs do not. The mature form of PhaC_{AR} is thought to accept a wider range of substrates. The requirement in the initiation would not be a critical drawback in the molecular design of block PHAs, because enzyme engineering enables the acquisition of homopolymer-synthesizing capacity [60]. We recently reported that a PhaC_{AR} derivative with reinforced activity toward 3HHx-CoA can synthesize a P(3HHx) homopolymer [61]. Given these relevant achievements, the enzymatic characteristics of PhaC_{AR} analyzed in this study provide direction(s) of its engineering for producing diverse PHA block copolymers.

Sequence regulation is a fascinating property of PhaC_{AR}. In this study, a block sequence was generated when the medium was supplemented with 2HB (Figs. 2 and 4). Previous studies have shown that homo-random block sequences are formed in the presence of GL units [35]. Collectively, 2-hydroxyacyl (2HA)-CoAs are thought to trigger block copolymerization. However, the role of 2HA-CoAs in block copolymer synthesis is yet to be fully understood at the molecular level. Further studies are needed to clarify this problem.

Conclusion

Unique sequence-regulating PHA synthase PhaC_{AR} can incorporate various HA units in the main-chain-length range of C₂₋₆. The substrate scope is the widest among characterized PHA synthases. Monomers play different roles in PHA synthesis depending on their main-chain-length. 3HA units (3HB or 3HP) are essential for the polymer synthesis by PhaC_{AR} and allow the incorporation of 2HB and LMC HA units as secondary components. The presence of a 2HB monomer presumably triggers block copolymerization, and 2HB units are incorporated into the polymer chain as a P(2HB) homopolymer segment. LMC HAs are incorporated into the P(3HB) backbone with a random sequence; thus, they are effective in controlling the crystallinity of the 3HB-based segment. These findings of regularity of the sequence control are useful for the molecular design of PHA block copolymers. Finally, PhaC_{AR} provides a versatile biosynthetic system for random and block copolyesters comprising 2-, 3-, 4-, 5-, and 6-hydroxyalkanoates.

Methods

Bacterial strain and plasmids

E. coli JM109 was used as the host strain (Table 6). The plasmids pBSP_{Re}phaC_{AR}pct and pBSP_{Re}phaC_{AR}pctalkK [36] were used for polymer production. These plasmids are the pBluescript KS⁺ derivatives with the ampicillin resistance gene. pBSP_{Re}phaC_{AR}pct contains the *phaC_{AR}* gene encoding the engineered chimeric PHA synthase, the *pct* gene encoding propionyl-CoA transferase

Table 6 Strain and plasmids used in this study

	Description	Source
Strain		
<i>Escherichia coli</i> JM109	<i>recA1, endA1, gyrA96, thi-1, hsdR17 (r_K⁻ m_K⁺), e14⁻(mcrA⁻), supE44, relA1, Δ(lac-proAB)/F⁺ [traD36, proAB⁺, lacI^q, lacZΔM15]</i>	Toyobo
Plasmid		
pBSP _{Re} phaC _{AR} pct	pBluescript KS ⁺ derivative containing the engineered chimeric PHA synthase gene <i>phaC_{AR}</i> and <i>pct</i> from <i>Megasphaera elsdenii</i> under the P _{Re} promoter from the <i>phb</i> operon in <i>Ralstonia eutropha</i> (<i>Cupriavidus necator</i>)	[33]
pBSP _{Re} phaC _{AR} pctalkK	pBSP _{Re} phaC _{AR} pct derivative containing <i>alkK</i> from <i>Pseudomonas putida</i>	[36]

from *Megasphaera elsdenii* under the P_{Re} promoter from the *phb* operon in *R. eutropha* [33]. In addition, $pBSP_{Re}phaC_{ARP}ctalkK$ harbors the *alkK* gene encoding MCL 3-hydroxyalkanoic acid CoA ligase from *Pseudomonas putida* [51].

Culture conditions for polymer production

E. coli JM109 harboring $pBSP_{Re}phaC_{ARP}ctalkK$ was cultivated in a 1.5 mL Luria–Bertani (LB) medium (10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract) containing ampicillin (100 mg/L) at 30 °C overnight as a preculture. The preculture (1 mL) was used to inoculate the LB medium (100 mL) containing glucose (20 g/L), ampicillin, and monomer precursors (see below) in a 500 mL flask. Cells were cultivated with reciprocal shaking (120 rpm) at 30 °C for 48 h, then collected by centrifugation (5000×g, 10 min, 4 °C), washed twice with pure water, and lyophilized.

Sodium (*R,S*)-3-hydroxybutyrate (3HB-Na), sodium (*R,S*)-2-hydroxybutyrate (2HB-Na), and sodium 3-hydroxypropionate (3HP-Na) were purchased from Tokyo Chemical Industry Co., Ltd. (TCI; Tokyo, Japan). Sodium 4-hydroxy-2-methylbutyrate (4H2MB-Na), sodium 5-hydroxyvalerate (5HV-Na), and sodium 6-hydroxyhexanoate (6HHx-Na) were prepared by hydrolyzing their corresponding lactones: α -methyl- ϵ -butyrolactone, δ -valerolactone, and ϵ -caprolactone (TCI). The lactones (5 g) were added to 100 mL of 1 N NaOH and hydrolyzed at 65 °C for 3 days. The solutions were neutralized by 6 N HCl to pH 7. The absence of polymerized products in the hydrolysates was confirmed by DOSY-NMR (Additional file 1: Fig. S1A–C).

Analysis of monomer composition

The polymers (2–5 mg/mL for 1H NMR, 20 mg/mL for ^{13}C NMR) were placed in screwed test tubes (round bottom), dissolved in 1 mL of $CDCl_3$, and heated at 60 °C for 15 min. The mixtures were then cooled to room temperature and filtered through a 0.2- μ m pore size polytetrafluoroethylene (PTFE) filter. NMR spectra were obtained using a JEOL ECS-400 spectrometer. The monomer composition of the synthesized polymer was determined by 1H NMR. The 400 MHz 1H NMR spectra were recorded in $CDCl_3$: P(3HB), δ 1.27 (d, 3H), 2.54 (dq, 2H), 5.26 (m, 1H). P(3HP), δ 2.67 (t, 2H), 4.37 (t, 2H). P(3HB-co-3HP), δ 1.28 (d, 3H), 2.44–2.64 (m, 2H), 2.67 (t, 2H), 4.37 (t, 2H), 5.26 (m, 1H). P(3HB-co-4H2MB), δ 1.17 (d, 3H), 1.28 (d, 3H), 2.00 (t, 1H), 2.54 (dq, 2H), 4.08–4.16 (m, 2H), 5.26 (m, 1H). P(3HB-co-5HV), δ 1.27 (d, 3H), 1.66 (t, 2H), 2.29–2.35 (m, 2H), 2.54 (dq, 2H), 5.26 (m, 1H). P(3HB-co-6HHx), δ 1.27 (d, 3H), 1.64 (t, 2H), 1.97–2.04 (m, 2H), 2.54 (dq, 2H), 4.06 (t, 2H), 5.26 (m, 1H).

P(2HB-co-3HP), δ 1.05 (t, 3H), 1.94–2.02 (m, 2H), 2.67 (t, 2H), 4.37 (t, 3H), 5.02–5.08 (m, 1H). P(2HB-co-3HB), δ 1.04 (t, 3H), 1.27 (d, 3H), 1.89–2.08 (m, 2H), 2.54 (dq, 2H), 5.06 (dd, 1H), 5.26 (m, 1H). P(2HB-co-3HB-co-3HP), δ 1.04 (t, 3H), 1.29 (dd, 3H), 1.93–2.06 (m, 2H), 2.45–2.55 (m, 2H), 2.62–2.68 (m, 2H), 4.32–4.38 (m, 2H), 5.06 (t, 1H), 5.25–5.34 (m, 1H). P(2HB-co-3HB-co-4H2MB), δ 1.04 (t, 3H), 1.10–1.18 (dd, 3H), 1.28 (d, 3H), 1.91–2.08 (m, 2H), 2.54 (dq, 2H), 4.11 (s, 2H), 5.07 (t, 1H), 5.26 (m, 1H). P(2HB-co-3HB-co-5HV), δ 1.04 (t, 3H), 1.28 (d, 3H), 1.66 (t, 2H), 1.89–2.08 (m, 2H), 2.22 (t, 2H), 2.54 (dq, 2H), 4.05–4.11 (m, 2H), 5.07 (dd, 1H), 5.26 (m, 1H). P(2HB-co-3HB-co-6HHx), δ 1.04 (t, 3H), 1.27 (d, 3H), 1.67 (br, 2H), 1.89–2.08 (m, 2H), 2.54 (dq, 2H), 4.03–4.11 (m, 2H), 5.07 (dd, 1H), 5.26 (m, 1H).

The incorporation of LMC units was also verified using GC and GC–MS. Approximately 1 mg polymer dissolved in 500 μ L of chloroform was combined with 500 μ L of 15:85 (v/v) sulfuric acid/methanol. The mixture was heated at 100 °C for 2 h in a glass tube with a screw cap to convert the polymer into low-molecular-weight derivatives. The reaction product was analyzed, as described previously [62]. The sample concentration was adjusted to approximately 2 mg/mL for GC and diluted to 100 μ g/mL for GC–MS.

Molecular weight measurement of polymers

The average molecular weight and polydispersity of the synthesized polymers were determined by size exclusion chromatography (SEC). Polymers extracted and purified from the *E. coli* cells were dissolved in chloroform to a concentration of 1–5 mg/mL. HPLC system (JASCO, Japan) equipped with two tandem K-806L columns (Shodex, Japan) was used for SEC analysis. The pump flow rate was 0.7 mL/min, the column temperature was 40 °C, and the sample injection volume was 100 μ L. The molecular weights of the polymers were calculated from calibration curves prepared using a commercially available polystyrene standard (Shodex).

Analysis of thermal properties

The glass transition temperature (T_g) and the melting temperature (T_m) of the synthesized polymers were analyzed using DSC. The polymers (5–10 mg) were confined in an aluminum pan using a pressing machine (METTLER TOLEDO™ Crucible Sealing Press). DSC 3 (METTLER TOLEDO) was used as an instrument. To confirm the polymer structure, T_m was measured after prolonged heating at an isothermal temperature. The measurement was performed under nitrogen atmosphere (flow rate: 100 mL/min) with the following

temperature controls: (1) cooling from 30 °C to −30 °C at 50 °C/min; (2) cooling from −30 °C to −50 °C at 20 °C/min; (3) heating from −50 °C to 210 °C at 20 °C/min; (4) cooling from 210 °C to −30 °C at 50 °C/min; (5) cooling from −30 °C to −50 °C at 20 °C/min; (6) isothermal heating at −50 °C for 5 min; and (7) heating from −50 °C to 210 °C at 20 °C/min.

Solvent fractionation

P(2HB-co-3HP) (30 mg) was dissolved in 1 mL of chloroform and incubated at 100 °C for 24 h. The solution was combined with 10 mL of diethylether in small steps, and the mixture was incubated at 4 °C for 3 h. The diethylether-soluble fraction was collected by passing through a PTFE membrane filter, and the polymer on the membrane was collected as the diethylether-insoluble fraction. A blend of P(2HB) and P(3HP) was treated in the same way.

Abbreviations

AlkK: MCL 3HA CoA ligase; CoA: Coenzyme A; DOSY NMR: Diffusion ordered spectroscopy NMR; DSC: Differential scanning calorimetry; GC: Gas chromatography; GL: Glycolate; HA: Hydroxyalkanoate; LA: Lactic acid; LMC: Long-main-chain; MCL: Medium-chain-length; NMR: Nuclear magnetic resonance; PCL: Polycaprolactone; PCT: Propionyl-CoA transferase; PHA: Polyhydroxyalkanoate; PhaC: PHA synthase; PLA: Polylactic acid; PTFE: Polytetrafluoroethylene; ROP: Ring-opening polymerization; SCL: Short-chain-length; SEC: Size exclusion chromatography; T_g : Glass transition temperature; T_m : Melting temperature; 2HB: 2-Hydroxybutyrate; 3HB: 3-Hydroxybutyrate; 3HHx: 3-Hydroxyhexanoate; 3HP: 3-Hydroxypropionate; 4HB: 4-Hydroxybutyrate; 4H2MB: 4-Hydroxy-2-methylbutyrate; 5HV: 5-Hydroxyvalerate; 6HHx: 6-Hydroxyhexanoate.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12934-022-01811-7>.

Additional file 1. Fig. S1. ^1H - ^1H DOSY-NMR of 4H2MB, 5HV, and 6HHx in D_2O . **Fig. S2.** GC analysis and Electron Ionization MS spectra of P(3HB-co-3HP), P(3HB-co-LMC HA)s, and relevant polymers synthesized by PhaC_{AR}. **Fig. S3.** ^{13}C NMR analysis of P(3HB-co-LMC HA)s. **Fig. S4A.** ^1H NMR of P(3HB-co-3HP), P(3HB-co-LMC HA)s P(2HB-co-3HP), P(2HB-co-3HB), P(2HB-co-3HB-co-3HP), P(2HB-co-3HB-co-LMC HA)s, and relevant polymers. **Fig. S5.** DSC thermograms of polymers in Table 3.

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Authors' contributions

KS performed experiments, analyzed data, and wrote the original version of the manuscript. TK, NI, and LP performed experiments. HT analyzed data and wrote the manuscript. MZ partly supervised the project and wrote the manuscript. KM designed the experiments, supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All datasets generated and analyzed during this study are included in this published article and its additional files.

Declarations

Competing interests

The authors declare that they have no competing interests.

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