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3 **Biosynthesis and Thermal Characterization of Polyhydroxyalkanoates**

4 **Bearing Phenyl and Phenylalkyl Side Groups**

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6 Shoji Mizuno,¹ Shiori Katsumata,¹ Ayaka Hiroe,¹ Takeharu Tsuge^{1,*}

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8 ¹*Department of Innovative and Engineered Materials, Tokyo Institute of Technology,*

9 *4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan*

10

11 *Corresponding author

12 Telephone: +81 45 924 5420; Fax: +81 45 924 5426; E-mail: tsuge.t.aa@m.titech.ac.jp

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16

Abstract

Polyhydroxyalkanoate (PHA) synthase 1 (PhaC1_{ps}) from *Pseudomonas* sp. 61-3 is a broad-substrate-specific enzyme. In this study, by employing *Ralstonia eutropha* PHB⁻4 recombinant expressing PhaC1_{ps}, a novel type of 3-hydroxybutyrate (3HB)-based PHA copolymer, poly(3HB-co-3-hydroxy-3-phenylpropionate) [P(3HB-co-3H3PhP)], bearing phenyl side groups, was synthesized. The 3H3PhP fraction was increased up to 8.9 mol% by feeding 3H3PhP precursors. As the phenyl side group was introduced into P(3HB), the melting temperature and the enthalpy of fusion decreased, while the glass transition temperature (T_g) increased, demonstrating a distinct thermal behavior as a result of the phenyl side group. Furthermore, PHAs bearing phenylalkyl side groups were synthesized from ω -phenylalkanoates by *Pseudomonas putida* KT2440, and their thermal properties were characterized. Based on the measured and predicted T_g values, the effect of the acyl chain length in the aromatic side group on T_g was investigated. This study demonstrates the role that aromatic side groups play in the thermal properties of PHAs.

1. Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that are synthesized as intracellular storage materials of carbon and energy and are necessary for the survival of bacteria. PHAs can be used as a raw material for manufacturing biodegradable plastics [1]. The most typical PHA produced by bacteria is poly[(*R*)-3-hydroxybutyrate] [P(3HB)]; P(3HB) is a highly crystalline material with poor elasticity. Therefore, 3HB-based PHA copolymers with appropriate flexibility and ductility as a result of reduced crystallinity are preferred for practical use [1]. As a second monomer for 3HB-based PHA copolymers, a variety of side chain structures such as ethyl, propyl, and longer *n*-alkyl groups have been reported [1,2]. When these comonomers are incorporated into the P(3HB) sequence, both the melting temperature (T_m) and glass transition temperature (T_g) of the polymer tend to decrease. P(3HB) has T_m of approximately 175 °C and T_g of approximately 5 °C, whereas P(3HB-*co*-5 mol% 3-hydroxyhexanoate), an example of a 3HB-based copolymer, has T_m of 138–147 °C and T_g of 0 °C [3].

It is recognized that the mechanical properties of P(3HB) and its copolymers gradually deteriorate with aging due to embrittlement by a process of secondary crystallization [4]. Crystal growth of polymers proceeds at a temperature above T_g but below T_m . Thus,

PHAs with T_g higher than ambient temperature can avoid deterioration by secondary crystallization. A few reports describe the biosynthesis of PHAs having relatively high T_g (up to 49 °C) [5–11]; however, a 3HB-based copolymer with increased T_g has not been reported.

In this study, our efforts were focused on the biosynthesis of 3HB-based copolymers that exhibit increased T_g as a result of the incorporated comonomer. Generally, an increase in the size of the side group in polymers causes steric hindrance and results in an increase in T_g . However, T_g depends not only on the size of the side group, but also on its rigidity. Based on the current knowledge of structure- T_g relationships, we attempted to copolymerize an aromatic monomer, 3-hydroxy-3-phenylpropionate (3H3PhP), which has a large and rigid side group, with 3HB (Fig. 1). In addition, *Pseudomonas putida* KT2440 was cultured to synthesize two types of poly(3-hydroxy- ω -phenylalkanoate)s [P(3HPhA)s], poly(3-hydroxy-5-phenylvalerate) [P(3H5PhV)] and poly(3-hydroxy-4-phenylbutyrate-*co*-3-hydroxy-6-phenylhexanoate) [P(3H4PhB-*co*-3H6PhHx)] (Fig. 1), and the effect of the acyl chain length in the aromatic side group on T_g was investigated.

2. Materials and methods

2-1 Materials

Racemic 3-hydroxy-3-phenylpropionic acid (3H3PhP), 3-phenylpropionic acid (3PhP), cinnamic acid (CA), 5-phenylvaleric acid (5PhV), and 6-phenylhexanoic acid (6PhHx) were purchased from Tokyo Kasei, Co. Ltd. (Tokyo, Japan). L-Phenylalanine (Phe) and kanamycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan).

2-2. Bacterial strains and gene knockout

For the synthesis of P(3HB-co-3H3PhP), *Ralstonia eutropha* PHB⁻4 (DSM541), which is a PHA negative mutant of *R. eutropha* H16 and is commonly used as a PHA production host, was transformed by the plasmid pBBR1⁺C1_{PS}AB_{Re} [12] carrying PHA synthase 1 gene (*phaC*_{PS}) from *Pseudomonas* sp. 61-3 and 3HB monomer supplier genes (*phaAB*_{Re}) from *R. eutropha* H16. Also, *Pseudomonas putida* KT2440 (ATCC47054) and *P. putida* KT2440Δ*phaG* were used as host strains for P(3HPhA) production in this study. The 3-hydroxyacyl-acyl carrier protein-coenzyme A (CoA) transferase gene (*phaG*) knockout in *P. putida* KT2440 was performed by a method

described by Schäfer et al [13]. The plasmid pNS2X-*phaG_{pp}*-UD carrying a knockout cassette consisting of *phaG_{pp}* upstream and downstream regions was constructed as follows: *phaG_{pp}* upstream and downstream regions were amplified by PCR using the genome DNA of *P. putida* KT2440 as a template and the primers phaG-UF (5'- CGG GCA TTT CAA CAA CAA GGT GAT C -3'), phaG-UR (5'- TGG CTA CAA GGC GCC GAG CCG CGT CAT CGA CTC CTG GCG C -3'), phaG-DF (5'- GCG CCA GGA GTC GAT GAC GCG GCT CGG CGC CTT GTA GCC A -3'), and phaG-DR (5'- GAG AGA AAC ATG CGT TGT CC -3'). The two obtained fragments [upstream (0.5 kb) and downstream (0.4 kb)] were used as templates, and an upstream-downstream fragment was amplified by overlap extension PCR. The PCR product (0.9 kb) was inserted into pNS2X-sacBR1, modified pMT5071 [14], at the *Swa*I site, and the resultant plasmid was named pNS2X-*phaG_{pp}*-UD. The knockout plasmid pNS2X-*phaG_{pp}*-UD was introduced into *P. putida* KT2440 by conjugational transfer [15], and the *phaG_{pp}* gene was deleted by homologous recombination as described previously [14], yielding *P. putida* KT2440Δ*phaG*.

2-3. PHA biosynthesis

PHA production was performed in 100 mL or 1 L of a mineral salt (MS) medium [16]

with carbon sources in a shaking flask at 30 °C for 40 h (*P. putida*) or 72 h (*R. eutropha*). For P(3HB-co-3H3PhP) synthesis by *R. eutropha*, fructose (20 g/L) and racemic 3H3PhP or its analogs were used as 3H3PhP precursors (Fig. 1). Kanamycin (50 mg/L) was added to the medium to maintain the expression plasmid. For P(3HPhA) synthesis by *P. putida*, butyric acid (3.5 g/L) was used as a carbon source for cell growth, and 5PhV and 6PhHx (3 g/L) were used as 3-hydroxy- ω -phenylalkanoate precursors [9]. After cultivation, the collected cells were washed with water or a water/hexane mixture to remove the remaining carbon source and then lyophilized.

2-4. HPLC analysis

The PHA content of the dried cells and the monomer composition were determined by high-performance liquid chromatography (HPLC). Prior to HPLC analysis, the dried cells were hydrolyzed under alkaline conditions as follows: approximately 10 mg of the dried cells were hydrolyzed by 2 mL of 1N KOH at 100 °C for 3 h, followed by neutralization with 2 mL of 1N HCl. By this alkaline hydrolysis, PHA was converted to monomeric 2-alkenoic acids [17]. The cell hydrolysates were filtered through a 0.45 μ m syringe filter and subjected to HPLC analysis using a Shimadzu LC-10Avp system (Kyoto, Japan) with an ion-exclusion column Aminex HPX-87H (Bio-Rad, CA, USA)

at 60 °C using 0.014N H₂SO₄ with 7% CH₃CN as a mobile phase. The chromatograms were recorded at 210 nm using a UV detector.

2-5. PHA characterization

The PHA polymers in cells were extracted by stirring with chloroform for 72 h at room temperature and purified by precipitation into methanol. After recovery of the PHA polymers, they were further purified by successive reprecipitation into hexane and methanol.

The number average molecular weight (M_n) and the weight average molecular weight (M_w) were determined by gel permeation chromatography (GPC) using a Shimadzu 10A GPC system. Chloroform was used as the eluent at a flow rate of 0.8 mL/min, and each sample (1 mg/mL) was applied. Polystyrene standards with a low polydispersity were used to plot a molecular weight calibration curve.

PHA films for thermal analysis were prepared by a solvent casting method. The extracted and purified PHA was dissolved in chloroform, and the polymer solution was poured into Petri dishes. The solvent was evaporated at room temperature, and the films were aged for at least three weeks to reach equilibrium crystallinity prior to analysis. For differential scanning calorimetry (DSC) analysis, 2–3 mg of the PHA film was

encapsulated in an aluminum pan and analyzed with a Perkin-Elmer Pyris 1 DSC (Perkin-Elmer, Waltham, MA, USA) in the temperature range of -50 to 200 °C at a heating rate of 20 °C/min under a nitrogen atmosphere.

The PHA bearing phenyl groups was analyzed by NMR spectroscopy. Each polymer (40 mg) was dissolved in CDCl₃ (1.0 mL) and subjected to 500 MHz ¹H NMR and 125 MHz ¹³C NMR analyses. The NMR spectra were recorded using a JEOL JNM-LA500 spectrometer as previously described [12].

3. Results and discussion

3-1. Biosynthesis of P(3HB-*co*-3HPhP) by recombinant *R. eutropha*

The biosynthesis of P(3HB-*co*-3HPhP) has not been reported in previous studies using natural PHA producers. To newly construct P(3HB-*co*-3HPhP)-producing bacteria, the broad-substrate-specific PHA synthase (PhaC1_{Ps}) from *Pseudomonas* sp. 61-3 was employed in *R. eutropha* PHB⁻4 strain. PhaC1_{Ps} is able to polymerize not only medium-chain-length 3HA (C6-C12), but also 3HB (C4) [16], while typical synthases from *Pseudomonas*, including *P. putida* synthase, are unable to polymerize 3HB. On the

other hand, it has been reported that typical *Pseudomonas* synthases are able to polymerize 3H4PhB and longer 3-hydroxy- ω -phenylalkanoate (3HPhA) monomers [5–9,18–21].

R. eutropha PHB⁻4 expressing PhaC1_{Ps} was grown on fructose by feeding 3H3PhP or its analogs to examine their utility as 3H3PhP precursors. 3H3PhP and its analogs (0.5 g/L) were fed at 0 h, 24 h, and 48 h of cultivation to give a total concentration of 1.5 g/L. The monomeric composition of PHA that accumulated in the cells after 72 h of cultivation is shown in Table 1.

From fructose as a sole carbon source, *R. eutropha* accumulated PHA consisting of 3HB as a major component and 3-hydroxyvalerate (3HV) and 3-hydroxy-4-methylvalerate (3H4MV) as minor components. This strain has a weak ability to produce 3HV and 3H4MV from fructose [22,23], thereby incorporating these monomer units into PHA.

By feeding 3H3PhP precursors other than L-phenylalanine, the cell growth was seriously inhibited, from 1.78 g/L to 0.43–0.97 g/L of dry cell. The PHA content in the cells was also reduced from 51 wt% to ~27 wt%. 3PhP was the most toxic precursor for cell growth and PHA accumulation (less than 1 wt%) of *R. eutropha* PHB⁻4.

By feeding CA and racemic 3H3PhP, their incorporation into PHA was observed as 3H3PhP fractions of 4.2 and 12.2 mol%, respectively. This result indicated that PhaC1_{Ps}

has the ability of polymerizing 3H3PhP because of its broad substrate specificity. It is well-known that PHA synthases only polymerize *R*-enantiomer hydroxyalkanoate monomers; thus, *R*-3H3PhP in the racemic 3H3PhP, when added into the culture medium as a precursor, would be selectively polymerized via *R*-3H3PhP-CoA as the substrate for PhaC1_{PS}. As for CA, after ligation with CoA, it would be *R*-specifically hydrated and then polymerized into PHA.

L-Phenylalanine, which is an aromatic amino acid with an identical carbon skeleton to 3H3PhP, enhanced cell growth because it was utilized as a carbon and nitrogen source. However, 3H3PhP was not detected in the PHA, suggesting that 3H3PhP cannot be derived from metabolites of L-phenylalanine degradation.

3-2. Characterization of P(3HB-*co*-3H3PhP)

The 3H3PhP-containing PHAs were extracted from *R. eutropha* PHB⁴ that was cultured with CA or racemic 3H3PhP (samples 2 and 3 in Table 1), and these polymers were subjected to NMR analysis.

Figure 2 shows the 500 MHz ¹H NMR spectrum of the 3H3PhP-containing PHAs synthesized with racemic 3H3PhP (sample 3). The signals at 7.2–7.4 ppm are attributed to the phenyl group. Signals at 6.1 ppm from the 3H3PhP methine proton and 2.9 ppm

from the 3H3PhP methylene proton were also present. Minor signals from 3HV and 3H4MV were detected at 1.9 ppm (3H4MV methine proton in the side chain), 1.7 ppm (3HV methylene proton in the side chain), and 0.9 ppm (3HV and 3H4MV methyl protons). From the intensity ratio of the methine in the main chain, the 3H3PhP fraction in the purified polymer was determined to be 8.9 mol% (Table 2). The total fraction of 3HV and 3H4MV units was determined to be 1.6 mol%. Because the 3HV and 3H4MV fractions are very small, this polymer is hereafter referred to as P(3HB-*co*-8.9 mol% 3H3PhP).

The 125 MHz ^{13}C NMR spectrum of P(3HB-*co*-8.9 mol% 3H3PhP) is shown in Fig. 3. Signals at 169 ppm from the 3H3PhP carbonyl carbon, 125–140 ppm from the phenyl group, 73 ppm from the 3H3PhP methine carbon, and 42 ppm from the 3H3PhP methylene carbon were detected. Thus, the NMR data provided convincing evidence for the presence of 3H3PhP. **Because of low sensitivity of ^{13}C NMR spectroscopy, the signals of 3HV and 3H4MV were hardly detected.**

The 3H3PhP-containing PHA synthesized with CA was also analyzed by ^1H NMR, and the 3H3PhP fraction was determined to be 4.1 mol%, as listed in Table 2. This polymer is hereafter referred to as P(3HB-*co*-4.1 mol% 3H3PhP).

The M_n of these P(3HB-*co*-3H3PhP)s were $6.5\text{--}9.7 \times 10^4$. The T_g values of the 4.1

mol%- and 8.9 mol%-containing polymers were 10.7 °C and 14.6 °C (Fig. 4), respectively, which are substantially increased from T_g of P(3HB) (sample 1, 7.9 °C). T_m and the enthalpy of fusion (ΔH_m), which is index of the degree of crystallinity, were detected to be 135–158 °C and 7.1–27.8 J/g, suggesting that these P(3HB-*co*-3H3PhP)s still contained P(3HB) crystal phase.

In our previous study, P(3HB-*co*-3HA) with 6 mol% 3HA (6–8 carbon atom length) showed T_m of 126 °C, T_g of -4 °C, and ΔH_m of 31 J/g [24]; P(3HB-*co*-3HHx) with 5 mol% 3HHx showed T_m of 138 °C, T_g of 0 °C, and ΔH_m of 45 J/g [3]. Compared to these polymers, P(3HB-*co*-4.1 mol% 3H3PhP) showed relatively high T_m (146 °C) and low ΔH_m (27.8 J/g). This implies that the phenyl side group strongly inhibited P(3HB) crystallization.

3-3. Characterization of P(3HPhA)s synthesized by *P. putida*

To investigate the size effect of the aromatic side group, P(3H3PhA)s were synthesized from 5PhV by *P. putida* KT2440 or from 6PhHx by *P. putida* KT2440 Δ *phaG*. After 40 h of cultivation, *P. putida* KT2440 grew to 1.9 g/L of dry cells with a PHA content of 58 wt%, and *P. putida* KT2440 Δ *phaG* grew to 1.4g/L with a PHA content of 21 wt%, where the PHA content was determined by gas chromatography as previously described

[16]. These polymers were extracted and characterized by NMR, DSC, and GPC, and the results are tabulated in Table 2. Sample 4, extracted from *P. putida* KT2440, was composed of 99.9 mol% 3H5PhV and 0.1 mol% 3-hydroxydecanoate (3HD); therefore, this polymer is hereafter referred to as P(3H5PhV). On the other hand, sample 5, extracted from *P. putida* KT2440 Δ *phaG*, was composed of 10.1 mol% 3H4PhB, 89.4 mol% 3H6PhHx, and 0.5 mol% 3HD; this polymer is hereafter referred to as P(3H4PhB-*co*-3H6PhHx). The 3H4PhB unit is generated by the β -oxidation cycle of *P. putida*, which is involved in fatty acid degradation. We also tried to synthesize P(3H4PhB-*co*-3H6PhHx) by using wild-type *P. putida* KT2440; however, the 3HD fraction was generally in the range of 5–26 mol% (data not shown) by the action of transferase encoded by *phaG*. This is the reason that *P. putida* KT2440 Δ *phaG* was constructed and employed as a PHA production host in this study.

The M_n values of P(3H5PhV) and P(3H4PhB-*co*-3H6PhHx) were 7.9×10^4 and 11.3×10^4 , respectively. The T_g values of P(3H5PhV) and P(3H4PhB-*co*-3H6PhHx) were 15.7 and 5.3 °C (Fig. 4), respectively. P(3H5PhV) showed relatively high T_g compared to P(3HB) and its copolymers. Both P(3H5PhV) and P(3H4PhB-*co*-3H6PhHx) did not show T_m or ΔH_m , suggesting that these polymers are amorphous, as previously reported [8].

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250 **3-4. Effect of the aromatic side group on T_g of PHA**

251 The copolymers have T_g values that lie between those of the corresponding pure
252 **homopolymers**. For a copolymer consisting of polymer 1 (T_{g1}) and polymer 2 (T_{g2}), the
253 resultant T_g (T_{gr}) is estimated by the Fox equation [25]:

$$254 \quad 1/T_{gr} = W_1/T_{g1} + W_2/T_{g2}$$

255 where the T_g values of the homopolymers are given in Kelvin, and W_1 and W_2 are the
256 weight fractions of polymer 1 and 2 in the copolymer, respectively. It has been
257 demonstrated that T_g of PHA copolymers follow the Fox equation [26,27].

258 We predicted T_g of P(3H3PhP) to be 62 °C (335 K) by the Fox equation using the
259 experimental data of three PHAs (Table 2, samples 1–3): P(3HB) (T_g =280.9 K,
260 W_{3H3PhP} =0), P(3HB-co-4.1 mol% 3H3PhP) (T_g =283.7 K, W_{3H3PhP} =0.068), and
261 P(3HB-co-8.9 mol% 3H3PhP) (T_g =287.6 K, W_{3H3PhP} =0.143). This estimated T_g is much
262 higher than ambient temperature and very close to that of poly(lactide) (T_g =60 °C) [11].

263 Also, P(3HB-co-3H3PhP) with 33 mol% 3H3PhP (W_{3H3PhP} =0.45) is expected to have T_g
264 of 30 °C, which is the same as the culture temperature of the PHA-producing bacteria.
265 Most bacterial PHAs have T_g lower than ambient temperature; thus, these polymers are
266 produced as flexible amorphous materials in the cells. However, PHAs with T_g

exceeding ambient temperature will be produced as rigid amorphous polymers. It might be considered that PHA synthases do not prefer to polymerize rigid amorphous PHA. If so, increases in the 3H3PhP fraction over 33 mol% may be a challenging task because of the increased rigidity of P(3HB-*co*-3H3PhP).

A number of studies have reported the thermal properties of biosynthesized aromatic PHAs [5–9,18–21,28–30]. Using previously reported T_g values and those observed and predicted in this study, the relationship between the average number of methylene in the aromatic side chain and T_g is shown in Fig. 5. Increasing the acyl chain length of the aromatic side group decreases T_g of PHA, probably as a result of an increase in the free volume. Thus, the effect of the increased size of the side group on T_g was compensated by the increased flexibility. Similar trends have been reported for medium-chain-length PHAs [31], poly(alkyl methacrylate)s [32], and poly(alkyl itaconate)s [32]. Because T_g is an important parameter for long-term stability of 3HB-based copolymers, as mentioned earlier, this observation provides a basis for the molecular design of PHA with regulated thermal properties.

4. Conclusions

P(3HB-*co*-3H3PhP) with a 3H3PhP fraction up to 8.9 mol% was synthesized for the first time by employing recombinant *R. eutropha* PHB⁻4 expressing the broad-substrate-specific PhaC1_{PS}. This copolymer showed increased T_g with increasing 3H3PhP fraction, unlike the alkane-based copolymers. From the prediction curve by the Fox equation, T_g of P(3H3PhP) is calculated to be 62 °C. Furthermore, P(3H5PhV) and P(3H4PhB-*co*-3H6PhHx) were synthesized by *P. putida* KT2440 strains, and the effect of acyl chain length of the aromatic side group on T_g was studied. The observed relationship suggested that the increased acyl chain length of the aromatic side group greatly decreases T_g of PHA, probably as a result of an increase in the free volume. The results of this study provide significant implications for understanding the role of aromatic side groups in the thermal behavior of PHAs.

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REFERENCES

- [1] Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci* 2000; 25: 1503-55.
- [2] Abe H, Doi Y. Side-chain effect of second monomer units on crystalline morphology, thermal properties, and enzymatic degradability for random copolyesters of (*R*)-3-hydroxybutyric acid with (*R*)-3-hydroxyalkanoic acids. *Biomacromolecules* 2002; 3: 133-8.
- [3] Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y. Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-*co*-(3-hydroxyhexanoate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkanoate synthase genes. *Macromol Biosci* 2004; 4: 238-42.
- [4] Iwata T, Doi Y. Mechanical properties of uniaxially cold-drawn films of poly[(*R*)-3-hydroxybutyrate] and its copolymers. *Macromol Symp* 2005; 224: 11-9.
- [5] Fritzsche K, Lenz RW, Fuller RC. An unusual bacterial polyester with a phenyl pendant group. *Makromol Chem* 1990; 191: 1957-65.
- [6] Curley JM, Hazer B, Lenz W, R. Fuller RC. Production of poly(3-hydroxyalkanoates) containing aromatic substituents by *Pseudomonas oleovorans*. *Macromolecules* 1996; 29: 1762-6.
- [7] Hazer B, Lenz RW, Fuller RC. Bacterial production of poly-3-hydroxyalkanoates containing arylalkyl substituent groups. *Polymer* 1996; 37: 5951-7.
- [8] Ward PG, O'Connor KE. Bacterial synthesis of polyhydroxyalkanoates containing aromatic and aliphatic monomers by *Pseudomonas putida* CA-3. *Int J Biol Macromol* 2005; 35: 127-33.
- [9] Song JJ, Choi MH, Yoon SC, Huh NE. Cometabolism of ω -phenylalkanoic acids with butyric acid for efficient production of aromatic polyesters in *Pseudomonas putida* BM01. *J Microbiol Biotechnol* 2001; 11: 435-42.
- [10] Shozui F, Matsumoto K, Motohashi R, Sun J, Satoh T, Kakuchi T, Taguchi S. Biosynthesis of a lactate (LA)-based polyester with a 96 mol% LA fraction and its application to stereocomplex formation. *Polym Degrad Stab* 2011; 96:

335 499-504.

336 [11] Yamada M, Matsumoto K, Uramoto S, Motohashi R, Abe H, Taguchi S. Lactate
337 fraction dependent mechanical properties of semitransparent
338 poly(lactate-co-3-hydroxybutyrate)s produced by control of lactyl-CoA
339 monomer fluxes in recombinant *Escherichia coli*. J Biotechnol 2011; 154:
340 255-60.

341 [12] Tsuge T, Yano K, Imazu S, Numata K, Kikkawa Y, Abe H, Taguchi S, Doi Y.
342 Biosynthesis of polyhydroxyalkanoate (PHA) copolymer from fructose using
343 wild-type and laboratory-evolved PHA synthases. Macromol Biosci 2005; 5:
344 112-7.

345 [13] Schäfer A, Tauch A, Kalinowski WJ, Thierbach G, Pühler A. Small mobilizable
346 multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18
347 and pK19: selection of defined deletions in the chromosome of
348 *Corynebacterium glutamicum*. Gene 1994; 145: 69-73.

349 [14] Tsuda M. Use of a transposon-encoded site-specific resolution system for
350 construction of large and defined deletion mutations in bacterial chromosome.
351 Gene 1998; 207: 33-41.

352 [15] Friedrich B, Hogrefe C, Schlegel HG. Naturally occurring genetic transfer of
353 hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J Bacteriol
354 1981; 147: 198-205.

355 [16] Kato M, Bao HJ, Kang CK, Fukui T, Doi Y. Production of a novel copolyester
356 of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by
357 *Pseudomonas* sp. 61-3 from sugars. Appl Microbiol Biotechnol 1996; 45:
358 363-70.

359 [17] Watanabe Y, Ichinomiya Y, Shimada D, Saika A, Abe H, Taguchi S, Tsuge T.
360 Development and validation of an HPLC-based screening method to acquire
361 polyhydroxyalkanoate synthase mutants with altered substrate specificity. J
362 Biosci Bioeng 2012; 113: 286-92.

363 [18] Kim YB, Lenz RW, Fuller RC. Preparation and characterization of
364 poly(β -hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with
365 mixtures of 5-phenylvaleric acid and *n*-alkanoic acids. Macromolecules 1991;

366 24: 5256-60.

367 [19] García B, Olivera ER, Miñambres B, Fernández-Valverde M, Cañedo LM,
368 Prieto MA, García JL, Martínez M, M. Luengo JM. Novel biodegradable
369 aromatic plastics from a bacterial source. J Biol Chem 1999; 274: No. 41,
370 29228-41.

371 [20] Kim DY, Kim YB, Rhee YH. Evaluation of various carbon substrates for the
372 biosynthesis of polyhydroxyalkanoates bearing functional groups by
373 *Pseudomonas putida*. Int J Biol Macromol 2000; 28: 23-9.

374 [21] Tobin KM, O'Connor KE. Polyhydroxyalkanoate accumulating diversity of
375 *Pseudomonas* species utilising aromatic hydrocarbons. FEMS Microbiol Lett
376 2005; 253: 111-8.

377 [22] Tanadchangsang N, Kitagawa A, Yamamoto T, Abe H, Tsuge T. Identification,
378 biosynthesis, and characterization of polyhydroxyalkanoate copolymer
379 consisting of 3-hydroxybutyrate and 3-hydroxy-4-methylvalerate.
380 Biomacromolecules 2009; 10: 2866-74.

381 [23] Saika A, Watanabe Y, Sudesh K, Abe H, Tsuge T. Enhanced incorporation of
382 3-hydroxy-4-methylvalerate unit into biosynthetic polyhydroxyalkanoate using
383 leucine as a precursor. AMB Express 2011; 1: 6.

384 [24] Tsuge T, Hamada Y, Watanabe Y, Tomizawa S, Yamamoto T, Abe H.
385 Characterization of biosynthesized P(3HB-co-3HA)s swellable in organic
386 solvents. Polym Degrad Stab 2010; 95: 1345-8.

387 [25] Fox, TG. Influence of diluent and of copolymer composition on the glass
388 temperature of a polymer system. Bull Am Phys Soc 1956; 1: 123-5.

389 [26] Park WH, Lenz RW, Goodwin S. Epoxidation of bacterial polyesters with
390 unsaturated side chains. I. Production and epoxidation of polyesters from
391 10-undecenoic acid. Macromolecules 1998; 31: 1480-6.

392 [27] Satkowski MM, Melik DH, Autran JP, Green PR, Noda I, Schechtman LA,
393 Physical and processing properties of polyhydroxyalkanoate (PHA) copolymers.
394 A. In Biopolymers; Doi Y, Steinbüchel A, Eds.; Wiley-VCH: Weinheim, 2001;
395 Vol. 3b, p 231-263.

396 [28] Abraham GA, Gallardo A, Roman JS, Olivera ER, Jodra R, García B,

- Miñambres B, García JL, Luengo JM. Microbial synthesis of poly(β -hydroxyalkanoates) bearing phenyl groups from *Pseudomonas putida*: Chemical structure and characterization. *Biomacromolecules* 2001; 2: 562-7.
- [29] Sandoval Á, Arias-Barrau E, Bermejo F, Cañedo L, Naharro G, Olivera ER, Luengo JM. Production of 3-hydroxy- ω -phenylalkanoic acids by a genetically engineered strain of *Pseudomonas putida*. *Appl Microbiol Biotechnol* 2005; 67: 97-105.
- [30] Shen R, Cai L, Meng D, Wu L, Guo K, Dong G, Liu L, Chen J, Wu Q, Chen G. Benzene containing polyhydroxyalkanoates homo- and copolymers synthesized by genome edited *Pseudomonas entomophila*. *Sci China Life Sci* 2014; 57: 4-10.
- [31] Abe H, Ishii N, Sato S, Tsuge T. Thermal properties and crystallization behaviors of medium-chain-length poly(3-hydroxyalkanoate)s. *Polymer* 2012; 53: 3026-34.
- [32] Cowie JMG, Henshall SAE, McEwen IJ, Veličković J. Poly(alkyl itaconates): 4. Glass and sub-glass transitions in the di-alkyl ester series, methyl to hexyl. *Polymer* 1977; 18: 612-6.

Figure legends

Fig. 1. Structures of the PHAs synthesized in this study (A) and the 3H3PhP precursors used in the synthesis of P(3HB-*co*-3H3PhP) (B). Methylene number (n) in poly(3-hydroxy- ω -phenylalkanoate) [P(3HPhA)] is as follows: n=3, poly(3H5PhV); n=2 and 4, poly(3H4PhB-*co*-3H6PhHx).

Fig. 2. 500 MHz ^1H NMR spectrum of P(3HB-*co*-8.9 mol% 3HPhP) (sample 3).

Fig. 3. 125 MHz ^{13}C NMR spectrum of P(3HB-*co*-8.9 mol% 3H3PhP) (sample 3).

Fig. 4. DSC traces of the PHAs during the second heating. Sample 1, P(3HB) synthesized by *R. eutropha* H16 (laboratory stock); Sample 2, P(3HB-*co*-4.1 mol% 3H3PhP); Sample 3, P(3HB-*co*-8.9 mol% 3H3PhP); Sample 4, P(3H5PhV); **Sample 5**, P(10.1 mol% 3H4PhB-*co*-3H6PhHx).

430 **Fig. 5.** The correlation between the average number (n) of methylene in the aromatic
431 side chain and T_g . Closed triangle, T_g predicted in this study; closed square, T_g values
432 measured in this study; open circle, previously published T_g values [5,8,9,27,30].
433

1 Table 1. PHA biosynthesis by recombinant *R. eutropha* PHB⁺4 expressing PhaC1_{Ps} from fructose (20 g/L) with 3H3PhP precursors

Additive ^a (3H3PhP precursor)	Dry cell wt (g/L)	PHA content (wt%) ^b	PHA composition (mol%) ^b				Sample No.
			3HB	3HV	3H4MV	3H3PhP	
None	1.78 ± 0.02	51 ± 1	98.8 ± 0.1	0.8 ± 0.0	0.3 ± 0.0	0	-
CA	0.68 ± 0.01	8 ± 0.2	92.1 ± 0.2	3.0 ± 0.1	0.6 ± 0.2	4.2 ± 0.2	2
3PhP	0.43 ± 0.03	Trace ^c	-	-	-	-	-
Racemic 3H3PhP	0.97 ± 0.03	27 ± 3	86.4 ± 0.7	1.0 ± 0.1	0.3 ± 0.0	12.2 ± 0.5	3
Phe	3.35 ± 0.19	45 ± 4	98.9 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0	-

2 Cells were cultured in MS medium containing fructose (20 g/L) with additive (0.5 g/L × 3) for 72 h at 30 °C.

3 ^aCA, cinnamic acid; 3PhP, 3-phenylpropionic acid, Phe, L-phenylalanine.

4 ^bPHA compositions and content in the cells were determined by HPLC. 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3H4MV,
5 3-hydroxy-4-methylvalerate; 3H3PhP, 3-hydroxy-3-phenylpropionate.

6 ^cless than 1 wt%.

7 Results are averages of three separate experiments.

9 Table 2. Thermal properties and molecular weight of the PHA samples

Sample No.	PHA composition (mol%) ^a						Thermal properties ^b			Molecular weight ^c	
	3HB	3H3PhP	3H4PhB	3H5PhV	3H6PhHx	Other	T_g (°C)	T_m (°C)	ΔH_m (J/g)	M_n ($\times 10^4$)	M_w/M_n
1 ^d	100	-	-	-	-	-	7.9	169	54.5	15.3	3.3
2	95.1	4.1	-	-	-	0.8	10.7	146, 158	27.8	6.5	3.5
3	89.5	8.9	-	-	-	1.6	14.6	135, 149	7.1	9.7	3.7
4 ^e	-	-	-	99.9	-	0.1	15.7	n.d. ^g	n.d.	7.9	2.2
5 ^f	-	-	10.1	-	89.4	0.5	5.3	n.d.	n.d.	11.3	2.7

10 ^a PHA compositions of the purified copolymer samples were determined by ¹H NMR. 3HB, 3-hydroxybutyrate; 3H3PhP,
 11 3-hydroxy-3-phenylpropionate; 3H4PhB, 3-hydroxy-4-phenylbutyrate; 3H5PhV, 3-hydroxy-5-phenylvalerate; 3H6PhHx,
 12 3-hydroxy-6-phenylhexanoate.

13 ^b T_g , glass-transition temperature (2nd heating); T_m , melting temperature (1st heating); ΔH_m , enthalpy of fusion (1st heating).

14 ^c M_n , number-average molecular weight; M_w , weight-average molecular weight; M_w/M_n , polydispersity.

15 ^d P(3HB) homopolymer synthesized by *R. eutropha* H16 (laboratory stock).

16 ^e P(3H5PhV) was synthesized by *P. putida* KT2440.

17 ^f P(3H4PhB-*co*-3H6PhHx) was synthesized by *P. putida* KT2440Δ*phaG*.

18 ^g Not detectable.

19

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5