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Engineering *in Vivo* Production of α-Branched Polyesters

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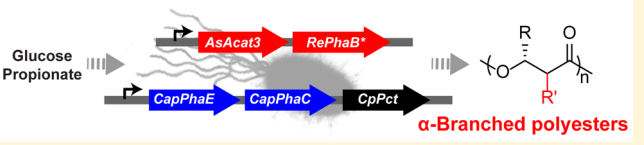
Supporting Information

ABSTRACT: Polymers are an important class of materials that are used for a broad range of applications, from drug delivery to packaging. Given their widespread use, a major challenge in this area is the development of technology for their production from renewable sources and efforts to promote their efficient recycling and biodegradation. In this regard, the synthesis of polyesters based on the natural polyhydroxyalkanoate (PHA) pathway offers an attractive route for producing sustainable polymers. However, monomer diversity in naturally occurring polyesters can be limited with respect to the design of polymers with material properties suitable for various applications. In this work, we have engineered a pathway to produce α-methyl-branched PHA. In the course of this work, we have also identified a PHA polymerase (CapPhaEC) from activated sludge from wastewater treatment that demonstrates a higher capacity for incorporation of α-branched monomer units than those previously identified or engineered. Production in *Escherichia coli* allows the construction of microbial strains that produce the copolyesters with 21−36% branched monomers using glucose and propionate as carbon sources. These polymers have typical weight-average molar masses (M_w) in the range (1.7−2.0) × 10^5 g mol^{-1} and display no observable melting transition, only relatively low glass transition temperatures from −13 to −20 °C. The lack of a melting transition indicates that these polymers are amorphous materials with no crystallinity, which is in contrast to the natural poly(hydroxybutyrate) homopolymer. Our results expand the utility of PHA-based pathways and provide biosynthetic access to α-branched polyesters to enrich the properties of bio-based sustainable polymers.



INTRODUCTION

Living systems are powerful factories for chemical production, utilizing a wide range of simple feedstocks from CO_2 to sugars, to produce an enormous breadth of organic and inorganic structures.[1−6] In addition to small molecules, living systems can also produce high molar mass macromolecules by polymerization, including not only genetically encoded products such as proteins and nucleic acids but also those produced by nontemplated chain growth such as lignin, cellulose, and hemicellulose.[7] Interestingly, many of these polymers have promising material properties but require further structural optimization to be broadly applicable.[8] For example, bacterial polyesters from the polyhydroxyalkanoate (PHA) family have long been known as a source for biodegradable plastics (Figure 1).[9] However, these PHA polyesters generally suffer from issues of brittleness, low thermal stability, poor mechanical properties, and processing difficulties.[10] In one striking example, a simple change from 3-hydroxybutyrate, the most commonly used PHA monomer, to lactate provided a polymer with a methyl branch and a shorter backbone. This small structural modification resulted in the development of a compostable plastic that is produced and utilized on a commercial scale.[11]

As aliphatic polyesters represent a key group of polymers that support many applications while typically being more easily degraded due to their ester linkage, we have focused on developing pathways to incorporate new monomer structures into this class of materials. Initial studies have shown that a variety of longer chain lengths as well as monomers that contain distal branch sites can be incorporated with wild-type PHA polymerases.[12,13] Additional work has shown that non-natural poly(2-, 4-, or 5-hydroxyalkanoates) can also be biosynthesized with mutant and some nonengineered polymerases.[14−16] Of note, the biosynthesis of aromatic polyesters, similar to poly(ethylene terephthalate) (PET), was recently achieved.[17] These studies, as well as others, have shown that structural characteristics such as chain length and branching can be key to improving relevant mechanical properties such as toughness, flexibility, and other properties related to manufacturing, processing, and applications.[14,18,19] In particular, PHA materials extracted from activated sludge containing an α-branch site have been shown to display lower degrees of crystallinity and broader melting temperature ranges than the most common bacterial PHA, poly(hydroxybutyrate) (PHB).[19] Furthermore, Tisma et al. have demonstrated that substitution of α-hydrogen atoms by alkyl groups in the polyester, poly(α-dimethylpropiolactone), reduced the rate of thermal degradation when compared to the unsubstituted poly(β-propiolactone).[20] This previous work suggests that the introduction of α-branching in PHA polymers may help mitigate the issues of brittleness and heat stability typically

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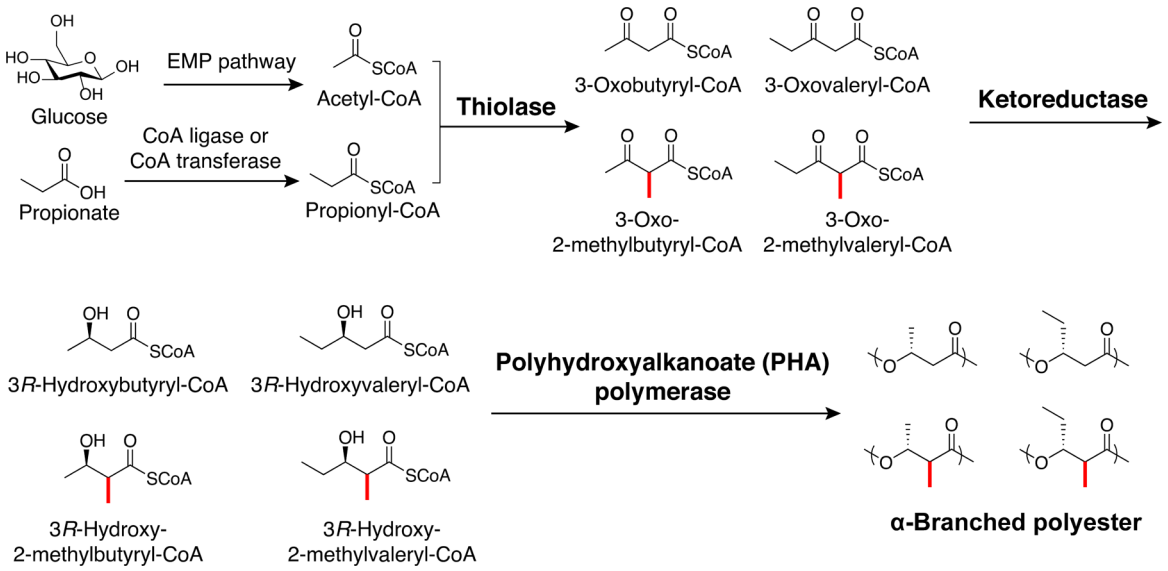

Figure 1. Design of biosynthetic pathway for α-branched PHA polyesters. Glucose and propionate can be transformed into universal building blocks acetyl-CoA and propionyl-CoA by the Embden–Meyerhof–Parnas (EMP) pathway and CoA ligase/CoA transferase, respectively. There are four possible combinations of these two acyl-CoA units via Claisen condensation by a thiolase. The resulting four 3-oxoacyl-CoAs are further reduced to generate 3*R*-hydroxyacyl-CoAs by a ketoreductase, which are then copolymerized by a polyhydroxyalkanoate (PHA) polymerase to form α-branched polyester. Thiolase and ketoreductase substrate selectivity plays a key role of controlling monomer availability for incorporation into the final copolymer by the PHA polymerase. α-Methyl branches have been highlighted in red and must be accommodated by all three enzymes.

associated with PHB homopolymer. On the basis of our previous work utilizing propionate as a building block to produce 3-hydroxyacid monomers with either an α-methyl branch or increased carbon chain tail at C_3,[21,22] we set out to engineer cells to produce aliphatic polyesters *in vivo* from these monomers.

To do so, we first focused on obtaining access to 3-hydroxyacids with an *R*-configuration of the hydroxyl substituent as PHAs made by enzymatic polymerization are found as stereopure 3*R*-hydroxyalkanoates. In contrast, the short branched-chain acid fermentation pathway we had previously identified from *Ascaris suum* produces 3*S*-hydroxyacids[21] as ketoreductases participating in energy metabolism often demonstrate this preference.[23] We were thus able to utilize the branched chain thiolase from *A. suum*, Acat3, but replaced Hadh2 with an engineered PhaB variant that could accommodate α-branching. In addition, we sought to identify a new PHA polymerase (PhaC) that could better utilize branched monomers. Although one PhaC from *Aeromonas caviae* has been identified that can incorporate this structural perturbation,[18] the level of α-branched monomers remained relatively low (2.0 ± 0.4%) in our work. On the basis of a report that an activated sludge sample could produce α-branched polyesters when fed propionate,[24] we were able to identify CapPhaEC from the metagenome,[25] which can incorporate the α-branched monomers at high levels (36 ± 9%). From these components, we engineered the production of PHAs with different incorporation levels of α-branched (3–36%) and linear C_4 vs C_5 (36–80%) monomers. Characterization of the polymers produced by fermentation showed that, in contrast to PHB homopolymer, the α-branched polyesters displayed no observable melting transition, only a low glass transition temperature, indicating that these polymers are amorphous materials with no crystallinity.

RESULTS AND DISCUSSION

Discovery of a PHA Polymerase with Relaxed Monomer Selectivity. Natural PHA polyesters are most typically composed of the 3*R*-hydroxybutyrate monomer unit, which is generated from two acetyl-CoAs by Claisen condensation followed by ketoreduction using thiolase and ketoreductase enzymes (Figure 1).[26] To explore the ability of the canonical PHA pathway from *Ralstonia eutropha*[27] to produce structurally modified polymers, we tested the ability of RePhaABC genes to utilize propionyl-CoA as a building block. Upon feeding glucose and propionate to provide acetyl-CoA and propionyl-CoA, respectively, we observed only 0.04 ± 0.02% molar ratio of branched monomers in the isolated polyester (Supporting Information, Figure S1). Given the preference of RePhaA for linear products, the branched chain AsAcat3 thiolase was then substituted in its place. This change led to a 36-fold increase in branched monomers incorporated into the PHA to a total molar ratio of 1.2 ± 0.2% (Figure S1). However, the production of free branched hydroxyacids was found to increase 340-fold when the thiolases were exchanged, indicating that the ability of RePhaB and/or RePhaC to incorporate the propionate-derived monomers into PHA was limiting (Figure S1).

We then set out to identify a ketoreductase–PHA polymerase pair that could better incorporate branched monomers. In this regard, it had been reported that α-branched polyesters could be produced by activated sludge.[24] However, many of the bacterial species found in activated sludge are unculturable, so we thus analyzed the metagenome (GOLD Analysis Project ID: Ga0074232)[25] to find new PHA clusters. A seven-gene cluster for PHA production, likely derived from the unculturable bacterium *Candidatus Accumulibacter phophatis* clade IIA str. UW-1, was found (Figure S2). In this cluster, three genes encode for PHA polymerases (CapPhaC1 and the two-subunit CapPhaEC) and the four

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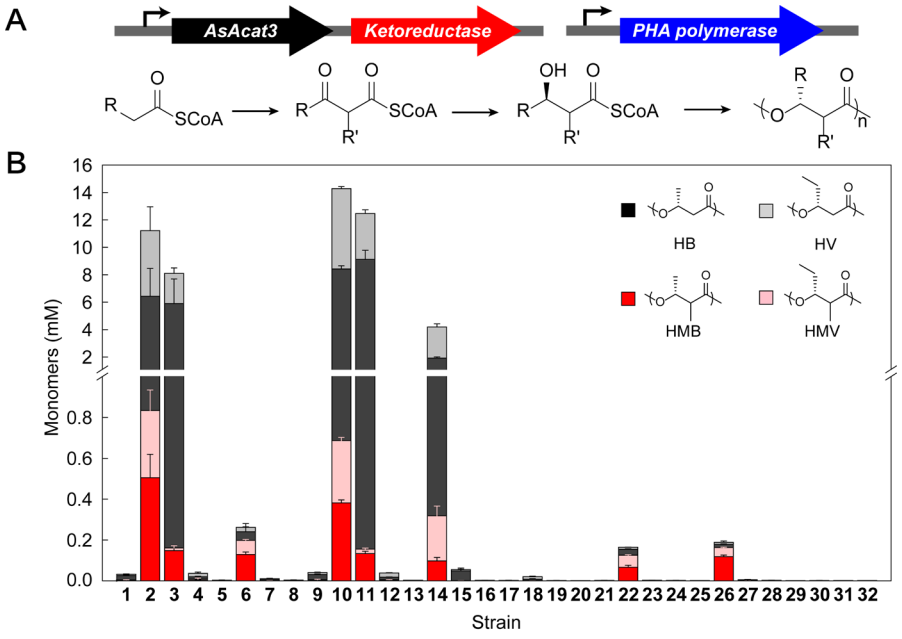

Strain	pT7-CapPhaC1	pT7-CapPhaEC	pT7-AcPhaPC*	pTrc33a
pAsAcat3-RePhaB	1	2	3	4
pAsAcat3-CapPhaB0	5	6	7	8
pAsAcat3-CapPhaB1	9	10	11	12
pAsAcat3-CapPhaB2	13	14	15	16
pAsAcat3-CapPhaB3	17	18	19	20
pCapPhaB0-CapPhaA	21	22	23	24
pAsAcat3-AsHadh2	25	26	27	28
pTrc99a	29	30	31	32

Figure 2. Screening combinations of ketoreductases and PHA polymerases for α-branched polyester production. (A) The branched polyester pathway is based on the AsAcat3 thiolase from *A. suum* (pTrc99a) with ketoreductase−PHA polymerase pairs (pTrc33a) varied. (B) Six ketoreductases and three PHA polymerases were combined and screened. The two vectors were cotransformed into *E. coli* BAP1[30] for polyester production. Strains were cultured for 2 days at 30 °C in TB containing 2% (w/v) glucose, 0.2% (w/v) sodium propionate, and 0.418% (w/v) MOPS in baffled flasks. Data are mean ± s.d. of biological replicates (n = 3). CapPhaA, putative thiolase from the *Candidatus A. phosphotis* cluster. AcPhaPC*, mutant PhaPC from *A. caviae*.[18]

genes between them were predicted to be thiolase (CapPhaA),[28] ketoreductase (CapPhaB0),[29] and two dehydratases with MaoC domain. Further homology search of the activated sludge metagenome using the RePhaB amino acid sequence as a search query resulted in 87 short-chain alcohol dehydrogenases candidates. Three sequences (CapPhaB1, CapPhaB2, and CapPhaB3; Table S1) were selected as candidates based on their location adjacent to a putative PHA-related gene (*phaR* or *phaA*).

The PHA expression system was assembled with synthetic genes encoding the thiolase (AsAcat3 or CapPhaA) and ketoreductase (CapPhaB0-3, RePhaB, and AsHadH2) as a single operon on the pTrc99a backbone (Figure 2A and Table S1). The three different PHA polymerases (CapPhaC1, CapPhaEC, and AcPhaC* which is a mutant PHA polymerase from *Aeromonas caviae* used to produce branched PHA from tiglic acid[18]) were then placed under the control of the T7*lac* promoter on the pTrc33a backbone. Plasmids for 18 different ketoreductase−PHA polymerase were constructed and transformed into *Escherichia coli* BAP1, a modified BL21(DE3) strain engineered to increase intracellular concentration of propionyl-CoA.[30] Strains **1**−**32** were cultured in TB broth containing glucose and propionate and tested for their ability to produce α-branched polymers (Figure 2B). The production screen shows that the CapPhaEC polymerase (strains **2**, **6**, **10**, **14**, **22**, and **26**) was the most active at incorporating α-branched monomers, with a 3-fold increase over AcPhaPC* (strains **3**, **7**, **11**, **15**, **23**, and **27**). In comparison, CapPhaC1 (strains **1**, **5**, **9**, **13**, **21**, and **25**) did not appear to be active with any ketoreductase. Of the ketoreductases, RePhaB surprisingly appeared to be the most active even compared to CapPhaB0, which is found in the gene cluster with CapPhaEC. AsHadH2 also demonstrated some visible activity (strain **26**) with CapPhaEC despite its preference for producing 3*S*-hydroxy branched products, possibly due to promiscuous production of 3*R*-configured products or by further metabolism resulting in racemization.

We then followed up the *in vitro* screening with *in vitro* biochemical experiments to explore the origin of the monomer distributions found in the PHA polymer products. A CapPhaEC expression plasmid containing a C-terminal His_6 tag of CapPhaC was constructed. By use of Ni-NTA affinity purification, the CapPhaEC complex was isolated cleanly, indicating that the two subunits bind tightly (Figure S3). 3*R*-Hydroxy-2-methylbutyryl-CoA was then prepared by chemoenzymatic synthesis (Figure S4) for polymerization assays[30] to test activity on the α-branched substrate in comparison to the canonical PHA polymerase RePhaC from *R. eutropha*. In comparing CapPhaEC against the canonical RePhaC, we observed that both enzymes demonstrate activity with the

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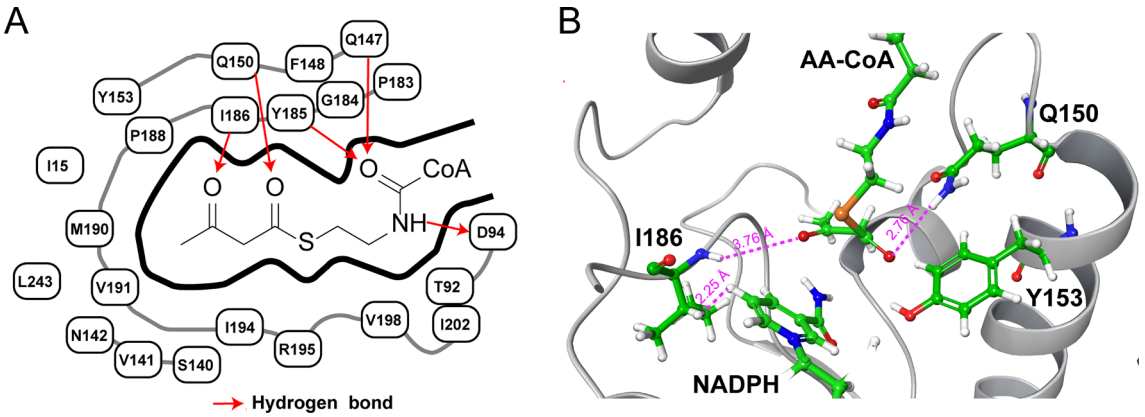
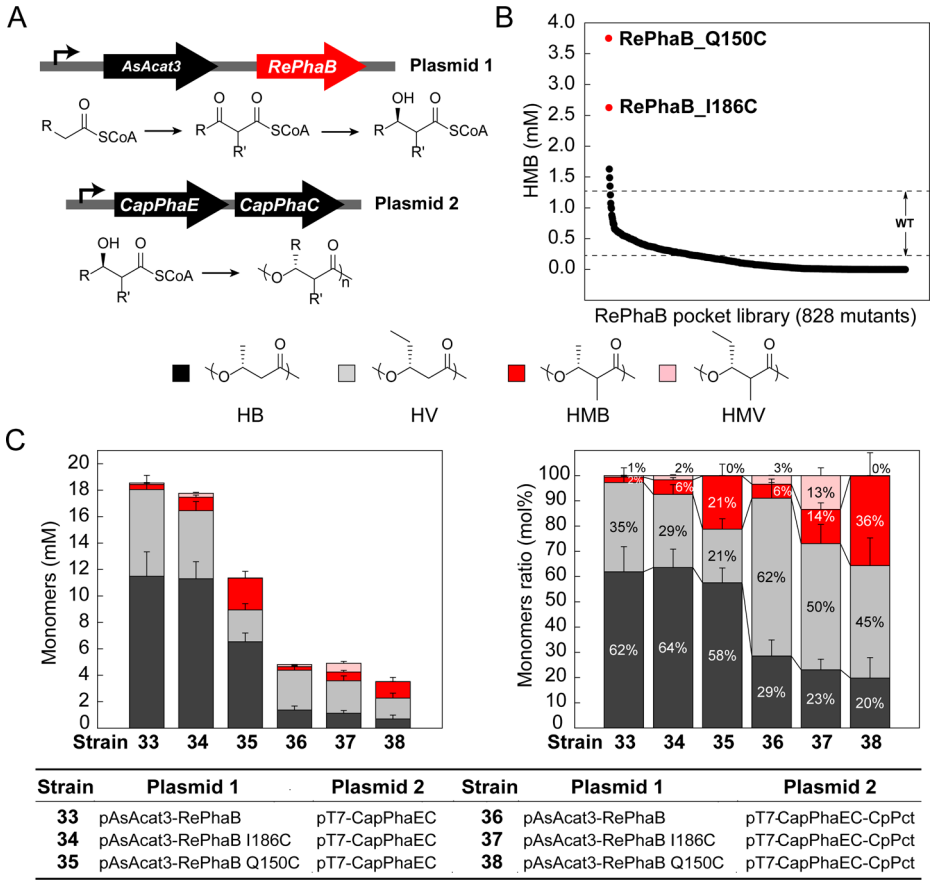


Figure 3. RePhaB pocket. (A) The pocket residues were selected based on two RePhaB structures (PDB No. 4N5M and 3VZS) with substrate acetoacetyl-CoA (AA-CoA), within a 5 Å distance around the acetoacetyl, cysteamine, and carbonyl of β-alanine moiety of substrate was used as the standard for pocket residues selection. Red arrows indicate possible hydrogen bonds. (B) 3D structure of RePhaB pocket showing the interaction of 3-oxo-butyryl-CoA with Q150 and I186. Y153 is believed to be the active residue which transfers the proton to the carbonyl group of substrate.[34] AA-CoA represents acetoacetyl-CoA.



Strain	Plasmid 1	Plasmid 2	Strain	Plasmid 1	Plasmid 2
33	pAsAcat3-RePhaB	pT7-CapPhaEC	**36**	pAsAcat3-RePhaB	pT7-CapPhaEC-CpPct
34	pAsAcat3-RePhaB I186C	pT7-CapPhaEC	**37**	pAsAcat3-RePhaB I186C	pT7-CapPhaEC-CpPct
35	pAsAcat3-RePhaB Q150C	pT7-CapPhaEC	**38**	pAsAcat3-RePhaB Q150C	pT7-CapPhaEC-CpPct

Figure 4. RePhaB pocket library screening for α-branched polyester production. (A) A two-plasmid system was constructed for production of α-polyester production. The first plasmid contains an operon containing a thiolase AsAcat3, a ketoreductase RePhaB driven by a single promoter. The second plasmid contains a PHA polymerase CapPhaEC. The RePhaB pocket library was made by NNK codon mutation based on the first plasmid. (B) Production performance of RePhaB pocket library toward α-branched monomer 3-hydroxy-2-methylbutyryl-CoA (HMB) in *E. coli* BAP1. (C) Production and characterization of monomer composition of PHA in engineered *E. coli* strains comparing WT, Q150C, and I186C RePhaB. A propionate-CoA transferase CpPct from *C. propionicum* was introduced in strains **36**, **37**, and **38** for further improvement of α-branched monomers ratio. Data are mean ± s.d. of biological replicates (n = 3).

linear 3*R*-hydroxybutyryl-CoA substrate as expected, but only CapPhaEC showed detectable activity with 3*R*-hydroxy-2-methylbutyryl-CoA (k_{cat}/K_M = (1.0 ± 0.2) × 10^3 M^{-1} s^{-1}) (Figure S3). It is interesting to note that the overall activity of CapPhaEC, measured by using the linear substrate (k_{cat}/K_M = (2.9 ± 0.2) × 10^5 M^{-1} s^{-1}), is close to 20-fold higher than that of RePhaC, making activity on noncanonical substrates easier to detect. While the relative ratio of activity to generate

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branched vs unbranched PHA is low (0.34%), the activity of CapPhaEC on branched substrates remains higher than other characterized PHA polymerases.[31] Furthermore, when branched and unbranched monomers are provided *in vivo*, CapPhaEC can incorporate the branched monomers at high molar ratios (strain **6**, 76 ± 6%; strain **22**, 76 ± 7%; strain **26**, 86 ± 5%), suggesting that the ketoreductase rather than CapPhaEC provides the more significant bottleneck for branch incorporation in this system.

It was interesting to note that strains containing CapPhaB0 (strains **6**, **7**, **22**, and **23**) did not support significant total PHA production given that it is located in the same gene cluster as CapPhaEC. We therefore purified His_6-CapPhaB0 (Figure S5) and characterized its activity with respect to 3*S*- and 3*R*-hydroxybutyryl-CoA oxidation. This comparison shows that activity toward 3*S*-hydroxybutyryl-CoA was 352-fold greater than 3*R*-hydroxybutyryl-CoA (Figure S5), indicating that the direct product of CapPhaB0 is not incorporated into PHA. However, it may be possible that the two MaoC dehydratases found in the gene cluster could be involved in its conversion to 3*R*-products. To test this possibility, two MaoC dehydratases on one expression vector were introduced into PHA pathways with different ketoreductases. No significant difference was observed compared with their controls, suggesting that the conversion between 3*S*- and 3*R*-product does not occur in this pathway (Figure S6). Taken together, we have constructed a functional pathway for α-branched polyester production (AsAcat3-RePhaB-CapPhaEC) and identified the ketoreductase as the bottleneck for incorporation of branched monomers into PHA *in vivo*.

Engineering the Ketoreductase RePhaB−Substrate Selectivity. *In vitro* steady-state kinetic characterization of RePhaB showed that it exhibits an ∼80-fold preference with respect to k_{cat}/K_M for the unbranched 3-oxobutyryl-CoA ((4.1 ± 0.8) × 10^7 M^{-1} s^{-1}) over the branched 3-oxo-2-methylbutyryl-CoA ((5.1 ± 0.3) × 10^5 M^{-1} s^{-1}) (Figure S7). Hence, enhancing the selectivity of RePhaB toward α-branched substrates could be an effective way to increase the ratio of α-branched monomers in the PHA polyesters. With this goal in mind, we performed saturation mutagenesis of the active site pocket residues[32] based on the published RePhaB crystal structures determined by X-ray diffraction (PDB No. 4N5M and 3VZS).[33,34] Twenty-two positions located within 5 Å of the acetoacetyl, cysteamine, and carbonyl of β-alanine moiety of the bound 3-oxobutyryl-CoA substrate (Figure 3) were identified and mutated by using primers containing NNK degenerate codons (Table S2). The mutants were screened *in vivo* by using a 24-deep well plate assay by pooling individual transformants corresponding to each position (37 colonies per pocket residue site in average) for a total of 828 RePhaB mutants. The PHA was extracted and hydrolyzed from each pooled set of mutants and analyzed for monomer composition by LC-QQQ MS (Figures S8 and S9).

Analysis of the mutants showed that ∼15% of the library exhibited higher straight-chain monomer incorporation (3-hydroxybutyrate, HB; 3-hydroxyvalerate, HV) into the PHAs compared to wild-type, whereas 4% of the library demonstrated improved branched monomer incorporation (3-hydroxy-2-methylbutyrate, HMB; 3-hydroxy-2-methylvalerate, HMV) (Figure S10). Of these, the RePhaB Q150C and I186C mutants appeared to be significantly improved (Figure 4B). Further validation under production conditions showed that these mutants did indeed increase incorporation of branched monomers to 21 ± 5% (Q150C) and 7.4 ± 1.2% (I186C) compared to wild-type (2.5 ± 0.5%) (Figure 4C,D). Interestingly, these two mutants appear to have different behaviors with Q50C improving only α-methyl branch incorporation (HMB) whereas I186C can increase accommodation for both the α-methyl branch and the longer C_5 branched monomer (HMB and HMV). Neither additional screening of these two positions to obtain full coverage nor combining the two mutations lead to identification of mutants with additional gains in branched monomer incorporation (Figure S11). However, changes in selectivity were observed with regard to incorporation of the linear C_4 (HB) vs C_5 (HV) monomers. RePhaB Q150L showed similar capability for branched monomer incorporation as the Q150C mutant but lower incorporation of the C_5 linear monomer, while the RePhaB I186Q showed improved incorporation of the C_5 linear monomer over the C_4 HB with almost complete loss of branched monomer incorporation.

Biochemical characterization of RePhaB Q150C shows that its accommodation of the α-branched substrate increased about 11-fold when comparing the relative ratios of the k_{cat}/K_Ms for 3-oxo-2-methylbutyryl-CoA:3-oxobutyryl-CoA (14 ± 9%) to that of the wild-type enzyme (1.3 ± 0.3%) (Figure S7). However, the magnitude of k_{cat}/K_M in the mutant decreased by 1 and 2 orders of magnitude for the branched vs unbranched substrate, respectively. Examining the structure of RePhaB, Q150 could possibly be involved in hydrogen bond with the carbonyl group of the substrate thioester, which could assist in positioning it for catalysis (Figure 3B). Exchange of glutamine for the smaller cysteine residue may allow space for the α-methyl group but could also perturb structural elements involved in catalysis. Despite this catalytic defect, *in vivo* production of PHA is maintained at reasonable level compared to wild-type (61 ± 13%, Figure 4C).

Isolation and Characterization of α-Branched Polyesters. With an improved ketoreductase in hand, we tried an additional strategy to increase the incorporation of α-branched monomers by introducing a propionate-CoA transferase from *Clostridium propionicum* (CpPct) to enhance propionyl-CoA supply into the pathway. With this addition, the incorporation of α-branched monomers was further improved to 27 ± 6% and 36 ± 9% for pathways with RePhaB I186C and RePhaB Q150C, respectively (Figure 4D). We then scaled up preparation of samples from various strains utilizing RePhaB Q150C, Q150L, I186C, and I186Q to characterize the properties of the resulting PHA polyesters. The modified PHAs were extracted from cells with chloroform[35] and analyzed by 1D- and 2D-NMR spectroscopy to further confirm the insertion of the branched monomers within the polymer (Table S3 and Figure S12). Compared to the control polyester samples that are composed of straight-chain monomers 3*R*-hydroxybutyrate and/or 3*R*-hydroxyvalerate, cross-peaks corresponding to the α-branched monomers, 3*R*-2-methyl-hydroxybutyrate and 3*R*-hydroxy-2-methylvalerate, were found in the polyester samples from strains containing the PhaB Q150C and I186C mutants (Figure S12).

Characterization of the polymers produced by fermentation shows that the copolyesters with 22%−32% branched monomers (Table S4; **1**, **2**, and **5**) have typical weight-average molar masses (M_w) in the range (1.7−2.0) × 10^5 g mol^{-1} with dispersity values ($Đ$) ranging from 2.1 to 2.2 (Figures S13 and S14). Thermogravimetric analysis indicates that the copolymers show 5% mass loss temperatures ($T_{d,5\%}$) in the range of

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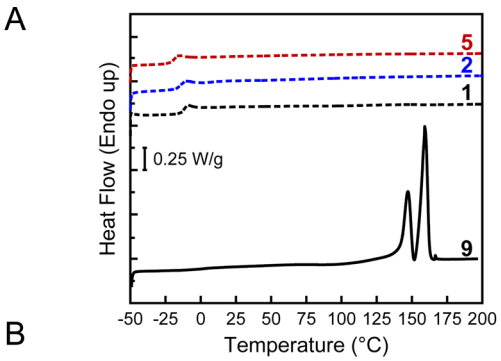
241−266 °C compared to a $T_{d,5\%}$ value of 227 °C for the commercial PHB homopolymer (Figure S15). The differential scanning calorimetry (DSC) thermograms of the PHA polyesters (samples **1**, **2**, and **5**) with at least 22 mol % of HMV or HMB display lowered glass transition temperatures (T_g = −13 to −20 °C) compared to typical values for PHB homopolymer and no observable melting transition, indicating that these polymers are amorphous materials with no crystallinity (Figure 5). Polymers derived from the RePhaB



Sample	PHA composition (mol%)				Thermal properties		
	HB	HMB	HV	HMV	T_g (°C)	T_m (°C)[a]	ΔH_m (J/g)[b]
1	40%	22%	38%	0%	-13		
2	49%	28%	23%	0%	-15		
5	15%	32%	52%	0%	-20		
9	100%	0%	0%	0%	0	146, 158	83.0

Figure 5. Thermal properties of α-branched polyesters. (A) Differential scanning calorimetry (DSC) traces of PHA samples **1**, **2**, and **5** produced by engineered *E. coli* strains in this work and control sample **9** (homopolymer PHB from Sigma-Aldrich). Traces are from the second heating cycle with a heating rate of 10 °C min^{-1} and are shifted vertically for clarity. (B) Samples information and thermal property values from DSC scan. HB, 3-hydroxybutyrate; HMB, 3-hydroxy-2-methylbutyrate; HV, 3-hydroxyvalerate; HMV, 3-hydroxy-2-methylvalerate; T_g, glass-transition temperature; T_m, melting temperature; ΔH_m, corrected enthalpy of fusion. [a]A blank space indicates that no transition was observed. [b]The corrected ΔH_m value reported subtracts the ΔH_c value from ΔH_m determined from instrumental software.

I186Q mutants with no α-branching but high linear C_5 content (HV, 62%) are similarly amorphous (Figure S16). As such, these amorphous α-branched polyesters are expected to exhibit mechanical behavior unlike the semicrystalline PHB homopolymer. Potential improvements, such as higher elasticity and flexibility due to a lack of crystallinity, would be useful in overcoming some of the limitations associated with the mechanical properties of PHB and achieving increased toughness, strength, and ductility in bioplastic-derived materials.[36]

CONCLUSIONS

Polyesters from the PHA family are a promising option in the search for more sustainable polymer materials as they have the potential to be both synthesized and broken down by living organisms. However, one challenge in their commercialization is that they have been optimized by evolution for carbon storage rather than industrial applications. As such, the tuning of their mechanical properties using alternative monomers could enable the development of PHA polymers for various commercial usages. Previous reports suggest that the introduction of α-branches into PHAs could both improve thermal stability for polymer processing and reduce issues related to brittleness by decreasing crystallinity.[18,19] In this work, we have engineered a PHA pathway that is capable of incorporating up to 36% α-branched monomers. This pathway utilizes a thiolase[21,22] that preferentially introduces an α-methyl branch as well as an engineered ketoreductase to accept the methyl substituent. Using the metagenome of activated sludge from wastewater treatment,[25] we were further able to identify a new PHA polymerase that showed a natural capability to produce α-branched polyesters. The enzyme CapPhaEC complex was shown to be able to polymerize 3*R*-hydroxy-2-methylbutyryl-CoA *in vitro* while the canonical PHA polymerase PhaC from *R. eutropha*[27] did not display any detectable activity.

The high molar mass α-branched polyesters produced by fermentation in this study display only a low glass transition temperature (−13 to −20 °C) and no observable melting transition, indicating that these polymers are entirely amorphous. These studies provide insight into the use of natural and engineered enzymes for developing new cellular routes for production of α-branched polyesters by fermentation of engineered microbes. Interestingly, characterization of these α-branched polyesters shows that their properties can be tuned, allowing for improved thermal properties compared to the natural PHB homopolymer as well as elimination of the crystallinity. Taken together, these α-branched polyesters show that non-native monomers can expand the material properties of PHAs in new directions and enable the production of new biopolymers from biomass.[37]

METHODS

Detailed procedures for plasmid construction, protein purification, acyl-CoA substrate synthesis, cell culture, biochemical assays, and polyester analysis are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b08585.

Materials and methods, DNA and protein sequences, plasmid construction, protein purification; acyl-CoA substrate synthesis, cell culture; biochemical assays, and polyester analysis (PDF)

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