microbial biotechnology

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Microbial synthesis of a novel terpolyester P(LA-*co*-3HB-*co*-3HP) from low-cost substrates

Yilin Ren,[1] Dechuan Meng,[1] Linping Wu,[2] Jinchun Chen,[1] Qiong Wu[1] and Guo-Qiang Chen[1,3,4,*]

[1]*Center for Synthetic and Systems Biology, School of Life Science, Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing 100084, China.*

[2]*Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, People's Republic of China.*

[3]*Center for Nano and Micro Mechanics, Tsinghua University, Beijing 100084, China.*

[4]*MOE Key Lab of Industrial Biocatalysis, Dept Chemical Engineering, Tsinghua University, Beijing 100084, China.*

Summary

Polylactide (PLA) is a bio-based plastic commonly synthesized by chemical catalytic reaction using lactic acid (LA) as a substrate. Here, novel LA-containing terpolyesters, namely, P[LA-*co*-3-hydroxybutyrate (3HB)-*co*-3-hydroxypropionate (3HP)], short as PLBP, were successfully synthesized for the first time by a recombinant *Escherichia coli* harbouring polyhydroxyalkanoate (PHA) synthase from *Pseudomonas stutzeri* (PhaC1$_{Ps}$) with 4-point mutations at E130D, S325T, S477G and Q481K, and 3-hydroxypropionyl-CoA (3HP-CoA) synthesis pathway from glycerol, 3-hydroxybutyryl-CoA (3HB-CoA) as well as lactyl-CoA (LA-CoA) pathways from glucose. Combining these pathways with the PHA synthase mutant phaC1$_{Ps}$ (E130D S325T S477G Q481K), the random terpolyester P(LA-*co*-3HB-*co*-3HP), or PLBP, was structurally confirmed by nuclear magnetic resonance to consist of 2 mol% LA, 90 mol% 3HB, and 8 mol% 3HP respectively. Remarkably, the PLBP terpolyester was produced from low-cost sustainable glycerol and glucose. Monomer ratios of PLBP could be regulated by ratios of glycerol to glucose. Other terpolyester thermal and mechanical properties can be manipulated by adjusting the monomer ratios. More PLBP applications are to be expected.

Received 5 September, 2016; revised 17 September, 2016; accepted 15 October, 2016. *For correspondence. E-mail: chengq@mail.tsinghua.edu.cn; Tel. +86-10-62783844; Fax +86-10-62794217.

Microbial Biotechnology (2017) **10**(2), 371–380
doi:10.1111/1751-7915.12453

Funding information

Plasmids pSEVA351 and pBHR68 were kindly donated by Professor Victor de Lorenzo of CSIC/Spain and Professor Alexander Steinbűchel of Műnster Univ/Germany respectively. This research was financially supported by the independent research program of Tsinghua University (grant no. 2015THZ10) and National Natural Science Foundation of China (grant no. 31430003). The Dutch Polymer Institute also provides partial funding to support this study.

Introduction

As increasing global warming and plastic pollution threaten human sustainability, materials from renewable biomass are attracting attention due to their biodegradability and environmentally friendliness. Polylactide (PLA) is a representative of bio-based biodegradable polyester synthesized via combination of microbial lactic acid (LA) fermentation and chemical polymerization of lactide (Sudesh and Iwata, 2008; Nampoothiri *et al.*, 2010; Park *et al.*, 2012a,b). PLA has been used in areas of biomedical implants, food packaging and drug delivery. However, the complicated synthetic process including fermentation for LA production, LA purification, lactide esterification and lactide ring-opening polymerization increases the cost, meanwhile the heavy metal residues in the final polymer products could limit its food or medical applications. In addition, PLA poor thermal and mechanical properties are also adverse to its large-scale applications.

Polyhydroxyalkanoates (PHA) are a family of diverse polyesters synthesized by a variety of bacteria as intracellular carbon and energy storage compounds (Li *et al.*, 2007; Chen and Hajnal, 2015; Koller and Rodríguez-Contreras, 2015). As biorenewable and biodegradable materials, the diversity of PHA provides different physical properties to suit various applications (Chen, 2009; Brigham and Sinskey, 2012; Koller, 2014). Copolymerization of LA with other hydroxyalkanoate (HA) monomers via microbial synthesis is one of the effective methods to improve the physical properties of PLA or PHA (Li *et al.*, 2010).

Recently, several engineered PHA synthases able to utilize lactyl-CoA (LA-CoA) and 3-hydroxybutyric-CoA (3HB-CoA) as substrates were reported (Taguchi *et al.*, 2008; Yang *et al.*, 2011; Ochi *et al.*, 2013). To deliver LA-CoA in recombinant *Escherichia coli*, propionyl-CoA transferases (Pct) in alanine fermentation pathway of

several organisms including *Clostridium propionicum* and *Megasphaera elsdenii* were expressed in host strains respectively. A 6 mol% LA fraction in the copolyester was achieved.[15] Subsequently, efforts were made on increasing the LA ratio in the copolyester by regulating the metabolic flux or evolving the PHA synthase (Jung *et al*., 2010; Yamada *et al*., 2010; Shozui *et al*., 2011), and also by the uses of different organisms in addition to commonly used *E. coli*, including *Corynebacterium glutamicum*, *Ralstonia eutropha* and *Sinorhizobium meliloti* (Song *et al*., 2012; Park *et al*., 2013; Tran and Charles, 2015). In addition, low-cost carbon substrates, such as glucose and xylose, were applied to synthesize LA-based copolyesters (Park *et al*., 2012a,b; Nduko *et al*., 2014; Salamanca-Cardona *et al*., 2014), which could reduce production cost and facilitate its industrialization.

As mentioned, PLA has the major deficiencies of poor flexibility, ductility and thermal resistance, and copolymerization is possibly effective to improve the PLA properties. Therefore, LA copolyesters were investigated with P(LA-*co*-3HB) as a representative (Taguchi *et al*., 2008; Jung *et al*., 2010; Yamada *et al*., 2010; Shozui *et al*., 2011; Yang *et al*., 2011; Ochi *et al*., 2013). However, mechanical properties of PHB are similar to PLA, therefore, other monomers such as 3-hydroxyvalerates (3HV), 3-hydroxyhexanoate (3HHx) and glycolate (GA) were introduced into the LA copolyesters for property improvements (Shozui *et al*., 2010a,b; Choi *et al*., 2016; Li *et al*., 2016). Remarkably, poly(3-hydroxypropionate) (P3HP), a relatively new PHA family member, has become very interesting due to its strong mechanical properties including an elongation at break of more than 600% and Young's modulus of 3 GPa (Andreeßen and Steinbüchel, 2010; Zhou *et al*., 2011a,b; Meng *et al*., 2012). Thus, 3HP monomers in LA copolyesters could very likely compensate for the shortcomings of PLA. This study attempted to biosynthesize a LA-containing terpolyester consisting of LA, 3HP and 3HB with improved properties over PLA homopolyester.

Results and discussion

Engineering a pathway for biosynthesis of P(LA-co-3HB-co-3HP) from unrelated carbon source

For the above-mentioned purpose, the substrate specificity of PHA synthase is the most important factor determining the monomer constituents incorporated into PHA. A mutant PHA synthase from *Pseudomonas stutzeri* strain 1317 ($PhaC1_{Ps}$) with 4-point mutations at E130D, S325T, S477G and Q481K was prepared as stated below, which could incorporate LA and other 3-HAs into PLBP terpolyester. In addition, a 3HP synthetic pathway combined with LA and 3HB pathways were constructed to supply the monomers for the terpolyester synthesis (Fig. 1).

The engineering pathway allows adjustments of 3HB, LA and 3HP monomer ratios in the terpolyesters by feeding various ratios of glucose to glycerol. When a higher LA ratio is needed, LA can be added to the culture to supply additional LA precursor.

Engineering a PHA synthase able to polymerize LA

The substrate specificity into PHA synthase is the most important factor determining the monomer constituents incorporated into PHA. Common PHA synthases are capable of polymerizing the 3-hydroxyalkanoates (3HAs) CoA with variable carbon chain lengths (Bernd, 2003). LA, which is the monomer of PLA, is a 2HA, which is not accepted by natural PHA synthases. To synthesize PLBP terpolyesters, a mutant of PHA synthase, able to incorporate LA and other 3-HAs at the same time was obtained via site-directed mutagenesis on type II PHA synthase $PhaC1_{Ps}$ of *P. stutzeri* 1317 which has already a versatile substrate specificity (Park *et al*., 2015). Five mutation sites on $phaC1_{ps}$ ($phaC2_{ps}$), including E130D,

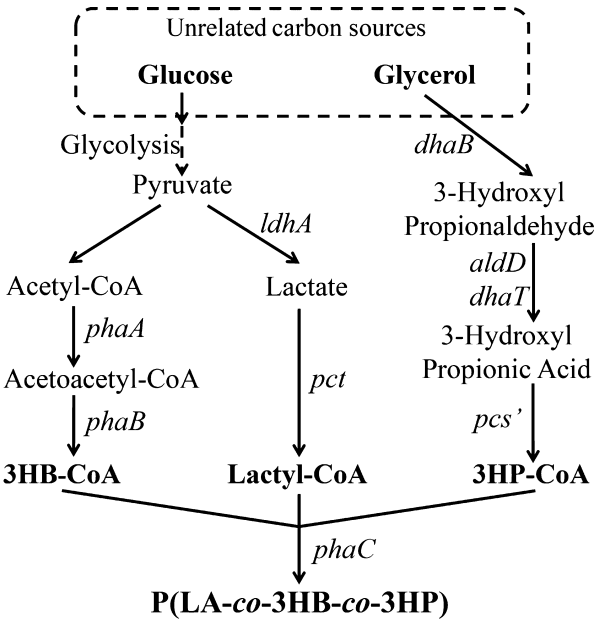

Fig. 1. Metabolically engineered pathways for production of terpolyester P(LA-*co*-3HB-*co*-3HP) or PLBP from unrelated carbon source. Enzymes encoded by each gene are described below: *phaA*, β-ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *ldhA*, lactate dehydrogenase; *pct*, propionyl-CoA transferase; *dhaB*, glycerol dehydratase; *dhaT*, 1,3-propanediol dehydrogenase; *aldD*, aldehyde dehydrogenase; *pcs*', propanoyl-CoA synthetase; *phaC*, PHA synthase from *Pseudomonas stutzeri* strain 1317 ($PhaC1_{Ps}$) with 4-point mutations at E130D, S325T, S477G and Q481K.

S325(326)T, F392(393)S, S477(478)G and Q481(482)K, were selected based on the alignment result with the previous LA-polymerizing enzymes (Fig. 2) (Taguchi *et al*., 2008; Jung *et al*., 2010; Yamada *et al*., 2010; Yang *et al*., 2011; Chuah *et al*., 2013; Ochi *et al*., 2013). With various combinations of these site mutations, up to 20 *phaC1*$_{Ps}$ and *phaC2*$_{Ps}$ variants were constructed (Table S1). To compare their activities towards LA polymerization, the 3HB-CoA synthetic pathway including genes of β-ketothiolase (*phaA*), NADPH-dependent acetoacetyl-CoA reductase (*phaB*) of *R. eutropha*, LA-CoA synthetic pathway including lactate dehydrogenase (*ldhA*) of *E. coli* and propionyl-CoA transferase (*pct*) of *Clostridium propionicum* were constructed into a series of plasmids termed pBLPCAB-X (X represents the specific *phaC* variant in the plasmid). pBLPCAB-Xs were derived from pBHR68 (kindly donated by Prof Steinbűchel of Műnster Univ/Germany), with the three genes of *ldhA*, *pct* and *phaC* variant inserted downstream the P_{re} promoter. The LA polymerization activities of typical *phaC* variants were determined by expressing the corresponding plasmids in *E. coli* S17-1 (Table 1).

When analysing the PHA accumulation capacity, single-point mutation on *phaC*$_{Ps}$ was found insufficient for LA polymerizations (data not shown). By combining Q481K and S325T mutations in *phaC1*$_{Ps}$, the recombinant started to synthesize P(2.81% LA-*co*-3HB) copolyester, confirming two-point mutant *phaC1*$_{Ps}$ (Q481K S325T) capable of polymerizing LA into PHA. Additional mutations on E130D and S477G to the above two points mutant increased the LA specificity as the LA ratio in the PHA copolyester produced by the recombinant expressing *phaC1*$_{Ps}$ (Q481K S325T E130D S477G) increased to over 5% (Table 1). However, mutation F392S had a negative effect on LA incorporation, as F392S added to *phaC1*$_{Ps}$ (Q481K S325T E130D S477G) reduced LA ratio in the copolyester to 3% (Table 1). Interestingly, *phaC2*$_{Ps}$ showed no activity towards LA-CoA no matter what point mutations were introduced. As a result, the LA polymerizing mutant enzyme PhaC1$_{Ps}$ (Q481K

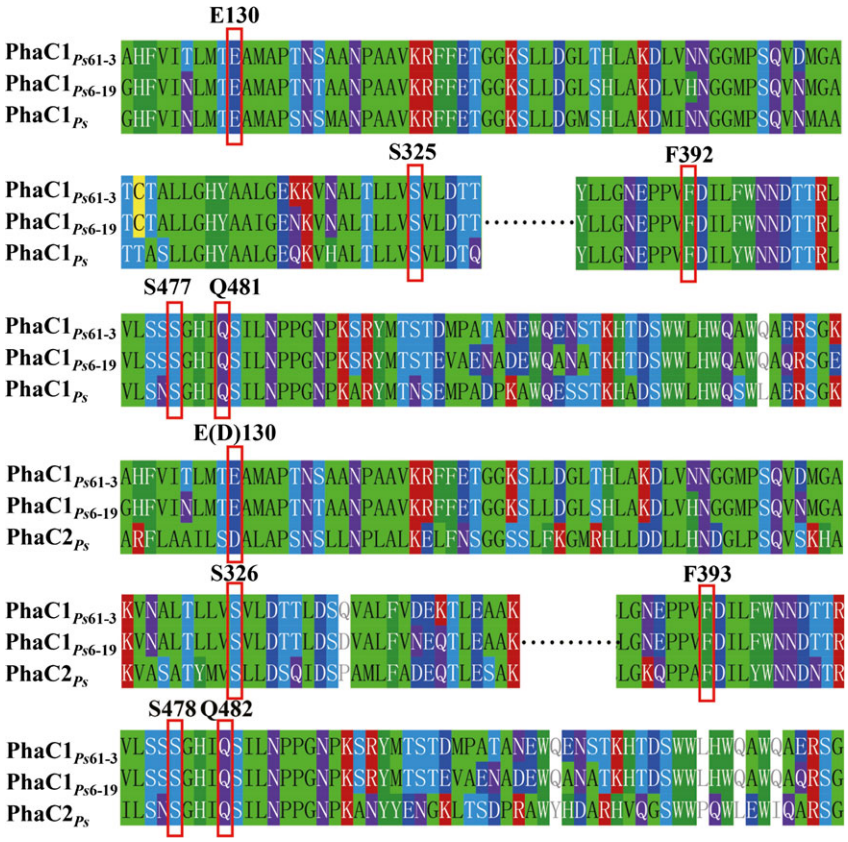

Fig. 2. Alignment of *phaC1*$_{Ps}$ and *phaC2*$_{Ps}$ with previous LA polymerizing enzymes reported (Taguchi *et al*., 2008; Yamada *et al*., 2010; Yang *et al*., 2011). *phaC1*$_{Ps}$ and *phaC2*$_{Ps}$ from *Pseudomonas stutzeri* were aligned with reported LA polymerizing enzymes which were *phaC1*$_{Ps61\text{-}3}$ from *Pseudomonas sp.* 61-3 and *phaC1*$_{Ps6\text{-}19}$ from *Pseudomonas sp.* MEBL6-19. Several constitutive sites were found dominating the LA polymerizing capacity (in the red frames). Abbreviations: phaC1$_{Ps}$, *Pseudomonas stutzeri* phaC1; phaC2$_{Ps}$, *Pseudomonas stutzeri* phaC2; phaC1$_{Ps61\text{-}3}$, *Pseudomonas sp.* 61-3 phaC1; phaC1$_{Ps6\text{-}19}$, *Pseudomonas sp.* MEBL6-19 phaC1; E, glutamic acid; D, aspartic acid; S, serine; F, phenylalanine; Q, glutamine. Five mutation sites on *phaC1*$_{ps}$ (*phaC2*$_{ps}$), including E130D, S325(326)T, F392(393)S, S477(478)G and Q481(482)K, were selected based on the alignment result with the LA polymerizing enzymes.

Table 1. LA polymerization activities of typical *phaC* variants.

E. coli	$phaC_{Ps}$ variants	CDM (g L^{-1})	PHA/CDM (wt%)	LA (mol%)
S-pBLPCAB1	$phaC1_{Ps}$	4.03 ± 0.27	39.22 ± 0.58	0
S-pBLPCAB1-2	$phaC1_{Ps}$ (Q481K S325T)	4.18 ± 0.13	40.12 ± 0.23	2.81 ± 0.30
S-pBLPCAB1-4	$phaC1_{Ps}$ (Q481K S325T E130D S477G)	3.13 ± 0.31	36.94 ± 0.38	5.01 ± 1.24
S-pBLPCAB1-5	$phaC1_{Ps}$ (Q481K S325T E130D S477G F392S)	3.01 ± 0.22	37.51 ± 0.23	3.01 ± 0.72
S-pBLPCAB2	$phaC2_{Ps}$	3.24 ± 0.09	41.50 ± 0.61	0
S-pBLPCAB2-2	$phaC2_{Ps}$ (Q482K S326T)	4.15 ± 0.43	37.71 ± 0.49	0
S-pBLPCAB2-3	$phaC2_{Ps}$ (Q482K S326T S478G)	3.76 ± 0.52	36.13 ± 0.77	0

Recombinant strains were cultivated for 48 h in shake flasks. The data are the averages of three parallel experiments.
LA, lactate; CDM, cell dry mass.

S325T E130D S477G) with the highest efficiency was obtained compared with other *phaC* wild type or mutant enzymes (Table 1 and Table S2). We therefore named the plasmid pBLPCAB1-4-containing $phaC1_{Ps}$ (Q481K S325T E130D S477G) plasmid pLA in further studies.

Construction of an effective PLBP synthetic system

Aimed to produce a novel terpolyester PLBP, three synthetic pathways for each constituent were constructed (Fig. 1), including PHB and PLA synthetic routes that were reported. The P3HP synthetic pathway was focused on as it would improve the mechanical properties of the terpolyesters.

P3HP could be synthesized from 1, 3-propanediol, glycerol and glucose (Andreeßen and Steinbüchel, 2010; Zhou *et al*., 2011a,b; Meng *et al*., 2015). Glycerol was chosen in this study to regulate 3HP ratio in the copolyesters, whereas glucose was the substrate for PLA and 3HB synthesis. The 3HP synthetic pathway from glycerol consisted of genes encoding glycerol dehydratase (*dhaB*) of *Klebsiella pneumoniae*, 1,3-propanediol dehydrogenase (*dhaT*) and aldehyde dehydrogenase (*aldD*) of *Pseudomonas putida* KT2442 and ACS domain of trifunctional propionyl-CoA synthetase (*pcs*') functioning as a CoA ligase of *Chloroflexus aurantiacus* (Andreeßen and Steinbüchel, 2010; Zhou *et al*., 2011a,b; Meng *et al*., 2012). The p3HP1p plasmid was constructed based on the pSEVA351 for P3HP production and contained genes of *dhaT*, *aldD*, *dhaB* and *pcs*' (Silva-Rocha *et al*., 2013). Gene fragments *dhaT-aldD*, *pcs* and *dhaB* were amplified from plasmid pZQ03, pDC02 and pZQ01 respectively. Subsequently, the pSEVA351 backbone was ligated with these three fragments by Gibson assembly. When strengthen the expression of dominant gene *dhaB* controlling glycerol utilization, the optimized plasmid p3HP2p exhibited enhanced efficiency in P3HP synthesis when it was co-expressed with pBHR68 in recombinant *E. coli* S17-1 (Table S3).

Finally, recombinant *E. coli* S-LA-3HP harbouring plasmids pLA and p3HP2p was obtained. When cultivated in LB medium supplemented with 20 g L^{-1} glucose and 10 g L^{-1} glycerol, a new terpolyester P(90.41 mol% 3HB-*co*-7.78 mol% 3HP-*co*-1.81 mol% LA) was successfully synthesized for the first time from unrelated carbon sources glucose and glycerol.

Nuclear magnetic resonance analyses of the PLBP terpolyester

The composition and monomer sequence distribution of P(3HB-*co*-3HP-*co*-LA) was confirmed by nuclear magnetic resonance (NMR) (Fig. 3). From the ^{1}H NMR spectra (Fig. 3A), there were not only well-characterized proton resonances of B(2), B(3) and B(4) in 3HB units (3HB abbreviated as B, 3HP monomer abbreviated as P and LA abbreviated as A) but also additional four proton resonances assigned to the 3HP units such as P(2), P (3) and LA units including A(2) and A(3) with identical intensities based on the previous studies (Park *et al*., 2013, 2015; Meng *et al*., 2015). The molar ratio of 3HB, 3HP and LA in the PHA copolyester was 90.41%, 7.78% and 1.81%, respectively, as calculated using the intensity of B(3), P(3) and A(2). The individual carbon species in 3HB, 3HP and LA monomer were also identified by specific ^{13}C NMR (Fig. 3B). The expanded spectra of individual splitting resonance of carboxyl carbon B(1), P (1) and A(1) in the copolyester were split into multiple peaks (Fig. 3C), which were assigned to the 3HB-centred (B(1)*B), 3HP-centred (P(1)*P), LA-centred (A (1)*A) and the three units comonomer sequences [B(1) *P(1)*A(1)] (N*M represents the interaction of monomer N and M) (Meng *et al*., 2015; Park *et al*., 2015). This phenomenon was due to different sequence arrangements of 3HB, 3HP and LA monomers in the polymer chains, which is common in random PHA copolyesters (Hu *et al*., 2011; Tripathi *et al*., 2012).

The tacticity distribution of tri-block copolyester was studied via ^{13}C NMR based on various stereo-sequences. The good resolution of methylene regions B (2) was chosen as an example for analysis. Two sharp peaks corresponding to B(2)*B and B(2)*P*A were observed (Fig. 3D). No split in each peak was visible, indicating that the stereo-sequence was isotactic. If the

polymer is syndiotactic or atactic, some diad or quadruple peaks should be observed in ^{13}C NMR (Kemnitzer *et al.*, 1993; Hocking and Marchessault, 1995). The above detailed analysis of ^{1}H NMR and ^{13}C NMR spectra clearly indicated that PHA was a random copolyester P(3HB-*co*-3HP-*co*-LA) with an isotactic microstructure.

Regulation of the monomer ratios in PLBP terpolyesters

Recombinant *E. coli* S17-1 harbouring genes described in Fig. 1 could produce terpolyesters PLBP consisting of LA, 3HB and 3HP when grown in a mixture of glucose and glycerol as substrates (Table 2). The terpolyesters PLBP were fully synthesized from unrelated carbon sources, which was more economic for PHA synthesis (Hermann-Krauss *et al.*, 2013; Povolo *et al.*, 2013). Compositions of monomers LA, 3HB and 3HP in PLBP could be adjusted by changing the glucose-to-glycerol ratios.

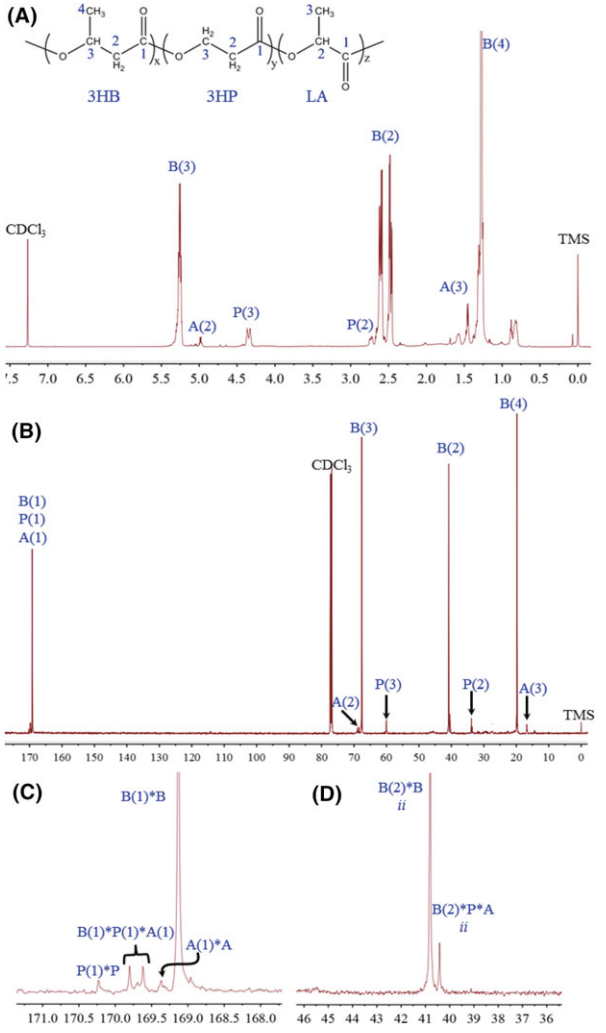

Fig. 3. NMR analysis of PLBP terpolyester. ^{1}H NMR spectra (A) and ^{13}C NMR spectra (B) of random copolyester P(3HB-*co*-3HP-*co*-LA) containing 90.41 mol% 3HB, 7.78 mol% 3HP and 1.81 mol% LA, respectively, and its expanded ^{13}C NMR spectra of carboxyl carbon [B(1), P(1), A(1)] area (C) and methylene regions (D) in the terpolyester. B, P and A refer to 3HB, 3HP and LA; numbering schemes were the same as molecular formulations of polyester indicated in the inset in (A). N*M represents the interaction of monomer N and M. "i" indicates "isotactic." Chemical shifts are in ppm and tetramethylsilane (TMS) was employed as an internal chemical shift standard.

Lactic acid ratio was as low as 1.81% in the terpolyester when PLBP was produced by the wild-type *E. coli* S17-1 harbouring genes described in Fig. 1. To increase the LA ratio in PLBP, the synthetic pathway was optimized and the competitive pathways of LA were deleted. Gene *ldhA* was inserted in pLA plasmid and was overexpressed to produce more LA as *ldhA* functions in converting pyruvate into LA (Jung *et al.*, 2010). However, it was found that overexpression of *ldhA* alone could not enhance the LA content in the terpolyester (Table S4) as a gene *dld* could convert LA back into pyruvate (Dym *et al.*, 2000; Choi *et al.*, 2016). Unexpectedly, the recombinant strain-containing plasmid pLA' without *ldhA*, replacing pLA plasmid of the PLBP synthetic system, synthesized a PLBP terpolyester with an increased LA ratio under the same culture conditions (Fig. S1.2). The deletion of *ldhA* in pLA plasmid resulted in a transcriptional change in those genes downstream the same promoter for the ones that were closer to the promoter (Fig. S1.1). It was proven that the transcriptional level of *pct* was correlated with the LA ratio in PLBP terpolyesters from the RT-PCR results (Fig. S1.2). It suggested that PCT-mediated CoA transfer reaction that transformed LA into LA-CoA was the time-limiting step of LA polymerization. Meanwhile, to improve LA production, a competitive pathway, pyruvate–formate pathway, was weakened. Gene *pflA*, the activator of pyruvate–formate lyase, was knocked out in *E. coli* S17-1 (Zhu and Shimizu, 2004; Shozui *et al.*, 2010b; Zhou *et al.*, 2011a,b). The gene *pflA* knockout lead to an obvious decrease in formate production of the strain, whereas the lactate concentration in the culture medium increased oppositely (Fig. S2).

Escherichia coli S17-1 Δ*pflA* harbouring two plasmids of pLA' and p3HP2p was constructed and studied in shake flasks using different substrates combinations (Table 2 and Fig. 4). Obviously, the LA ratio in PLBP terpolyester produced by this recombinant was increased compared with the wild-type *E. coli* S17-1. The substrates added to the culture were utilized simultaneously indicating the synthesis of random copolyesters (Fig. 4). In general, several tendencies could be summarized from the data: there was a positive correlation between LA ratio and glucose concentration. Especially when glucose concentration was decreased to 10 g L^{-1}, the LA ratio was down to 0.79% because most of the glucose

Table 2. PLBP production using various substrate concentrations by *E. coli* S17-1 *ΔpflA* harbouring two plasmids of pLA' and p3HP2p.

Glu (g L^{-1})	Gly (g L^{-1})	LA (g L^{-1})	CDM (g L^{-1})	PHA (wt%)	3HP (mol%)	LA (mol%)
20	10	0	3.70 ± 0.12	40.94 ± 0.44	9.85 ± 1.43	10.74 ± 2.22
20	5	0	3.88 ± 0.3	45.17 ± 1.08	8.04 ± 0.77	13.10 ± 1.23
20	2	0	4.79 ± 0.08	53.31 ± 2.12	5.42 ± 0.8	9.18 ± 0.31
30	10	0	5.21 ± 0.72	59.04 ± 1.07	8.93 ± 0.87	11.53 ± 2.15
10	10	0	3.05 ± 0.41	38.67 ± 1.7	12.03 ± 0.39	0.79 ± 0.55
20	10	2	1.43 ± 0.19	45.06 ± 3.50	10.27 ± 0.69	23.54 ± 1.95
20	10	5	0.98 ± 0.23	42.67 ± 0.56	8.95 ± 2.3	27.78 ± 0.72

The recombinant was cultivated in LB medium supplemented with different concentrations of glucose (glu), glycerol (gly) and LA for 48 h in shake flasks. The data are the averages of three parallel experiments.
CDM, cell dry mass; PHA, the terpolyester; 3HP, 3-hydroxypropionate; LA, lactate.

was utilized for cell growth; when extra D, L-LA was added into the medium, LA ratio increased sharply to more than 27%, whereas the cell dry mass (CDM) was deceased oppositely due to the toxicity of LA; as the substrate of P3HP, glycerol affected 3HP ratio in PLBP terpolyester in a similar relationship as that between glucose and LA ratio. Interestingly, 3HP ratio reached its peak value of 12.03% when glucose was reduced to 10 g L^{-1}. Synthesis of PLBP terpolyester with variable compositions was achieved.

At a high glucose concentration of 30 g L^{-1}, cells grew to over 5.2 g L^{-1} containing close to 60% PHA, this was both the highest cell growth and the highest PHA accumulation compared with other low glucose concentration (Table 2). The terpolyester consisted of 9% 3HP, 12% LA and 79% 3HB. The result indicated that high concentration of glucose favoured formation of cell mass and PHA, especially PHB. At the lowest glycerol concentration of 2 g L^{-1}, 3HP had the lowest content of 5% in the terpolyester. LA addition improved LA ratios in the terpolyester, but it had negative effect on cell growth (Table 2). Hence, several strategies exist to change the composition of the monomer level by adjusting the ratio of fed carbon sources.

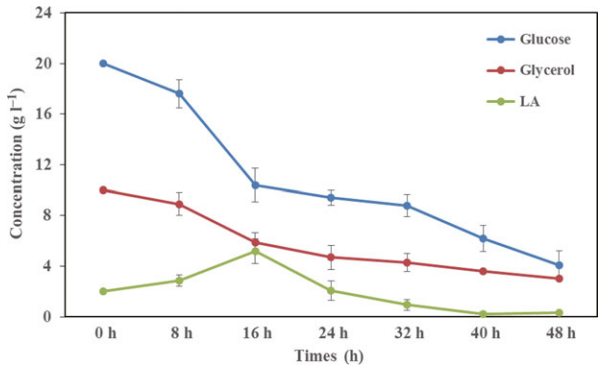

Fig. 4. The concentration of glucose, glycerol and lactate in shake-flask studies. *E. coli* S17-1 *ΔpflA* harbouring two plasmids of pLA' and p3HP2p was cultured in LB medium supplemented with 20 g L^{-1} glucose, 10 g L^{-1} glycerol and 2 g L^{-1} lactate. Blue line, glucose; red line, glycerol; green line, lactate. Error bars represent the standard deviation of experiments conducted in triplicates.

Physical characterization of PLBP with different monomer compositions

Two types of PLBPs were extracted and the compositions of them were determined by GC. The physical properties of PLBPs including molecular mass, thermal properties and mechanical properties were studied (Table 3). The weight average molecular mass of PLBP was less than 2×10^5, which was lower compared with its homopolyester and P(LA-*co*-3HB) copolyester, yet approximated the same as weight average molecular mass of PLBV terpolyesters (Shozui *et al.*, 2010b). In addition, PLBPs inherited the ductility of P3HP as it had an improved elongation at break of over 100% when the 3HP ratio reached 15%. The thermal parameters including T_m, T_g and ΔH_m of PLBP were in between of those of individual homopolyesters (PLA, P3HB and P3HP) as blocks. Interestingly, PLBPs had two T_m and the lower one was close to the T_m of PHB. Possibly, the presence of 3HB-rich segments in the terpolyester led to this phenomenon. The incorporation of 3HP into P(LA-*co*-3HB) significantly improved the tensile strength and elongation at break compared with its copolyester P(LA-*co*-3HB).

Conclusions

Recombinant *E. coli* S17-1 *ΔpflA* consisting of three synthetic pathways of lactyl-CoA, 3-hydroxypropionyl-CoA and 3-hydroxybutyryl-CoA was able to polymerize the three monomers to form random terpolyester P(LA-*co*-3HB-*co*-3HP) or PLBP under the catalysis of $phaC1_{Ps}$ (Q481K S325T E130D S477G) cloned from *P. stutzeri* strain 1317 with four specific point mutations. The terpolyester compositions can be controlled by changing the ratios of three substrates including glucose, glycerol and LA. The terpolyesters have changing properties depending on monomer ratios,

especially an enhanced elongation at break compared with the homopolyesters of 3HB and LA as well as their copolyesters.

Experimental procedures

Microorganism, plasmid and shake-flask culture conditions

All the microorganisms and plasmids used in this study are listed in Table 4. *E. coli* Trans 1T1 and JM109 were used as the host strains for genetic manipulation. *E. coli* S17-1 was used for polymer production (Simon *et al.*, 1983). All the *E. coli* strains were cultured in LB medium. LB medium contains (g L^{-1}): 10 tryptone, 5 yeast extract and 10 NaCl. Glycerol and glucose were added into LB medium as carbon sources. The seed cultures stored at −80°C were inoculated into LB medium and cultivated at 37°C for 12 h at 200 rpm/min on a rotary shaker for reactivation (BBT-14-BJQH042, INFORS HT, Hong Kong, China). Subsequently, the seed cultures were inoculated into 500 mL conical flasks containing 50 mL LB medium with an inoculation volume ratio of 5%.

Table 3. Physical characterization of various PLBP terpolyesters.

Monomer ratio[a] (mol %)			Molecular mass[b]			Mechanical properties[c]			Thermal properties[d]		
LA	3HB	3HP	M_w (10^4)	M_n (10^4)	M_w/M_n	Tensile strength (MPa)	Young's modulus (MPa)	Elongation at break (%)	T_g (°C)	T_m (°C)	ΔH_m (J g^{-1})
100[e]	0	0	20	–	–	52 ± 2	1020	2	60	153	9.2
0	100[f]	0	–	–	–	18 ± 0.7	1470 ± 78	3 ± 0.4	7.1	131.8	–
0	0	100[g]	–	30	–	28.3	333.3	683.5	−21.5	78	54
15[h]	85	0	82	34.2	2.4	10 ± 0	194 ± 5	75 ± 2	−9, 19	149, 167	0.6, 3.2
1.8	90.4	7.8[i]	15.3	9.1	1.68	18 ± 2	332.8 ± 8.5	15.3 ± 4.5	−5.9	129, 150	3.3, 32
7.2	79.8	13[j]	11.7	7.6	1.54	12.5 ± 1.3	231.4 ± 9.7	100.9 ± 12	−2	132, 154	2.7, 39

[a]Determined by gas chromatography.
[b]M_w, weight-averaged Molecular mass; M_n, number-averaged Molecular mass; M_w/M_n; polydispersity; the unit of M_w and M_n is Da.
[c]The values are the averages of at least three independent measurements.
[d]T_g, glass-transition temperature; T_m, melting temperature; ΔH_m, enthalpy of fusion.
[e]PLA was chemically synthesized (Zaman *et al.*, 2011).
[f]P3HB was synthesized by bacteria (Li *et al.*, 2011).
[g]P3HP was synthesized by bacteria (Zhou *et al.*, 2011a,b).
[h]P(LA-*co*-3HB) was produced by recombinant *E. coli* (Yamada *et al.*, 2011).
[i]Sample weight of P(90.4 mol% 3HB-co-7.8 mol% 3HP-co-1.8 mol% LA) was 18.2 mg.
[j]Sample weight of P(79.8 mol% 3HB-co-13 mol% 3HP-co-7.2 mol% LA) was 17.5 mg.

Table 4. Strains and plasmids used in this study.

Strains/plasmids	Description	Reference/source
E. coli Trans1-T1	Expression host	TransGen Biotech
E. coli S17-1	*recA*, harbours the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi-1*	Simon *et al.* (1983)
S-NC	*E. coli* S17-1 harbours pBluescript SK^- plasmid	This study
S-BL	*E. coli* S17-1 harbours pBL plasmid	This study
S-LA	*E. coli* S17-1 harbours pLA plasmid	This study
S-LA'	*E. coli* S17-1 harbours pLA' plasmid	This study
pBHR68	Derivative of pBluescript SK^- containing the 5.2-kb *SmaI/EcoRI* fragment comprising the PHA operon from *Ralstonia eutropha*	Spiekermann *et al.* (1999)
pSEVA351	Cloning vector, RSF1010 replicon, Cm^R	Silva-Rocha *et al.* (2013)
pBluescript SK^-	The commonly used commercial plasmid	TransGenBiotech
pZQ 03	Derivative of pBHR68, *phaC* and *pcs*' under the control of *lac* promoter, Amp^R	Zhou *et al.* (2011a,b)
pDC02	Derivative of pBHR68, *phaC*, *dhaB*, *gpp*, *gpd*, *gdrAB* and *pduP* under the control of P_{Re} promoter derived from *Ralstonia eutropha pha* operon, Amp^R	Meng *et al.* (2015)
pLA	Derivative of pBHR68, *phaC1*$_{Ps}$ (S325T Q481K E130D S477G), *pct* and *ldhA* was inserted into backbone	This study
pLA'	Derivative of pBHR68, *phaC1*$_{Ps}$ ((S325T Q481K E130D S477G) and *pct* was inserted into backbone	This study
p3HP1p	Derivative of pSEVA351, *dhaB*, *dhaT*, *aldD* and *pcs*' was inserted into backbone downstream 1 P_{re} promoters	This study
p3HP2p	Derivative of pSEVA351, *dhaB*, *dhaT*, *aldD* and *pcs*' was inserted into backbone with 2 P_{re} promoters	This study
pBL	Derivative of pBluescript SK^-, *ldhA* was inserted into backbone	This study

When an antibiotic selection pressure was required, the medium was supplemented with ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}) or chloramphenicol (34 μg mL^{-1}). To increase the LA ratio in the terpolyester, various amounts of D, L-LA (1, 2 or 5 g L^{-1}) were added into the LB medium along with NaOH-modulating pH to neutral. When P3HP was the constituent of the polymer, 5 μM vitaminB_{12} (VB_{12}) was added into the medium to maintain the activity of glycerol dehydratase (*dhaB*).

PHA analysis, extraction and purification

Cells were harvested by centrifugation (CR21 GIII; Hitachi, Tokyo, Japan) at 10 000 rpm/min for 8 min, then washed with distilled water and centrifuged again. CDM were measured after the concentrated cells were lyophilized at −65°C with five times air pressure (LGJ-10C; SiHuanKeXue, Beijing, China). Thirty to fourty milligrams of lyophilized cells was used for the transesterification reaction in which 2 mL of transesterification mixture and 2 mL of chloroform were added in each transesterification test tube (Kato *et al*., 1996). After a 4 h transesterification at 100°C, the PHA content and monomer compositions of the cells were assayed by gas chromatograph (GC-2014; Shimadzu, Suzhou, China) (Ouyang *et al*., 2007). The intracellular polymers were extracted using a Soxhlet extractor (Soxtec 2050; Foss, Hilleroed, Denmark). The extracted PHA was purified via precipitation when mixed with the 10-folds volume of ice-cold ethanol and dissolved in chloroform for film casting.

Metabolic flux analysis

Concentrations of LA, glycerol and glucose were determined using a high-performance liquid chromatography (HPLC) (LC-20A; Shimadzu) equipped with an ion exchange column (Aminexs HPX-87H; Bio-Rad, 7.8 × 300 mm^2, Hercules, California, USA) and a refractive index detector (RID-10A; Shimadzu). Gene *pct* transcriptional level was assayed using RT-PCR. Total RNA was extracted from recombinant *E. coli* strains using RNA prep pure Cell/Bacteria Kit (Tiangen, Beijing, China). cDNA was synthesized using Fastquant RT Kit (Tiangen) and then real-time PCR (RT-PCR) was carried out for mRNA analysis with SuperReal PreMix (SYBR Green; Tiangen). 16S rRNA was used as the inner standard. The experimental procedures were described (Lv *et al*., 2015).

NMR analysis on PHA

The ^1H and ^{13}C spectra were obtained using a JEOL JNM-ECA 600 NMR spectrometer to measure the polymer compositions, the chemical microstructures and the monomer sequences. Tetramethylsilane was used as the internal standard.

Molecular mass and other properties of PHA

Molecular mass was determined via gel permeation chromatography (GPC Spectra System P2000; Shimadzu) equipped with a Shimadzu HSG60 column at 40°C. The melting temperature (T_m), enthalpy of fusion (ΔH_m) and glass-transition temperature (T_g) were measured via differential scanning calorimetry (DSC-60; Shimadzu) in a temperature ranging from −80°C to 200°C under a nitrogen atmosphere of 50 mL min^{-1}. Thermal stabilities of the materials were studied by a thermogravimetric analyser (TA-Q50; TA Instrument, New Castle, Delaware, USA). Three to five milligrams of each sample was loaded at temperature ranging from 10 to 400°C in a nitrogen atmosphere of 50 mL min^{-1} (Shen *et al*., 2009). Mechanical properties were studied using a materials testing machine (INSTRON 3365; Instron, Grove City, Ohio, USA) at room temperature at a speed of 5 mm min^{-1}.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Site specific mutations of two PHA synthases $phaC_{Ps}$ variants.

Table S2. Comparison of various LA polymerizing enzymes.

Table S3. P3HP synthetic ability of *E. coli* harboring p3HP1p and p3HP2p plasmids, respectively.

Table S4. Effects of expressing gene *ldhA* on LA synthesis.

Fig. S1.1. Comparison of plasmids structure between pLA and pLA'.

Fig. S1.2. Relationship between *pct* transcriptional level and LA ratio in the terpolymer.

Fig. S2. Formations of extracellular formate and lactate by *E. coli* S17-1 (A) and the Δ*pflA* mutant (B) under aerobic conditions.

