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Research Article

Biosynthesis of Poly(3HB-*co*-3HP) with Variable Monomer Composition in Recombinant *Cupriavidus necator* H16

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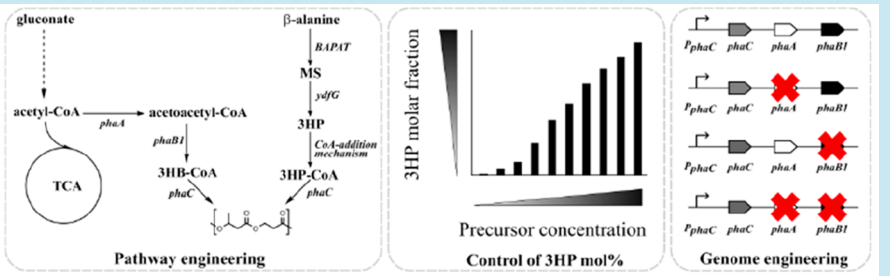

ABSTRACT: Polyhydroxyalkanoates are attractive alternatives to traditional plastics. However, although polyhydroxybutyrate (PHB) is produced in large quantities by *Cupriavidus necator* H16, its properties are far from ideal for the manufacture of plastic products. These properties may be improved through its coproduction with 3-hydroxypropionate (3HP), which leads to the formation of the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) (poly(3HB-*co*-3HP). To achieve this, a pathway was designed to enable *C. necator* H16 to convert *β*-alanine to 3HP. The initial low levels of incorporation of 3HP into the copolymer were overcome by the overproduction of the native propionyl-CoA transferase together with PHA synthase from *Chromobacterium* sp. USM2. Following optimization of 3HP incorporation into the copolymer, the molar fraction of 3HP could be controlled by cultivation in medium containing different concentrations of *β*-alanine. Between 0 and 80 mol % 3HP could be achieved. Further supplementation with 2 mM cysteine increased the maximum 3HP molar fraction to 89%. Additionally, the effect of deletions of the *phaA* and *phaB*1 genes of the *phaCAB* operon on 3HP molar fraction were investigated. A *phaAB1* double knockout resulted in a copolymer containing 91 mol % 3HP without the need for cysteine supplementation.

KEYWORDS: *polyhydroxyalkanoates, poly(3-hydroxybutyrate-co-3-hydroxypropionate), Cupriavidus necator H16, PHA synthase, synthetic pathway*

Approximately 380 million tons of plastic was manufactured in 2015, resulting in the generation of around 140 million tons of plastic waste.[1] Traditional plastics are recalcitrant in nature, leading to accumulation in the environment and damage to natural ecosystems. Furthermore, the supply of fossil fuels from which plastics are produced is finite. As such, alternative materials available from renewable feedstocks are needed. Polyhydroxyalkanoates (PHAs) are a class of polymers accumulated intracellularly by various bacteria when encountering conditions of nutrient limitation and carbon excess.[2] PHAs represent attractive alternatives to traditional plastics as the thermal and mechanical properties are similar. Additionally, PHAs are biodegradable and can be produced by the microbial fermentation of renewable carbon sources such as sugars, oils, and gases, rather than fossil fuels.[3−8] As a result, PHAs have attracted significant research and industry interest.

The most widely studied PHA is poly(3-hydroxybutyrate) (PHB). The enzyme components of the biosynthetic pathway responsible for PHB production in the model organism *Cupriavidus necator* H16 (*C. necator* H16) are encoded by the *phaCAB* operon.[9] First, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA in a reaction catalyzed by a *β*-ketothiolase (PhaA). The acetoacetyl-CoA is then reduced to 3-hydroxybutyryl-CoA (3HB-CoA) by an NADPH-dependent acetoacetyl-CoA reductase (PhaB1). Lastly, polymerization of 3HB-CoA into PHB is facilitated by the PHA synthase (PhaC). Understanding of the PHB biosynthetic pathway and advances in metabolic engineering have also allowed production of PHB in non-natural producers such as *Escherichia coli*.[10] The properties of PHB, while similar to plastic, are not ideal.[11] Thermal degradation occurs at temperatures close to the melting point, and high crystallinity results in a brittle material.[12,13] Consequently, the widespread

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application of PHB-based products has yet to be realized. The properties of PHAs can be altered by changing the monomeric composition of the polymer. More than 160 hydroxyalkanoate (HA) monomers have been identified to date.[14] Furthermore, PHA synthases exhibit relatively broad substrate specificity, allowing incorporation of different HA monomers into the polymer, resulting in a copolymer.[15] As a consequence, the potential for producing PHAs with improved properties is vast. Copolymers consisting of 3-hydroxybutyrate (3HB) and a second monomer, such as 3-hydroxypropionate (3HP), 3-hydroxyvalerate (3HV), or 3-hydroxyhexanoate (3HHx) have been previously produced.[16−18] In particular, poly(3HB-*co*-3HP) copolymers showed decreased crystallinity, melting temperature, and glass transition temperature, when compared to PHB.[13,19−24]

The biosynthesis of poly(3HB-*co*-3HP) was first described in 1991.[19] Following cultivation of *C. necator* H16 with 3HP, 1,5-pentanediol, or 1,7-heptanediol, it was possible to accumulate poly(3HB-*co*-3HP). The 3HP molar fraction, however, was limited to 7 mol % 3HP and under. Subsequently it was shown that *C. necator* H16 could be engineered to produce poly(3HB-*co*-3HP) from fructose by expression of genes encoding malonyl-CoA reductase and a 3HP-CoA synthetase domain of propionyl-CoA synthetase from *Chloroflexus aurantiacus*.[16] In this case poly(3HB-*co*-3HP) was produced using fructose as the sole carbon source; however, the highest 3HP molar fraction achieved by *C. necator* H16 was 1 mol % 3HP. While even low 3HP molar fraction influences the properties of poly(3HB-*co*-3HP) compared to PHB, more desirable properties can be obtained with higher 3HP content.[25] These studies indicate that while *C. necator* H16 is capable of incorporating 3HP into the growing copolymer chain, the process is inefficient and only low 3HP content is attained.

Various routes to 3HP-containing copolymers have been described.[16,26−30] One of the most common is the glycerol-utilizing pduP pathway, first described by Andreessen et al.[31] The pduP route describes a pathway by which glycerol is first converted to 3-hydroxypropionaldehyde by glycerol dehydratase, using vitamin B12 as a cofactor. The 3-hydroxypropionaldehyde is subsequently converted to 3HP-CoA by propionaldehyde dehydrogenase. Lastly, 3HP-CoA is polymerized by the activity of a PHA synthase. The pathway takes its name from the propionaldehyde dehydrogenase, which is encoded by the *pduP* gene,[32] and has been widely used for the production of poly(3HP) and poly(3HB-*co*-3HP).[27,31,33−35] The pduP pathway is reliant on glycerol dehydratase, which requires vitamin B12 as a cofactor. As most bacteria do not naturally produce vitamin B12, this expensive cofactor must be added to the medium. While this has been avoided by using organisms which synthesize vitamin B12,[36,37] the biosynthetic pathway for this cofactor is absent in *C. necator* H16.[38]

Another option is the malonyl-CoA route, which has been used to produce poly(3HB-*co*-3HP) in *C. necator* H16, and poly(3HP) in *E. coli*.[16,39] In this case, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase, then malonyl-CoA is reduced to 3HP by malonyl-CoA reductase. The 3HP must then be activated to 3HP-CoA, which can be facilitated by overexpression of a CoA ligase or CoA transferase. As before, incorporation of 3HP-CoA into the copolymer is then catalyzed by the action of a PHA synthase. Unlike the pduP pathway, which is restricted to glycerol as the carbon source, the malonyl-CoA pathway presents the advantage of being able to use any carbon source. However, the activity of malonyl-CoA reductase is reduced, since the optimum growth temperature of *C. necator* H16 is 30 °C, while malonyl-CoA reductase exhibits highest activity at around 55−57 °C.[16,39]

A more recently described pathway for the production of 3HP is via β-alanine.[40,41] In this route, β-alanine derived from the tricarboxylic (TCA) cycle is converted to malonic semialdehyde and then to 3HP. The β-alanine pathway has previously been used for the production of poly(3HP) in *E. coli*.[28,42] Since the 3HP is derived from the TCA cycle, a wide range of carbon sources can be used. While a full β-alanine route which provides 3HP from the TCA has not yet been established in *C. necator* H16, it was chosen to investigate the ability of *C. necator* H16 to produce poly(3HB-*co*-3HP) when grown in medium supplemented with β-alanine (Figure 1).

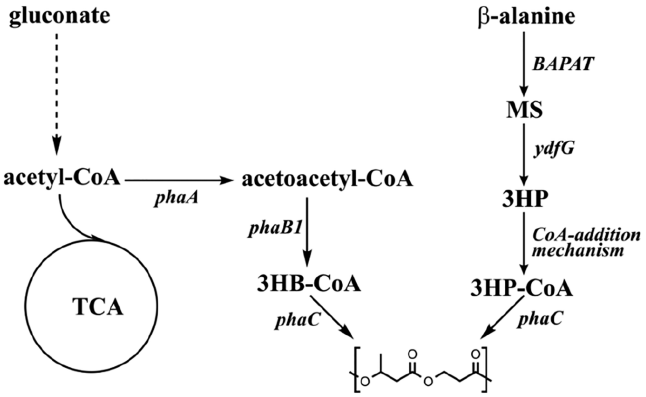

Figure 1. Schematic for the biosynthesis of poly(3HB-*co*-3HP) by engineered *C. necator* H16. The 3HP monomer is produced by conversion of exogenously supplemented β-alanine via malonic semialdehyde (MS). Activation of 3HP to 3HP-CoA is facilitated by a CoA-addition mechanism. Gluconate is metabolized to acetyl-CoA which is converted to PHB by the action of the enzymes encoded by the *phaCAB* operon. Incorporation of 3HP monomer units into the growing polymer chain results in the biosynthesis of poly(3HB-*co*-3HP).

In this work, the biosynthesis of poly(3HB-*co*-3HP) using engineered *C. necator* H16 cultivated in medium containing β-alanine is reported. Strains containing the engineered pathway were able to produce poly(3HB-*co*-3HP) with a range of 3HP molar content between 0 and 80 mol % 3HP depending on the amount of β-alanine supplied. Additional supplementation with cysteine was used to increase 3HP molar fraction to 89 mol %. Deletion of genes of the *phaCAB* operon was shown to have a positive effect on 3HP molar fraction; simultaneous deletion of both *phaA* and *phaB1* resulted in up to 91 mol % 3HP in the copolymer without the need for cysteine supplementation.

RESULTS AND DISCUSSION

Operon Structures of Plasmids Tested for Improved Synthesis of Poly(3HB-*co*-3HP). To improve the ability of *C. necator* H16 to incorporate 3HP into the polymer, several plasmids were constructed and tested (Table 1). A systematic approach was employed. First, a pathway was assembled that would enhance the conversion of exogenously supplied β-alanine to 3HP. Previously it was shown that the expression of genes encoding a β-alanine pyruvate aminotransferase

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Table 1. Strains and Plasmids[a]

strain or plasmid	description	source or reference
Escherichia coli		
DH5α	Used in plasmid construction	52
S17−1	Donor in conjugative plasmid transfer	53
Cupriavidus necator		
H16	Wild type	DSMZ, Germany
Δ3	Derivative of H16; Δ*mmsA1* Δ*mmsA2* Δ*mmsA3*	54
Δ3A	Derivative of Δ3; Δ*phaA*	This study
Δ3B1	Derivative of Δ3; Δ*phaB1*	This study
Δ3AB1	Derivative of Δ3; Δ*phaAB1*	This study
Plasmids		
pMTL71301	Broad host range plasmid; pBBR1 origin of replication, Tet^r	55
peyfP	pMTL71301 derivative; P_{phaC}, *eyfP*	This study
pCNCM0	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$	This study
pCNCM12	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, $prpE_{Cn}$, P_{trp}	This study
pCNCM13	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, pct_{Cn}, P_{trp}	This study
pCNCM14	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, $prpE_{Ec}$, P_{trp}	This study
pCNCM15	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, $atoAD_{Ec}$, P_{trp}	This study
pCNCM17	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, $phaC_{Cn}$, pct_{Cn}, P_{trp}	This study
pCNCM21	pMTL71301 derivative; P_{phaC}, $BAPAT_{Cv}$, $ydfG_{Ec}$, T_{rrnB}, $phaC_{Cs}$, pct_{Cn}, P_{trp}	This study
pMTL70641	Suicide vector; *sacB* and Tet^r	55
pCNCM_phaA	pMTL70641 carrying deletion cassette for *phaA*	This study
pCNCM_phaB1	pMTL70641 carrying deletion cassette for *phaB1*	This study
pCNCM_phaAB1	pMTL70641 carrying deletion cassette for *phaAB1*	This study

[a]*Cs*: *Chromobacterium sp*. USM2. *Cv*: *Chromobacterium violaceum*. *Cn*: *Cupriavidus necator* H16. *Ec*: *Escherichia coli*.

(BAPAT) and an NADP-dependent 3-hydroxyacid dehydrogenase (ydfG) was sufficient for the production of 3HP from β-alanine in yeast, as well as during the production of poly(3HP) in *E. coli*.[28,40,42] Accordingly, a pathway to the 3HP monomer from β-alanine was assembled comprising genes coding for a putative BAPAT from *Chromobacterium violaceum*, and YdfG from *E. coli*. The respective genes, $BAPAT_{Cv}$ and $ydfG_{Ec}$, were positioned downstream of the promoter of the *phaC* gene (P_{phaC}), resulting in plasmid pCNCM0.

Since PHA synthases act strictly on hydroxyacyl-CoA (HA-CoA) substrates,[43] it was hypothesized that increased activation of 3HP to 3HP-CoA would improve incorporation of 3HP into the copolymer. Therefore, the effect of overproducing a "CoA-addition" mechanism on 3HP incorporation was tested. The effectiveness of four different enzymes in converting 3HP to 3HP-CoA in *C. necator* H16 were evaluated. These were propionyl-CoA synthetase ($PrpE_{Cn}$) and propionyl-CoA transferase (Pct_{Cn}) from *C. necator* H16, as well as propionyl-CoA synthetase ($PrpE_{Ec}$) and acetoacetyl-CoA acetyltransferase ($AtoAD_{Ec}$) from *E. coli*. Pct_{Cn} has been previously shown to catalyze the transfer of CoA from acetyl-CoA to 3HP[44,45] and exploited for the production of copolymers containing medium-chain-length hydroxyalkanoate monomers.[46] While $PrpE_{Ec}$ has been used previously for production of 3HP-containing polymers,[37,39,42] both $PrpE_{Cn}$ and $AtoAD_{Ec}$ have been used in the production of 3HV-containing polymers as a mechanism for activating propionate to propionyl-CoA.[47,48] Each of the respective CoA-addition mechanisms were individually cloned downstream of the promoter of the *trp* gene (P_{trp}), resulting in plasmids pCNCM12−15. The P_{trp} promoter has been utilized in *C. necator* H16 for the production of PHAs, where it was shown to be stronger than the P_{phaC} promoter[49]

Lastly, to increase incorporation of 3HP into the copolymer, two different PHA synthases were evaluated. As the *C. necator* H16 PHA synthase has previously been shown to have a much lower substrate specificity for 3HP than 3HB,[50,51] its overproduction is likely to result in higher levels of 3HP incorporation into the copolymer. Overexpression of its encoding gene was, therefore, tested. For comparative purposes, the overproduction of a PHA synthase from *Chromobacterium* sp. USM2 (*C.* sp. USM2) was also explored. During this work it was found that Pct_{Cn} was the most effective CoA-addition mechanism. Accordingly, the genes encoding the two PHA synthases were positioned individually downstream of pct_{Cn}, resulting in plasmids CNCM17 and Δ3_CNCM21, respectively. The PHA synthase of *C. necator* H16 has previously been used extensively for the production of various 3HP-containing polymers.[26,27,29,33] On the other hand, the PHA synthase from *Chromobacterium* sp. USM2 was used in the production of poly(3HP).[35] The components and arrangement structure of the synthetic operons in the constructed plasmids is shown in Figure 2.

Construction of a 3HP-Producing Strain of *C. necator* H16. The first aim was to produce 3HP from exogenously supplied β-alanine. Therefore, plasmid pCNCM0 was constructed, which encodes $BAPAT_{Cv}$ and $ydfG_{Ec}$. To avoid consumption of any 3HP synthesized, a previously described mutant strain of *C. necator* H16 was chosen as the host for this study.[54] This strain, *C. necator* H16 Δ3, is unable to use 3HP as a carbon source. Transformation of Δ3 with plasmid pCNCM0 resulted in strain Δ3_CNCM0 which should be able to produce 3HP when grown in medium supplemented with β-alanine, facilitated by the expression of $BAPAT_{Cv}$ and $ydfG_{Ec}$. As a control, a plasmid expressing *eyfP* under the control of the P_{phaC} promoter was constructed and transformed into Δ3, resulting in the control strain Δ3_efyP. Strains Δ3_CNCM0 and Δ3_efyP were cultivated in SGMM supplemented with 50 mM β-alanine. HPLC analysis revealed secretion of approximately 40 mM 3HP into the supernatant by strain Δ3_CNCM0, while no 3HP was observed in strain Δ3_eyfP (Figure 3). Since 3HP was detected in the supernatant of strain Δ3_CNCM0, this suggested that it was not being incorporated the PHB polymer. Indeed, only 1.27 mol % 3HP was detected in the copolymer produced by strain Δ3_CNCM0. As expected, the polymer produced by strain Δ3_eyfP was a PHB homopolymer (Table 2).

PHA synthases act strictly on HA-CoA substrates.[43] Therefore, 3HP must be activated to 3HP-CoA before incorporation into the polymer by a PHA synthase. The results presented in Table 2 showed the molar fraction of 3HP was low, suggesting insufficient conversion of 3HP to 3HP-CoA. Previous studies have also reported low 3HP molar fractions as a result of inadequate generation of 3HP-CoA.[16,36] This has been overcome by increasing the capacity of the cell to add a CoA moiety to 3HP.[56]

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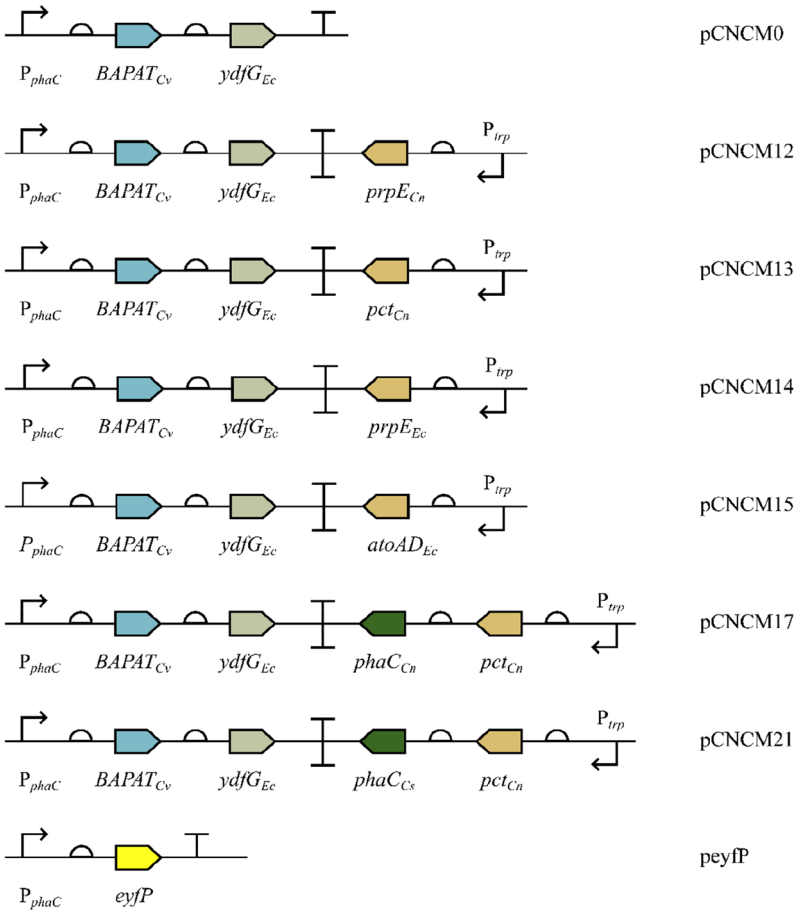

Figure 2. Plasmids tested for the production of poly(3HB-*co*-3HP). Plasmid pCNCM0 facilitated the conversion of *β*-alanine to 3HP. Plasmids pCNCM12−pCNCM15 were constructed to test increased activation of 3HP to 3HP-CoA. Plasmids pCNCM17 and pCNCM21 compared the ability of two PHA synthases to incorporate 3HP into the copolymer. Plasmid peyfP was used as a control plasmid.

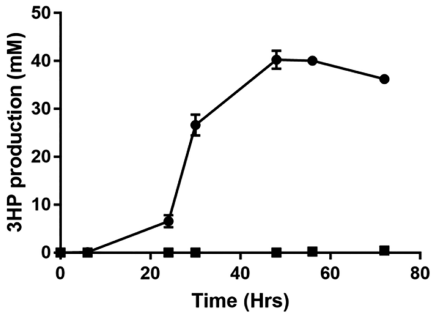

Figure 3. Production of 3HP from *β*-alanine using engineered *C. necator* H16. Δ3_eyfP (■), Δ3_CNCM0 (●). All data are averages from triplicate experiments.

Table 2. Polymer Produced during Cultivation of Engineered *C. necator* H16 in Sodium Gluconate SGMM Supplemented with 50 mM *β*-Alanine[a]

strain	CDW (g/L)	PHA (wt %)	PHA (g/L)	3HB (mol %)	3HP (mol %)
Δ3_eyfP	4.25	71.88	3.05	100	–
Δ3_CNCM0	2.00	17.74	0.35	98.73	1.27

[a]All data are averages from triplicate experiments.

Increasing the Conversion of 3HP to 3HP-CoA. It was seen that strain Δ3_CNCM13, expressing pct_{Cn} as the CoA addiction mechanism, exhibited increased 3HP molar fraction, achieving approximately 11.5 mol % 3HP. This represented a 10-fold increase in 3HP molar fraction compared to strain Δ3_CNCM0. Expression of the propionyl-CoA synthetases of *C. necator* H16 and *E. coli* in strains Δ3_CNCM12 and Δ3_CNCM14, respectively, did not increase 3HP molar fraction, and no growth was observed during cultivation of strain Δ3_CNCM15, expressing $atoAD_{Ec}$ (Table 3).

Propionyl-CoA synthetases are ATP-dependent. Insufficient availability of ATP under the growth conditions employed offers one explanation as to why the production of these enzymes failed to improve incorporation of 3HP into the polymer. Alternatively, since these synthetases use free CoA,

Table 3. Impact of Expression of CoA-Addition Mechanism on Composition of Poly(3HB-*co*-3HP) Produced by Engineered *C. necator* H16[a]

strain	CDW (g/L)	PHA (wt %)	PHA (g/L)	3HB (mol %)	3HP (mol %)
Δ3_CNCM12	3.54	34.05	1.2	98.1	1.9
Δ3_CNCM13	2.81	12.81	0.36	88.5	11.5
Δ3_CNCM14	4.13	41.06	1.67	98.4	1.6
Δ3_CNCM15	–	–	–	–	–

[a]Strains were cultivated in SGMM supplemented with 50 mM *β*-alanine for 48 h. All data are averages from triplicate experiments.

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the availability of this cofactor represents another possible reason as to why the molar ration of 3HP incorporated in the copolymer was not increased. Strain Δ3_CNCM15, which received the *E. coli atoAD*$_{Ec}$ gene encoding acetoacetyl-CoA acetyltransferase, failed to grow. A similar detrimental effect on the growth of the host was observed following cloning of the propionyl-CoA transferase (Pct$_{Cp}$) of *Clostridium propionicum*.[57] The authors attributed the growth defects to excessive reduction of the intracellular acetyl-CoA pool, as Pct$_{Cp}$ can use acetyl-CoA as a CoA donor. Since AtoAD$_{Ec}$ can also use acetyl-CoA as a CoA donor, its overproduction in strain Δ3_CNCM15, as a consequence of placing its encoding gene under the control of the strong P$_{trp}$ promoter, may similarly explain the growth inhibition observed here. Pct$_{Cp}$ has since been used on several occasions for production of 3HP-containing polymers.[30,36,56] As such it is possible that AtoAD$_{Ec}$ can be used for activation of 3HP to 3HP-CoA provided expression is moderated through the use of a weaker promoter system.

Taken together, of the four different CoA transferases analyzed, the propionyl-CoA transferase of *C. necator* H16 was shown to be the most effective means of converting 3HP to 3HP-CoA. Its use resulted in a 3HP molar fraction of 11.5 mol % in the PHA copolymer. Nonetheless, the proportion of 3HP remained relatively low.

Comparison of Two PHA Synthases for Improved 3HP Incorporation. Strains Δ3_CNCM17 and Δ3_CNCM21 both produced a copolymer with a dramatically increased 3HP molar fraction in comparison to strain Δ3_CNCM13, achieving approximately 77 and 80 mol % 3HP, respectively (Table 4). Additionally, the PHA content of the cells and PHA titer were increased relative to strain Δ3_CNCM13.

Table 4. Impact of the Combined Overproduction of Propionyl-CoA Synthetase and PHA Synthase on the Composition of Poly(3HB-*co*-3HP) Produced by Engineered *C. necator* H16[a]

strain	CDW (g/L)	PHA (wt %)	PHA (g/L)	3HB (mol %)	3HP (mol %)
Δ3_CNCM17	3.33	14.30	0.47	22.66	77.34
Δ3_CNCM21	3.96	19.27	0.76	20.29	79.71

[a]Strains were cultivated in SGMM supplemented with 50 mM β-alanine for 48 h. All data are averages from triplicate experiments.

It was notable that the use PHA synthase of *Chromobacterium* sp. USM2 (strain Δ3_CNCM21) resulted in 60% more PHA compared to the *C. necator* H16 synthetase used in strain Δ3_CNCM17. The difference in PHA titer is most likely attributable to the higher specific activity of the *Chromobacterium* sp. USM2 PHA synthase. Thus, the *Chromobacterium* sp. USM2 synthetase has an activity of approximately 238 U/mg, while the PHA synthase of *C. necator* H16 was measured at 40 U/mg.[50,58] On the basis of this, plasmid pCNCM21 was used in all subsequent experiments.

Production of Poly(3HB-*co*-3HP) with Different Monomer Compositions. Data from previous studies has shown that modulating the ratio of monomer to polymer in a copolymer can significantly change its properties in terms of, for instance, opacity, transparency, and adhesiveness.[59,60] Accordingly, the molar fraction of 3HP in poly(3HB-*co*-3HP) copolymers has been shown to influence crystallinity, melting temperature, and glass transition temperature.[13,19−24] Given that 3HP in strain Δ3_CNCM21 is produced from β-alanine, altering the concentration of this amino acid supplement in media provides a simple route to controlling the composition of the copolymer being produced. This was tested by growing Δ3_CNCM21 in SGMM containing between 0 to 40 mM exogenous β-alanine. The polymers produced under these conditions contained between 0 and 78 mol % 3HP (Table 5). Notably, supplementation with 40 mM β-alanine resulted in approximately the same 3HP molar fraction as had been achieved when cultures were supplemented with 50 mM β-alanine. One possible reason for this is that the amount of acetyl-CoA, which is required as the CoA donor molecule for activation of 3HP to 3HP-CoA, was insufficient at these higher β-alanine concentrations.

Table 5. Production of Poly(3HB-*co*-3HP) with a Range of 3HP Content Following Cultivation of Strain Δ3_CNCM21 in SGMM Supplemented with Different Concentrations of β-Alanine (± Cysteine) for 48 h

β-alanine (mM)	cysteine (mM)	CDW (g/L)	PHA (wt %)	PHA (g/L)	3HB (mol %)	3HP (mol %)
0	–	2.92	65.97	1.92	>99.9	Trace
5	–	4.41	60.17	2.24	97.76	2.24
10	–	5.10	50.76	2.59	92.89	7.11
15	–	5.31	39.24	2.08	83.4	16.60
20	–	4.99	29.91	1.49	63.28	36.72
25	–	5.16	24.03	1.23	50.17	49.83
30	–	5.03	22.16	1.11	32.99	67.01
40	–	4.14	16.97	0.70	21.37	78.63
50[a]	–	3.96	19.27	0.76	20.29	79.71
50	2	4.76	17.51	0.83	11.16	88.84

[a]Data taken from Table 4. All data are averages from triplicate experiments.

To attempt to further increase incorporation of 3HP into the copolymer, it was hypothesized that supplementation with cysteine could be used. Cysteine is a precursor to CoA-biosynthesis and supplementation with this amino acid has previously been used to increase the titers of compounds requiring either CoA or acetyl-CoA in their biosynthesis, including PHB.[61−64] Accordingly, strain Δ3_CNCM21 was grown in SGMM supplemented with 2 mM cysteine in addition to 50 mM β-alanine. The poly(3HB-*co*-3HP) produced contained 89 mol % 3HP (Table 5).

Effect of phaA, phaB1, and phaAB1 Deletions on 3HP Content in Copolymer. The preceding experiments demonstrated that a maximum 3HP content in the polymer of approximately 80 mol % was achievable with the most effective enzyme combination and with supplementation of the media with β-alanine. This could be further increased through the addition of cysteine to the medium (Table 5). One potential strategy for increasing the 3HP molar fraction without the need for cysteine supplementation would be to reduce flux toward 3HB-CoA biosynthesis. Genome analysis of *C. necator* H16 has revealed the presence of at least 15 β-ketothiolases and three acetoacetyl-CoA reductases.[38,65,66] Previous work has exploited alteration of the *phaCAB* operon to reduce the capacity of *C. necator* H16 to produce 3HB-CoA, thereby favoring incorporation of a second monomer.[5,65,67−69] Such an approach has previously been exploited for the improved incorporation of 3-hydroxyhexanoate, 3-mercapto-

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propionate, and 3-hydroxyvalerate into growing polymer chains.[5,65,67,69] To suppress 3HB-CoA formation, a range of knockouts targeting the *phaCAB* operon were therefore generated. Single knockout of the *phaA* and *phaB1* genes resulted in strains Δ3A and Δ3B1, respectively, while deletion of both *phaA* and *phaB1* produced strain Δ3AB1 (Figure 4).

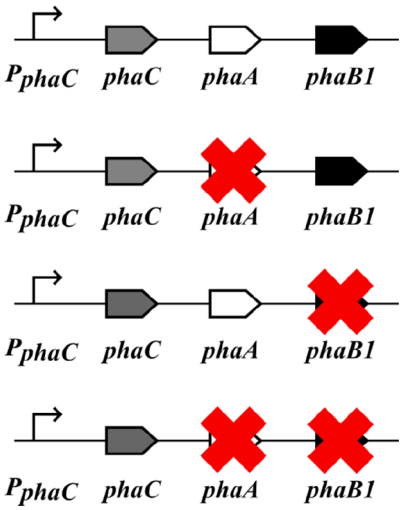

Figure 4. Deletions carried out to suppress formation of 3HB-CoA. From top to bottom; wild-type *phaCAB* operon, deletion of *phaA*, deletion of *phaB1*, deletion of *phaA* and *phaB1*.

The effect of the various *phaCAB* operon alterations is shown in Table 6. In control strains, PHB production was

Table 6. Production of Poly(3HB-*co*-3HP) by *C. necator* H16 Strains with Reduced Capacity to Form 3HB-CoA[a]

strain	CDW (g/L)	PHA (wt %)	PHA (g/L)	3HB (mol %)	3HP (mol %)
Δ3A_CNCM21	3.48	33.15	1.15	16.44	83.56
Δ3B1_CNCM21	2.93	28.15	0.83	10.82	89.18
Δ3AB1_CNCM21	3.01	29.50	0.88	8.89	91.11
Δ3A_eyfP	3.23	72.13	2.33	100	0
Δ3B1_eyfP	2.15	18.05	0.38	100	0
Δ3AB1_eyfP	1.86	16.04	0.30	100	0

[a]Strains were cultivated in SGMM supplemented with 50 mM β-alanine for 48 h. All data are averages from triplicate experiments.

either slightly or significantly reduced depending on the deletion. Deletion of *phaA* alone resulted in a minor decrease in PHB titer. Compared to strain Δ3_eyfP, which produced more than 3g/L of PHB, strain Δ3A_eyfP produced 2.33g/L PHB, although the PHA content of both strains was approximately 70%. In contrast, deletion of *phaB1* significantly reduced PHB titer with strain Δ3B1_eyfP producing only 0.38g/L of PHB. Deletion of both *phaA* and *phaB1* (strain Δ3AB1_eyfP) resulted in a more dramatic outcome, with a decrease in PHA titer to 0.30g/L.

As for experimental strains, reducing flux toward 3HB-CoA resulted in increased 3HP molar fraction. Strain Δ3A_CNCM21 produced poly(3HB-*co*-3HP) with around 84 mol % 3HP, accounting for approximately 33% CDW. Additionally, the PHA titer was increased to 1.15g/L. Further increases in 3HP molar fraction were observed in strains containing a *phaB1* deletion, although PHA content and titer was reduced in comparison to the *phaA* knockout strain. Strain Δ3B1_CNCM21 produced a copolymer containing 89 mol % 3HP, whereas strain Δ3AB1_CNCM21 produced a copolymer with 91 mol % 3HP. Prior to this, the highest molar fraction of 3HP achieved was 89 mol % 3HP through supplementation of the media with 2 mM cysteine.

Reducing the capacity of *C. necator* H16 to produce 3HB-CoA therefore resulted in further enhancement of 3HP molar fraction in the copolymer. Suppressing acetyl-CoA flux toward PHB biosynthesis could have also increased the acetyl-CoA pool available for activation of 3HP to 3HP-CoA by Pct_{Cn} as a secondary benefit. It was reported that increased acetyl-CoA availability following deletion of *phaA* resulted in greater 3HHx content when *C. necator* H16 was engineered to produce poly(3HB-*co*-3HHx).[69] Excluding those already described in the *phaCAB* operon, at least 14 homologues of *phaA* and two homologues of *phaB1* have been identified in *C. necator* H16, indicating a significant capacity for 3HB-CoA biosynthesis.[65,66] Despite this, deletion of *phaA* or *phaB1* either individually or together resulted in an increase in 3HP content in the copolymer.

Previous studies have shown *phaB3*, a homologue of *phaB1*, is only active under certain growth conditions, while *phaB2* is inactive. For example, although a *phaB1* deletion strain was capable of producing PHB during growth on fructose as a consequence of possessing a functional *phaB3* gene, PhaB3 activity was not detected during growth on trioleate or palm oil.[66,70] It was also shown that upregulation of *phaB3* occurred when *C. necator* H16 was cultivated in gluconate supplemented with 3,3′-thiodipropionic acid, compared to gluconate alone.[71] Deletion of all three *phaB* homologues results in accumulation of less than 20% of CDW as PHA.[66] During growth on gluconate in the course of this work, strains lacking *phaB1* also accumulated less than 20% CDW (Table 6). In the conditions tested, *phaB3* is, therefore, presumed to be inactive.

Production of Poly(3HB-*co*-3HP) from Batch Cultivation. Of all the recombinant strains tested so far, strain Δ3A_CNCM21 had exhibited a good balance of PHA content and PHA titer along with high 3HP molar fraction. As such, it was chosen to cultivate strain Δ3A_CNCM21 in a bioreactor to further investigate the results obtained during the flask cultivation. Strain Δ3A_CNCM21 was therefore cultivated in a 2.5 L bioreactor using SGMM supplemented with 50 mM β-alanine as before.

Following 42 h of cultivation, cell dry weight (CDW) reached 6.25 g/L of which 25.54% (wt %) was PHA, corresponding to a PHA titer of 1.6 g/L. Additionally, the poly(3HB-*co*-3HP) copolymer produced by strain Δ3A_CNCM21 contained approximately 91.19 mol % 3HP. This was higher than strain Δ3A_CNCM21 had achieved from flask cultivation and was equivalent to the 3HP content of the copolymer produced by strain Δ3AB1_CNCM21.

CONCLUSION

In the present study we demonstrated that it is possible to engineer *C. necator* H16 to produce a poly(3HB-*co*-3HP) polymer through the overexpression of genes encoding the enzymes necessary to convert exogenously supplied β-alanine into 3HP. Although the two enzymes used, a β-alanine pyruvate aminotransferase (BAPAT) from *C. violaceum* and an *E. coli* NADP-dependent 3-hydroxyacid dehydrogenase (ydfG), catalyzed the production of approximately 40 mM 3HP from 50 mM β-alanine, only 1.27 mol % 3HP was

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incorporated into the polymer. The 3HP molar fraction could be increased by either the overexpression of pivotal genes involved either in the conversion of 3HP to 3HP-CoA (propionyl-CoA transferase) or the synthesis of PHA itself (PHA synthase), the inclusion of additional media supplements (cysteine), or the suppression of 3HB-CoA biosynthesis by appropriate gene deletion. Through a combination of all these measures it was possible to produce poly(3HB-*co*-3HP) copolymers containing over 80 mol % 3HP, with the highest 3HP molar fraction achieved being 91 mol % 3HP.

Nonetheless, PHA content and titer were relatively low, and decreased as 3HP content of the copolymer increased. Low PHA yield is a key limiting factor to commercialization of PHAs as an alternative to traditional plastics.[72] Previous research has shown that PHA titer can be increased by a range of strategies including engineering of promoters, ribosomal binding sites, cell morphology, and PHA synthase substrate specificity.[8,73−76] Future work therefore could be focused on increasing the PHA content and titer of the cells by employing a variety of the approaches mentioned.

In this study, only the downstream part of the β-alanine pathway was investigated, and β-alanine was supplied exogenously rather than derived from the TCA cycle. While this approach was sufficient for our purposes, supplementation of β-alanine at large scale will add to copolymer production costs. An alternative solution is to develop the upstream part of the β-alanine pathway, in which β-alanine can be derived from the TCA cycle. Previously this approach has been used to overproduce β-alanine using recombinant *E. coli*.[77] By combining the upstream and downstream parts of the β-alanine pathway, it is then possible to produce 3HP and 3HP-containing copolymers from a range of carbon sources without the need to provide exogenous β-alanine.[28,40−42] Previously, it has been shown that *C. necator* H16 is capable of growing on a wide array of carbon sources, including various waste streams such as used cooking oil, spent coffee grounds, and CO_2-rich off gases.[78−80] As such a future strain of *C. necator* H16, which has been engineered to overproduce β-alanine and therefore 3HP from the TCA cycle, could theoretically produce poly(3HB-*co*-3HP) from sources such as CO_2 without the need to supplement β-alanine. Use of waste carbon sources is expected to decrease the cost of PHA production in comparison to using more expensive and highly refined carbon sources such as sugars.[14] To that end and to avoid supplementation of β-alanine, subsequent work will focus on increasing the supply of β-alanine from the TCA cycle.

MATERIALS AND METHODS

Microorganisms, Plasmids, and Genetic Methods. General cultivation of *C. necator* H16 was carried out in lysogeny broth (LB) at 30 °C. *E. coli* was cultivated in LB at 37 °C. When acting as a recipient during conjugation, *C. necator* H16 was cultivated in low-salt LB medium.[81] Where necessary, tetracycline was added to a concentration of 15 μg/mL for both *E. coli* and *C. necator* H16, and gentamycin was added to a concentration of 10 μg/mL for *C. necator* H16 cultivations.

All strains and plasmids used are listed in Table S1. The nucleotide sequence of plasmids pCNCM0, pCNCM12, pCNCM13, pCNCM14, pCNCM15, pCNCM17, pCNCM21, pCNCM_phaA, pCNCM_phaB1, and pCNCM_phaAB1 have been deposited in the public version of the JBEI registry (https://public-registry.jbei.org) under the accession numbers JPUB_018762, JPUB_018763, JPUB_018764, JPUB_018765, JPUB_018766, JPUB_018767, JPUB_018768, JPUB_018769, JPUB_018770, and JPUB_018771, respectively. The PHA synthase from *Chromobacterium* sp. USM2 was synthesized without codon optimization by Genscript Biotech (Leiden, Netherlands) Supporting Information.

PCR was performed using Q5 High-Fidelity 2× Master Mix (NEB) and GoTaq Green Master Mix (Promega) according to the manufacturers' protocol. Q5 High-Fidelity 2× Master Mix was used for amplification of sequences for cloning, while GoTaq Green Master Mix was used for screening purposes. Assembly of plasmids was carried out using NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs Inc. (NEB)). Plasmid DNA was isolated using the Monarch Plasmid Miniprep Kit (NEB). Restriction enzymes were purchased from NEB and Thermo Fisher Scientific. Gel-purified PCR products were extracted using the Monarch DNA Gel Extraction Kit (NEB). Chemically competent *E. coli* were transformed via heat shock.[82] Plasmids were transferred to *C. necator* H16 via conjugation using *E. coli* S17−1 as a donor strain.[81]

Knockout plasmids were constructed using a suicide vector encoding a copy of the *sacB* gene to allow for counter-selection using sucrose. Approximately 700 bp of the upstream and downstream regions flanking the gene of interest were amplified and assembled into a deletion cassette using the HiFi DNA Assembly Kit (NEB).

HPLC Analysis of 3HP, PHA Production, and Characterization. 3HP concentration in the supernatant was determined by HPLC using a previously described method.[54]

Engineered strains of *C. necator* H16 were streaked on solid SGMM agar containing tetracycline. From these plates, precultures were prepared in 50 mL Falcon tubes containing 5 mL LB medium supplemented with tetracycline. The precultures were grown overnight at 30 °C with shaking at 200 rpm. Production of PHA by engineered strains of *C. necator* H16 was carried out in 250 mL Erlenmeyer flasks containing 30 mL of sodium gluconate minimal medium (SGMM) supplemented with β-alanine. In all experiments, overnight precultures were used to inoculate flasks to a starting OD_{600} of 0.05. In all experiments, SGMM was supplemented with 50 mM β-alanine, unless otherwise stated.

Batch cultivation was carried out in a 2.5 L bioreactor (New Brunswick BioFlo/CelliGen 115, Eppendorf AG, Hamburg, Germany) with a working volume of 1.5 L. SGMM supplemented with 50 mM β-alanine was used as the culture medium. Culture conditions were maintained as follows: temperature was maintained at 30 °C, pH 6.9, and dissolved oxygen concentration at 30%.

SGMM was composed of 2% sodium gluconate (w/v), $Na_2HPO_4{\cdot}12H_2O$ 9 g/L, KH_2PO_4 1.5 g/L, NH_4Cl 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $CaCl_2{\cdot}2H_2O$ 20 mg/L, Fe(III)NH_4-citrate 1.2 mg/L, and trace element solution (SL7) 1 mL/L. SL7 was prepared to the following composition; 25% (w/v) HCl 1.3 mL/L, H_3BO_3 62 mg/L, $CoCl_2{\cdot}6H_2O$ 190 mg/L, $CuCl_2{\cdot}2H_2O$ 17 mg/L, $MnCl_2{\cdot}4H_2O$ 100 mg/L, $Na_2MoO_4{\cdot}2H_2O$ 36 mg/L, $NiCl_2{\cdot}6H_2O$ 24 mg/L, and $ZnCl_2$ 70 mg/L. The pH was adjusted to 6.9. Engineered strains of *C. necator* H16 were cultivated aerobically at 30 °C with shaking at 200 rpm.

Bacterial culture was centrifuged and washed prior to lyophilization, after which the cell dry weight was measured.

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The PHA content of the cells was determined by gas chromatography/mass spectrometry after propanolysis in chloroform (Agilent Technologies, GC 6890N, MS5 973N).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00283.

Further details on knockout plasmid construction and DNA sequence of the PHA synthase from *Chromobacterium* sp. USM2 (PDF)

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Author Contributions

C.M. and K.K. designed the study. C.M. performed the experiments. C.M., K.K., and N.P.M. analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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