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MINI-REVIEW

Polyhydroxyalkanoic acids from structurally-unrelated carbon sources in *Escherichia coli*

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Abstract Polyhydroxyalkanoates (PHAs) that contain varied monomers with different chain lengths/structures were normally synthesized when a structurally-related precursor was present. The biosynthesis of PHAs from unrelated carbon sources in microorganisms including *Escherichia coli* met many challenges in the past. Recently, with the development of metabolic engineering and synthetic biology, the production of PHAs from unrelated carbon sources obtained a breakthrough. Polyesters containing 2-hydroxypropionate, 3-hydroxypropionate, 4-hydroxybutyrate, 3-hydroxyvalarate, and medium-chain-length 3-hydroxyalkanoate monomers can all be synthesized in *E. coli* by integrating exogenous or endogenous pathways and/or genes. This review will summarize the progresses in this area. In addition, the strategies that lead to the production of PHAs with varied monomers and high polymer content in the cell are discussed.

Keywords Polyhydroxyalkanoates · Unrelated carbon source · *Escherichia coli* · PHA · Metabolic engineering

Introduction

Polyhydroxyalkanoates (PHAs) are carbon and energy storage materials synthesized by a variety of bacteria that grows in the presence of excess carbon sources and if another macroelement (N, O, P, S) is depleted at the same time (Anderson and Dawes 1990; Steinbüchel et al. 1995). These polymers have attracted extensive interest as environmentally friendly, biodegradable alternatives to petroleum-based plastics (Chen 2009). More than 150 types of PHAs with various monomer constituents have been reported till now (Hazer and Steinbüchel 2007; Steinbüchel and Valentin 1995).

Although *Escherichia coli* do not accumulate PHAs under natural conditions, it was supposed to be an ideal host for the production of PHAs due to the absence of an intracellular depolymerization system and the presence of convenient metabolic engineering tools (Li et al. 2007a). The most typical polyester, poly-β-3-hydroxybutyrate (P3HB), was first heterologously synthesized in *E. coli* in 1988 (Schubert et al. 1988; Slater et al. 1988). Since then, many strategies have been developed to improve the polymer content in the host and to facilitate the isolation of the polymers (Li et al. 2007b). P3HB is naturally accumulated by microorganisms from structurally unrelated carbon sources, while most other polyesters (either homopolymers or copolymers) that contain the monomers such as 3-hydroxypropionate (3HP), 4-hydroxybutyrate (4HB), and 5-hydroxyvalarate etc. cannot be accumulated under natural conditions from unrelated carbon sources (Steinbüchel and Valentin 1995). Structurally-related precursors which were used to synthesize PHAs are usually high cost, toxic to cells, and insoluble in water. Therefore, scientists have made many efforts to create new metabolic pathways for such monomers synthesis in *E. coli* from unrelated carbon source by combining genes from various organisms. This review summarized the efforts that have been done in this area (Table 1).

Polyesters containing the 3HV monomers

Besides P3HB, polyesters containing the 3HV monomers were also found in the early days. In initial P(3HB-*co*-3HV) (PHBV) production, propionic acid or pentanoic acid had to be supplied together with glucose (Byrom 1987; Fidler and Dennis 1992; Law et al. 2004). Since propionic acid is toxic to cells, researchers tried to find a propionate-independent pathway. Early efforts on *Cupriavidus necator* proved that the

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Table 1 Summary of PHAs that was produced in recombinant *E. coli* from unrelated carbon sources

Strains	Carbon source	Genes involved in monomers synthesis	PHA composition	Reference
E. coli JM109	Glucose	pct_{Me}, engineered $PhaC1_{Ps6\text{-}19}$	P(94 mol%3HB-co-6 mol% LA)	Taguchi et al. 2008
E. coli W3110	Glucose	pct_{Me}, engineered $PhaC1_{Ps6\text{-}19}$	P(53 mol% 3HB-co-47 mol% LA)	Yamada et al. 2009
E. coli XL1-Blue Δ*ackA PldhA*:: *Ptrc* Δ*ppc* Δ*adhE Pacs*::*Ptrc*	Glucose	engineered $phaC1_{Ps6\text{-}19}$, pct_{Cp},	PLA	Jung et al. 2010
E. coli XL1-Blue Δ*ackA PldhA*:: *Ptrc* Δ*ppc* Δ*adhE Pacs*::*Ptrc*	Glucose	engineered $phaC1_{Ps6\text{-}19}$, pct_{Cp}, $phbAB_{Cn}$	P(30 mol%3HB-co-70 mol%LA)	Jung et al. 2010
E. coli XL1-Blue	Glucose	engineered $phaC1_{Ps6\text{-}19}$, pct_{Cp}, $phbAB_{Cn}$	P(36 mol%3HB-co-6~64 mol%LA)	Yang et al. 2010
E. coli HMS174(DE3)	Glycerol	$dhaB1_{Cb}$, $pduP_{Se}$, $phbC_{Cn}$	P3HP	Andreeßen et al. 2010
E. coli BL21(DE3)	Glucose	$accABCD_{Ec}$, mcr_{Ca}, $prpE_{Ec}$, $phbC_{Cn}$	P3HP	Wang et al. 2012a
E. coli DH5α	Glucose	$sucD_{Ck}$, $4hbD_{Ck}$, $orfZ_{Ck}$, $phbCAB_{Cn}$	P(97.2 mol%3HB-co-2.8 mol%4HB)	Valentin and Dennis 1997
E. coli JM109 Δ*sad* Δ*gabD*	Glucose	$sucD_{Ck}$, $4hbD_{Ck}$, $orfZ_{Ck}$, $phbCAB_{Cn}$	P(89 mol%3HB-co-11 mol%4HB)	Li et al. 2010
E. coli DH5α	Glucose	$gabT_{Ec}$, $gadA_{Ec}$, $gadB_{Ec}$, $orfZ_{Ck}$, $phbC_{Cn}$	P4HB	Song et al. 2005
E. coli JM109 Δ*sad* Δ*gabD*	Glucose	$sucD_{Ck}$, $4hbD_{Ck}$, $orfZ_{Ck}$, $phbC_{Cn}$, *phaP*	P4HB	Zhou et al. 2012
E. coli DH5α Δ*prpC* Δ*scpC* Δ*pta*	Xylose	$thrA^{C1034T}thrBC_{Ec}$, $ilvA_{Cg}$, $phbCAB_{Cn}$	P(82.5 mol%3HB-co-17.5 mol%3HV)	Chen et al. 2011
E. coli S17-1	Glucose	$phaG_{Pp}$, $phaC1_{Pa}$	P-3-hydroxydecanoate (P3HD)	Rehm et al. 2001
E. coli LS5218 Δ*fadE*	Glucose	$phaG_{Pp}$, engineered $PhaC1_{Ps61\text{-}3}$, CoA ligase $PP0763_{Pp}$	mcl-PHA	Wang et al. 2012b
*E. coli*Δ*fadB*Δ*fadR*	Glucose	*tesA*, $phaC2_{Po}$	P-3-hydroxyoctanoate (P3HO)	Klinke et al. 1999
E. coli LS1298Δ*fadB*, *E. coli* RS3097	Glucose	$phaG_{Uc}$, $phaC1_{Pa}$	P3HD	Rehm et al. 2001
E. coli MG1655 Δ*araBAD* Δ*fadR* Δ*fadAB* Δ*fadIJ* *E. coli* MG1655Δ*araBAD* Δ*fadR* Δ*fadAB* Δ*fadIJ*:: F(Ptrc_BTE)	Glucose	BTE_{Uc}, $phaJ1\sim4_{Pa}$, $phaC2_{Pa}$, $PP0763_{Pp}$	mcl-PHA	Agnew et al. 2012
E. coli JM109	Glucose	Engineered $fabH_{Ec}$, $phaC_{Ac}$ or engineered $phaC1_{Ps61\text{-}3}$	scl-mcl PHA (mainly C_4-C_6)	Nomura et al. 2004
E. coli JM109	Glucose	Engineered $fabH_{Ec}$, $FabG_{Ec}$, engineered $phaC1_{Ps61\text{-}3}$	scl-mcl PHA (C_4-C_{12})	Nomura et al. 2005

propionate-independent pathway is possible but with a very low 3HV fraction (Eschenlauer et al. 1996; Steinbüchel and Pieper 1992). In 2002, Aldor et al. constructed a recombinant *Salmonella enterica* serovar Typhimurium strain (Aldor et al. 2002). In this strain, a methylmalonyl-CoA mutase gene *sbm* and a methylmalonyl-CoA decarboxylase gene *ygfG* from *E. coli* were subcloned therefore, succinyl-CoA that derived from the tricarboxylic acid (TCA) cycle, can be converted to propionyl-CoA. The resulting *S. enterica* strain was proved to accumulate PHBV with a 30 mol% 3HV fraction in the copolymer from glycerol. However, in recombinant *E. coli*, only a 4 % 3HV fraction was obtained in the copolymer even when threonine was added to the medium (Eschenlauer et al. 1996). We recently engineered a metabolic pathway in *E. coli* via threonine to synthesize PHBV from glucose. By deregulating the feedback inhibition of the threonine and overexpressing three threonine synthesis genes $thrA^{C1035T}BC$, this strain can first accumulate threonine, then convert the threonine to 2-ketobutyrate by threonine deaminase IlvA, which was incorporated from *Cornybacterium glutamicum*. To increase the propionyl-CoA content, the competitive pathways of catalytic conversion of propionyl-CoA to 3-hydroxyvaleryl-CoA were blocked (Chen et al. 2011). To further increase the 3HV fraction in the copolymer, the threonine biosynthesis pathway should be optimized. In addition, new pathways leading to the formation of 3HV is also valuable. Nevertheless, it seems that it is not possible to synthesize the PHV homopolymer in *E. coli*.

Polyesters containing the 4HB monomers

In 1988, Doi et al. discovered that *C. necator* (formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus*, *Wautersia eutropha*, and *Hydrogenomonas eutropha*) was able to synthesize P(3HB-*co*-4HB) when 4-hydroxybutyric or 4-chlorobutyric acid was fed as the second carbon source in the medium (Doi et al. 1988). Later, researchers found that many bacteria including *Comamonas acidovorans* (Lee et al. 2004; Park et al. 2005), *Alcaligenes latus* (Kang et al. 1995), *Comamonas testosteroni* (Renner et al. 1996), and *Hydrogenophaga pseudoflava* (Choi et al. 1999) can all accumulate P(3HB-*co*-4HB) and/or P4HB homopolymer. The capability of polyesters containing the 4HB monomers formation in wild-type microorganisms inspired researchers to explore the possibility of P4HB synthesis in recombinant *E. coli*. In 1996, a 4-hydroxybutyrate CoA transferase gene *orfZ* was discovered from *Clostridium kluyveri*, a non PHAs producer, that can transfer the CoA to 4-hydroxybutyrate (Söhling and Gottschalk 1996), which made it possible for the heterologous P4HB biosynthesis in *E. coli*. By cloning *orfZ* gene from *C. kluyveri*, researchers first synthesized P4HB homopolymer in recombinant *E. coli* in the presence of precursor substrate 4HB (Hein et al. 1997; Song et al. 1999). Then, by introducing the whole succinate degradation pathway genes and P3HB biosynthesis genes *phaCAB* from *C. necator*, recombinant *E. coli* was proved to accumulate P(3HB-*co*-4HB) directly from glucose (Valentin and Dennis 1997). In this recombinant *E. coli* succinate, a TCA cycle intermediate was converted to 4-hydroxybutyryl-CoA via succinyl-CoA: CoA transferase, succinic semialdehyde dehydrogenase (SucD), 4-hydroxybutyrate dehydrogenase (4hbD), and 4-hydroxybutyryl-CoA via: CoA transferase. This was the first example that a copolymer was heterologously synthesized in *E. coli* from unrelated carbon source. However, the 4HB fraction in the initial synthesized polymer was very low. Inactivation of *E. coli* native succinate semialdehyde dehydrogenase genes *sad* and *gabD* improved the 4HB content up to 11 % in the copolymer, resulting to the highest P(3HB-*co*-4HB) production in *E. coli* from glucose to this day (Li et al. 2010). Meanwhile, the biosynthesis of 4HB homopolymer from unrelated carbon source was achieved. In 2005, Song et al. engineered a 4HB biosynthesis pathway via glutamate in *E. coli* using glucose as a sole carbon source with 0.78 g/L yield of P4HB (Song et al. 2005). Significant level of P4HB can now be obtained from glucose via succinate degradation pathway in *sad* and *gabD* genes deficient strain of *E. coli* JM109 that coexpressed with four PHA binding proteins PhaP1, PhaP2, PhaP3, and PhaP4, respectively. Over 68 % P4HB (11.5 g/L) of the cell dry weight was produced in a fed-batch fermentation process (Zhou et al. 2012).

Polyesters containing the LA monomers

Polylactic acid (PLA), the unnatural polyester, is usually synthesized by chemical polymerization of fermented product, lactic acid (2-hydroxypropionic acid, 2HP). Due to its promising market and drawbacks during the chemical synthesis process (Södergård and Stolt 2002), people had tried to synthesize this polymer directly by microbial fermentation. Taking advantage of the wide substrate specificity of PHA synthase, enzymes/mutants that can incorporate LA monomer were created and selected. Recombinant *E. coli* expressing the mutated propionate CoA-transferase gene *pct* from *Megasphaera elsdenii*, which allows generation of (D)-lactyl-CoA in the cell, and the mutated type II PHA synthase gene *phaC*1 from *Pseudomonas* sp. 61-3 was proved to synthesize LA copolymer directly by fermentation from glucose. The polymer composition is 94 mol% 3HB and 6 mol% LA, and the polymer content in the cell is 19 % of the dry cell weight. In the following experiments, this group enriched the LA fraction in the copolymer from the previous 6 mol% to 47 mol% by anaerobic cultivation (Taguchi et al. 2008; Yamada et al. 2009). Employing a *pct* gene from *Clostridium propionicum* and *phaC*1 from *Pseudomonas* sp. MBEL6-19, Lee Sang Yup group reported that PLA homopolymer could be produced up to 11 wt% from glucose (Jung et al. 2010). Several PhaC1$_{Ps6\text{-}19}$ variants with mutations were investigated with respect to their PLA biosynthesis capability in wild-type *E. coli* XL1-Blue (Yang et al. 2011). Together with β-ketothiolase and acetoacetyl-CoA reductase genes *phbAB*, the recombinant strain can accumulate P(3HB-*co*-LA) with 6~64 mol% LA fraction in the copolymer from glucose (Park et al. 2008; Yang et al. 2010). Then, they further engineered the host by knocking out the *ackA* (acetate kinase), *ppc* (phosphoenolpyruvate carboxylase), and *adhE* (acetaldehyde/alcohol dehydrogenase) genes and by replacing the promoters of the *ldhA* (*D*-lactate dehydrogenase) and *acs* (acetyl-CoA synthetase) genes with the *trc* promoter to increase the metabolic fluxes at the systems level. The final P(3HB-*co*-LA) copolymer contains up to 70 mol% LA fraction with 46 wt% polymer content (Jung et al. 2010). Using this in vivo PLA biosynthesis system, they can also synthesize PHAs containing 2-hydroxybutyrate monomer (Park et al. 2012). The conversion of chemical synthesis process to fermentative process opened up a new way for PLA production, and this process can also generated polyesters that contains both LA and 3HB fractions. The synthesis of copolymer containing LA and other types of monomers is expected.

Polyesters containing the 3HP monomers

Polyesters containing the 3HP monomers could be produced when 3HP, α,ω-alkanediols, or acrylate was added as

carbon source (Green et al. 2002; Nakamura et al. 1991; Valentin et al. 2000). Since this kind of polymer was supposed to have higher rigidity, ductility, and stability, many efforts have been made in *E. coli* for the production of P3HP or its copolymer with high content and controllable compositions (Meng et al. 2012; Zhou et al. 2011). In 2008, P(3HP-*co*-3HB) synthesis from unrelated carbon sources was first achieved in engineered *C. necator* by introducing malonyl-CoA reductase (Mcr) and the 3HP-CoA synthetase domain of trifunctional propionyl-CoA synthase (Acs) from *Chloroflexus aurantiacus*. Strains harboring the two heterologous genes synthesized P(3HB-*co*-3HP) only with 0.2–2.1 mol% of 3HP fraction (Fukui et al. 2009). In 2010, an engineered *E. coli* was developed by introducing the genes of glycerol dehydratase (DhaB1) from *Clostridium butyricum*, propionaldehyde dehydrogenase (PduP) from *S. enterica* serovar Typhimurium LT2, and PHA synthase (PhbC) of *C. necator*. This recombinant *E. coli* accumulated up to 1.42 g/L P3HP homopolymer in fed-batch fermentation from unrelated carbon source, glycerol (Andreeßen et al. 2010). In the same year, production of P3HP from glucose in recombinant *E. coli* was achieved by cloning the genes *accABCD* encoding acetyl-CoA carboxylase and genes for Mcr, propionyl-CoA synthetase (PrpE), and PhbC from *C. necator* (Wang et al. 2012a). However, the polymer content was 1.32 g/L and only 0.98 % wt/wt of the cell dry weight. Nevertheless, this attempt shows the feasibility of engineering a P3HP biosynthetic pathway using a structurally-unrelated carbon source in *E. coli*. Further effort towards the improvement of the P3HP production should be made. Since several 3HP synthetic pathways were designed and large quantity of 3HP was produced from unrelated carbon sources, many experiences involved in 3HP production in *E. coli* should be used for reference (Cho et al. 2010; Jiang et al. 2009; Rathnasingh et al. 2009; Rathnasingh et al. 2012).

Polyesters containing the 3HA monomers

Medium-chain-length PHAs (mcl-PHA) are naturally synthesized through fatty acid de novo biosynthesis pathway or β-oxidation pathway from Pseudomonads (Timm and Steinbüchel 1990). In 1997, recombinant *E. coli* expressed the *phaC*1 gene from *Pseudomonas aeruginosa* and was found to produce mcl-PHA from related carbon source fatty acid through a β-oxidation pathway by knocking out the *fadB* gene (Langenbach et al. 1997; Qi et al. 1997). Later, by introducing with a transacylase gene *phaG* from *Pseudomonas putida*, *E. coli* was proved to accumulate 2 to 3 % mcl-PHA of cellular dry weight through fatty acid de novo biosynthesis from glucose (Rehm et al. 2001). The transacylase *phaG* catalyzes the transfer of the (*R*)-3-hydroxy-acyl moiety from the acyl-carrier-protein (ACP) thioester to CoA making the intermediates from de novo fatty acid biosynthesis into the substrates for mcl-PHA biosynthesis. However, due to the low efficiency of *PhaG*, further improvement of mcl-PHA production in this pathway was made. A recent study suggested that *PhaG* is not a 3-hydroxyacyl-ACP: CoA transferase as reported but a 3-hydroxyacyl-ACP thioesterase. Based on this, they overexpressed a predicted mcl-fatty acid CoA ligase PP0763 from *P. putida* together with *P. putida PhaG* and the engineered *Pseudomonas* sp. 61-3 PhaC1 in *E. coli*. The new-generated strains can accumulate 11.6 % mcl-PHA of the cell dry weight (about 400 mg/L mcl-PHA) when grown on glucose as a sole carbon source (Wang et al. 2012b). Meanwhile, some researchers tried to combine fatty acid de novo biosynthesis with β-oxidation by using an acyl-ACP thioesterase, which hydrolyzes acyl-ACPs and produces enhanced intracellular free fatty acid. Then, fatty acid is channeled into β-oxidation pathway to form (*R*)-3-hydroxyacyl-CoA. Coexpression of the cytosolic thioesterase I gene *tesA* and a PHA synthase gene (*phaC*2 from *Pseudomonas oleovorans*) in *E. coli*Δ*fadB*Δ*fadR* resulted in the synthesis of mcl-PHA composed mainly of 3-hydroxyoctanoate from the glucose (Klinke et al. 1999). Another acyl-ACP thioesterase from *Umbellularia californica* can also lead to the formation of mcl-PHA in *E. coli fad* mutants (Rehm and Steinbüchel 2001). To improve the production of mcl-PHA, a deep-going reinvent was carried out. They found that *E. coli* Δ*fadRABIJ* expressed with the same acyl-ACP thioesterase (named by BTE), *P. aeruginosa* PhaC2, and enoyl-CoA hydratase (phaJ3), CoA ligase PP0763 from *P. putida* produced mcl-PHA with over 15 % CDW, (Agnew et al. 2012). Mutated 3-ketoacyl-ACP synthase III genes *fabH* and 3-ketoacyl-ACP reductases (*FabG*) genes from *E. coli* can channeled the de novo fatty acid to mcl-PHA biosynthesis but via a different point (Nomura et al. 2004). In this case, FabH channeled the 3-ketoacyl-ACP to 3-ketoacyl-CoA, while *FabG* enhanced the conversion of 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA (Nomura et al. 2005).

Some PHAs such as P(3HB-*co*-3HHx) can be synthesized from unrelated carbon source in wild-type bacteria (Fukui et al. 2002; Qiu et al. 2005) but not in recombinant *E. coli* yet. To realize this, the metabolic pathways in wild-type bacteria should be made clear first.

Perspective

Since the PHA synthase has wide-substrate specificity, which enabled the incorporation of various monomers into PHA polymer, many efforts have been done to synthesize various PHAs with different properties in *E. coli*. However, most of the natural microorganism can only synthesize the PHAs with varied monomers under laboratory conditions by adding structurally-related precursors (Steinbüchel and

Lütke-Eversloh 2003). These structurally-related precursors are usually high cost, poorly miscible with water, toxic to bacteria at relatively low concentrations, hard control for fed batch, and/or higher oxygen demand than carbohydrates. Therefore, more and more researchers have moved their interest onto inexpensive and renewable unrelated carbon sources such as glucose. Increasing the efficiency of their use in PHAs biosynthesis is critical to the overall economics. (Figure 1 shows the known metabolic pathways so far which is constructed for PHAs biosynthesis in recombinant *E. coli* from unrelated carbon source.)

Therefore, the principal task in this area is to exploit the metabolic potential of *E. coli* for the production of PHAs with tailor-made monomer composition from unrelated carbon source. Pathways could be constructed via heterologous and/or combinatorial expression of genes from different organisms. In this case, synthetic biology offered us a conceptual and technological framework to speed up the creation of new metabolic enzymes and/or pathways (Lee et al. 2012). Some new creating noninherent pathways for the synthesis of fuels or chemicals (Dellomonaco et al. 2011; Felnagle et al. 2012) could be used as a bridge to link unrelated carbon source with PHAs production. Second, improvement of the PHAs content in the cell is also important. The metabolic flux which leads to the PHAs synthesis can be maximized by optimizing the physiological state of the cell at the systems level. For this purpose, omics technology and/or systems biology provide many tools. Third,

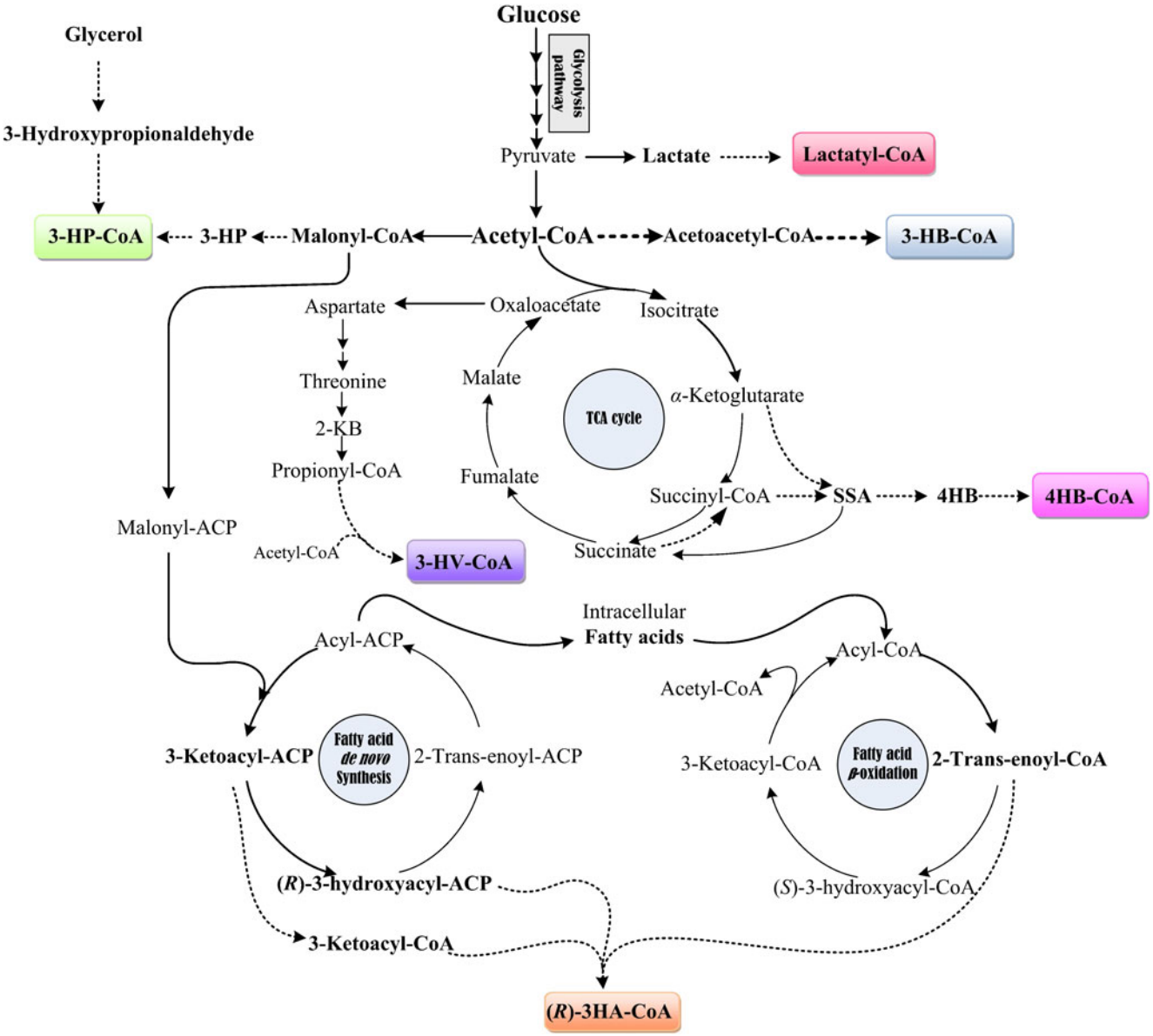

Fig. 1 Scheme showing the metabolic pathways leading to the formation of various PHA monomers in recombinant *E. coli*. Dot line indicates the engineered pathways

most of unrelated carbon sources used for PHAs production is glucose and employment of other cheap, renewable, unrelated carbon source for PHAs production is necessary.

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