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NOTE

Biosynthetic Pathway for Poly(3-Hydroxypropionate) in Recombinant *Escherichia coli*

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Poly(3-hydroxypropionate) (P3HP) is a biodegradable and biocompatible thermoplastic. In this study, we engineered a P3HP biosynthetic pathway in recombinant *Escherichia coli*. The genes for malonyl-CoA reductase (*mcr*, from *Chloroflexus aurantiacus*), propionyl-CoA synthetase (*prpE*, from *E. coli*), and polyhydroxyalkanoate synthase (*phaC1*, from *Ralstonia eutropha*) were cloned and expressed in *E. coli*. The *E. coli* genes *accABCD* encoding acetyl-CoA carboxylase were used to channel the carbon into the P3HP pathway. Using glucose as a sole carbon source, the cell yield and P3HP content were 1.32 g/L and 0.98% (wt/wt [cell dry weight]), respectively. Although the yield is relatively low, our study shows the feasibility of engineering a P3HP biosynthetic pathway using a structurally unrelated carbon source in bacteria.

Keywords: poly(3-hydroxypropionate), malonyl-CoA reductase, propionyl-coenzyme A synthetase, polyhydroxyalkanoate synthase, recombinant *E. coli*

The discovery of polyhydroxybutyrate (PHB) by Lemoigne in 1926 precipitated the identification of more than 150 polyhydroxyalkanoate (PHA) monomer structures (Steinbüchel and Lutke-Eversloh, 2003). As intracellular storage compounds for energy and carbon, PHAs are produced by various bacteria when cells are cultivated in the presence of an excess carbon source, while another nutrient limits growth. In efforts to develop new polymers, engineered strains have been constructed to produce novel PHA homopolymers and block copolymers using metabolic engineering approaches (Chen, 2009; Fukui *et al.*, 2009; Andreeßen and Steinbüchel, 2010; Liu *et al.*, 2011).

Poly(3-hydroxypropionate) (P3HP) is a biodegradable and biocompatible thermoplastic exhibiting high rigidity, ductility, and exceptional tensile strength in drawn films (Andreeßen and Steinbüchel, 2010). This polymer can be chemically synthesized via the ring-opening polymerization of β-propiolactone (Gresham *et al.*, 1948; Yamashita *et al.*, 1996). However it is not commercially feasible as β-propiolactone is a human carcinogen and too costly. Thus far, there has been no report demonstrating that a natural organism can synthesize P3HP.

Information on PHA copolymers containing 3HP monomer were summarized by Andreeßen and Steinbüchel (2010). As 3HP is not a common compound in most metabolic pathways, most of those copolymers were produced when the engineered strains were fed with structurally related carbon sources such as 3-hydroxypropionate (3HP), α,ω-alkanediols, and acrylate (Nakamura *et al.*, 1991; Kang *et al.*, 1993; Valentin *et al.*, 2000; Green *et al.*, 2002). The discovery of 3HP cycle in the green, non-sulfur thermophile, *Chloroflexus aurantiacus*, made it possible to produce 3HP from sugar *in vivo*. In this pathway, malonyl-CoA is reduced to free 3HP by a unique malonyl-CoA reductase (MCR) in an NADPH-dependent manner (Hugler *et al.*, 2002). Fukui *et al.* (2009) introduced the malonyl-CoA reductase and the 3HP-CoA synthetase domain of trifunctional propionyl-CoA synthase from *C. aurantiacus* into a well-known PHB-producing strain *Cupriavidus necator* (formerly *R. eutropha*), and this strain synthesized 0.77 g/L poly(3HB-*co*-3HP) copolymers with 2.1 mol% of the 3HP fraction using fructose as sole carbon source.

The first report about P3HP homopolymer biosynthesis arose in 2010 (Andreeßen *et al.*, 2010). Andreeßen *et al.* (2010) developed the conversion of glycerol into P3HP by introducing the genes for glycerol dehydratase of *Clostridium butyricum*, propionaldehyde dehydrogenase of *Salmonella enterica* serovar Typhimurium LT2, and PHA synthase of *Ralstonia eutropha* into a recombinant *E. coli* strain. P3HP was accumulated up to 1.42 g/L in fed-batch fermentation.

Compared with the P3HP pathway using glycerol, the method utilizing MCR was expected to have some advantages. Acetyl-CoA is a common intermediate of sugar metabolism, so that various sugars derived from lignocellulosic biomass can be used as raw material for P3HP production. Furthermore, the production of P3HP from glucose is redox neutral. The conversion of glucose to each mol of acetyl-CoA generates 2 mol of NADH, while subsequent reduction of malonyl-CoA to 3HP utilizes 2 mol of NADPH, which is the product of $NADP^+$ reduction by NADH and pyridine nucleotide

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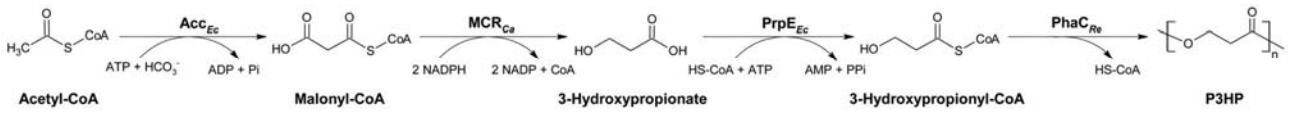

Fig. 1. Pathway for P3HP biosynthesis in the recombinant *E. coli*. Acc_{Ec}, acetyl-CoA carboxylase of *E. coli*; MCR_{Ca}, malonyl-CoA reductase of *C. aurantiacus*; $PhaC_{Re}$, PHA synthase of *R. eutropha*; $PrpE_{Ec}$, propionyl-CoA synthetase of *E. coli*.

transhydrogenase PntAB (Clarke and Bragg, 1985).

To construct a recombinant *E. coli* strain producing P3HP, we developed a new strategy using MCR. Fukui *et al.* (2009) suggested that lower activity of 3HP-CoA synthetase than that of malonyl-CoA reductase caused the limited incorporation of 3HP units into the copolymers. Low activity of this thermophilic enzyme could be a result of low temperature (30°C for *C. necator* growth). To achieve higher 3HP-CoA synthetase activity at 37°C (for *E. coli* growth), the propionyl-CoA synthetase from *E. coli* was overexpressed. In addition, the acetyl-CoA carboxylase converting acetyl-CoA into malonyl-CoA was used to channel the carbon into the P3HP pathway.

Seven genes required in the MCR route were assembled into operons and cloned into two compatible plasmids. Plasmid pA-*accADBC* (Fig. 2, Cao *et al.*, 2011) carries the *accADBC* genes encoding subunits of acetyl-CoA carboxylase. Plasmid pHP304 was constructed as follows: the PHA synthase gene *phaC1* from *R. eutropha* H16 (Friedrich *et al.*, 2006) synthesized by Genray Company (China) was inserted into the *Nde*I and *Bam*HI sites of the pET21a(+) vector to generate plasmid pHP301. Then, a PCR fragment containing the propionyl-CoA synthetase gene *prpE*, generated with primers 129 (5′-CAGGATCCGGAGGAATAAACCATGTCTTTTA GCGAATTTTATC-3′) and 153 (5′-CAGAAGCTTACTCT TCCATCGCCTGGC-3′) and *E. coli* BL21(DE3) genomic DNA as template, was digested with *Bam*HI and *Hin*dIII and then ligated between the same restriction sites of pHP301 to construct pHP302. Next, a PCR fragment containing the malonyl-CoA reductase gene *mcr,* generated with primers 125 (5′-GGCAGATCTCAGCGGAACAGGACGAC-3′) and 126 (5′-CCCTCGAGGAATTTACACGGTAATCGC-3′) and the pTrc99A-*mcr* plasmid (Kroeger *et al.*, 2011) as template, was digested with *Bgl*II and *Xho*I and then ligated between the same restriction sites of expression vector pCOLADuet-1 to construct pHP303. Finally, the *Hin*dIII-*Xho*I fragments from pHP303 containing the T7 promoter and *mcr* coding region were purified from an agarose gel, and ligated with pHP302 digested with *Hin*dIII and *Xho*I. The resulting plasmid was named as pHP304 (Fig. 2), and its structure confirmed by restriction enzyme digestion and DNA sequencing.

Expression of the cloned genes was determined by SDS-PAGE and by Coomassie brilliant blue staining as shown in Fig. 3A, and the gel was analyzed using Quantity One software (Bio-Rad, USA). All proteins, except PrpE, were observed as distinct bands with the expected molecular weights.

Enzymatic activities of MCR and PrpE were determined *in vitro*, using crude extracts obtained by sonication of cells of *E. coli* BL21(DE3)/pHP304, using cells of *E. coli* BL21(DE3)/pET21a(+) as a negative control. The malonyl-CoA reductase activity was measured as described (Kroeger *et al.*, 2011). The malonyl-CoA is reduced to 3HP with an apparent activity of 0.23±0.02 μmol/min/mg protein at 37°C, which presented about 60% of the enzyme activity at 57°C (0.38± 0.01 μmol/min/mg protein, Fig. 3B). Considering the high expression level of MCR (>10% of the total soluble proteins, Fig. 3A), its activity was quite low.

The propionyl-CoA synthetase activity was quantified as described (Horswill and Escalante-Semerena, 1999). The crude cell extract catalyzed the formation of 3HP-CoA at an apparent activity of 1.1±0.1 μmol/min/mg protein, which is much lower than that when propionate was used as substrate (3.6±0.4 μmol/min/mg protein) (Fig. 3C). The velocity of 3HP-CoA synthesis was almost 5-fold higher than that of malonyl-CoA reduction.

For P3HP accumulation, control strain *E. coli* BL21(DE3)/pET21a(+) and *E. coli* BL21(DE3)/pHP304 were cultivated in baffled shaking flasks containing minimal medium (30 mM potassium phosphate buffer (pH 7.0), 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L yeast extract, and 20 g/L glucose). The recombinant cells were induced at ~0.6 OD_{600} with 0.2 mM IPTG. IPTG (0.2 mM), biotin (40 mg/L), and

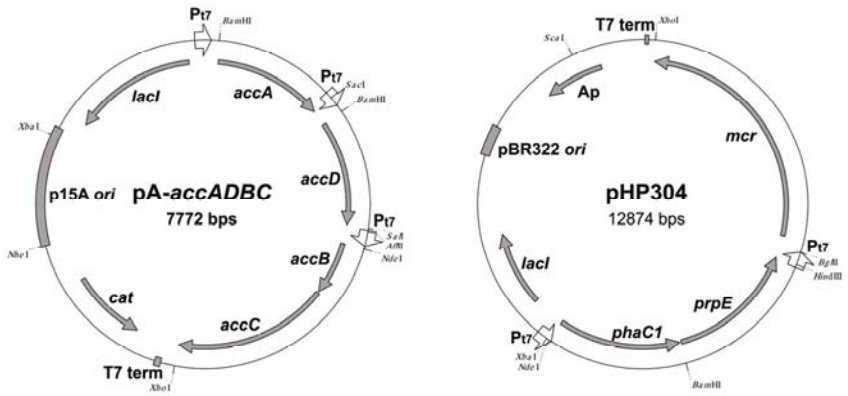

Fig. 2. Physical maps of plasmids pA-*accADBC* and pHP304. Pt7, phage T7 promoter; T7 term, phage T7 terminator; *ori*, origin of replication; *cat*, chloramphenicol resistance gene; Ap, ampicillin resistance gene; *lacI*, *lac* repressor protein.

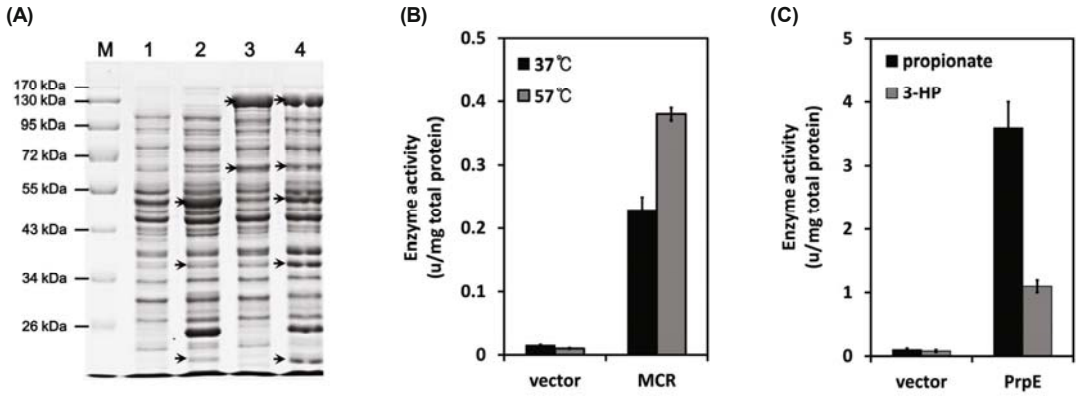

Fig. 3. Expression and enzyme activity of heterologous proteins in *E. coli* cells. (A) Coomassie brilliant blue-stained SDS-PAGE of crude cell lysate from different constructs. Lanes: 1, *E. coli* BL21(DE3)/pACYCDuet-1/pET21a; 2, *E. coli* BL21(DE3)/pA-*accADBC*; 3, *E. coli* BL21(DE3)/pHP304; 4, *E. coli* BL21(DE3)/pA-*accADBC*/pHP304; M, PageRuler Prestained Protein Ladder (Fermentas). The positions of overexpressed AccC (49.3 kDa), AccA and AccD (35 kDa and 33.3 kDa), AccB (16.7 kDa), MCR (132 kDa), and PhaC1 (64 kDa) are indicated by arrows. (B) Enzyme activity of malonyl-CoA reductase MCR at 37°C (black) and 57°C (grey). One unit of activity (u) was defined as the protein amount required for the synthesis of 1 μmol 3HP in 1 min. (C) Enzyme activity of propionyl-CoA synthase PrpE when propionate (black) and 3HP (grey) was used as substrate. One unit of activity (u) was defined as the protein amount required for the synthesis of 1 μmol acyl-CoA in 1 min.

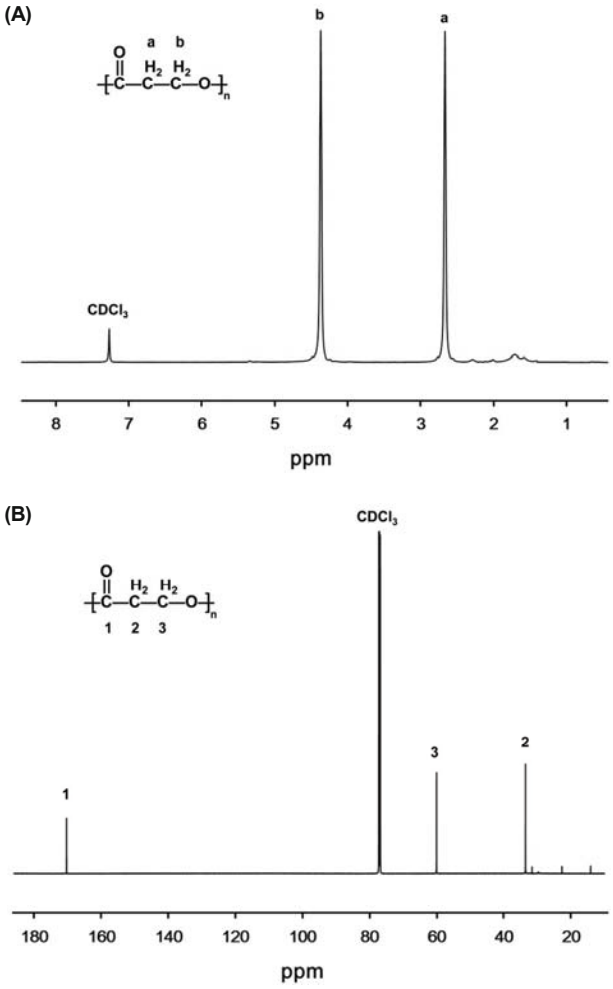

Fig. 4. The 600 MHz ^{1}H (A) and ^{13}C (B) NMR spectra of P3HP synthesized by *E. coli* BL21(DE3)/pA-*accADBC*/pHP304 in $CDCl_3$ solution.

$NaHCO_3$ (20 mM) were added periodically every 12 h until 60 h. After 72 h cultivation, the cells were harvested by centrifugation and lyophilized. PHA was extracted from lyophilized cells with hot chloroform in a Soxhlet apparatus, and precipitated by ice-cold ethanol as described (Lageveen *et al.*, 1988). While the control strain produced no PHA, a trace amount of PHA was detected in the strain carrying pHP304. It was speculated that a low level of cellular malonyl-CoA impeded production of P3HP. Given that overexpression of acetyl-CoA carboxylase elevates cellular malonyl-CoA concentration (Zha *et al.*, 2009), plasmid pA-*accADBC* was transformed into *E. coli* BL21 (DE3)/pHP304. When *acc*, *mcr*, *prpE*, and *phaC1* were coexpressed, accumulation of PHA within the cells was observed by fluorescence microscopy (Ostle and Holt, 1982). Grown with glucose as a sole carbon source, the recombinant cells produced 1.32 g/L cell dry weight containing 0.98% (wt/wt [cell dry weight]) PHA.

10 μg of the extracted PHA was degraded by methanolysis in the presence of sulfuric acid (Brandl *et al.*, 1988). The resulting methyl ester was analyzed using a 5975C Series GC/MSD system (Agilent, USA). GC analyses of the methyl ester of extracted polymer showed a specific peak with a retention time of R_T=5.72 min, which is identical to the retention time of the methyl 3HP standard. This particular peak was extracted by electron impact for MS analysis. Once again, the extracted peak spectrum showed a mass of 103 Da, corresponding to the mass of ionized 3HP methyl ester. Dissociation of this ion led to other masses, such as 31, 43, 55, and 73 Da. Comparison with the NIST database confirmed that the acid methanolysis product of extracted polymer is the methyl 3HP. The chemical structure of P3HP was confirmed by NMR analyses (Fig. 4). The ^{1}H and ^{13}C NMR spectra were recorded at room temperature in d-chloroform ($CDCl_3$) (20 mg/ml) as described (Wang *et al.*, 2009) using an AVANCE III 600 NMR spectrometer (Bruker, Switzerland).

The molecular weight of P3HP was determined using gel permeation chromatography (DAWN HELEOS II, Wyatt,

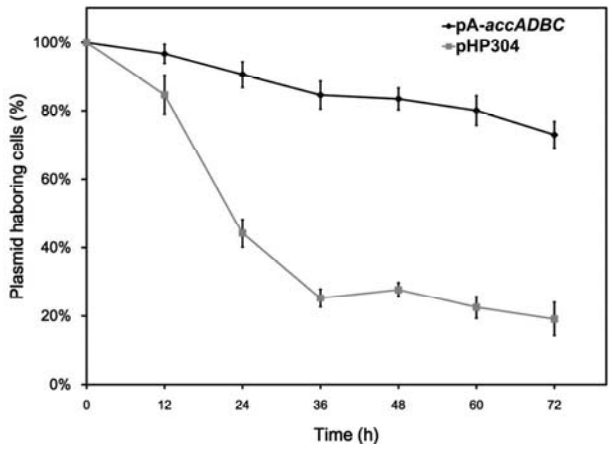

Fig. 5. **Plasmid stability of *E. coli* BL21(DE3)/pA-*accADBC*/pHP304.**

USA) as described (Liu *et al.*, 2011). The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of the P3HP produced by the recombinant *E. coli* were 6.58×10^4 Da and 1.08×10^5 Da, respectively. Differential scanning calorimetry (DSC) data were recorded using Diamond DSC (Pekin Elmer, USA) as described (Arai *et al.*, 1999), and the melting temperature (T_m), glass transition temperature (T_g) , and enthalpy of fusion (ΔH_m) of P3HP were 77°C, 7°C, and 101.6 J/g, respectively.

Compared with other strains producing PHA, our strain has a low content of P3HP. In one aspect, this was caused by loss of plasmids. To verify plasmid stability, diluted culture was spread onto LB plates without any antibiotics, and with ampicillin or chloramphenicol, and grown overnight. The number of colonies on the plates without antibiotics was set to 100%. After 72 h of cultivation, 73% of cells carried plasmid pA-*accADBC*, and only 19% possessed plasmid pHP304 (Fig. 5). The loss of plasmid pHP304 was much higher than pA-*accADBC*, and it was assumed that the segregational instability of pHP304 was caused by two reasons. First, pHP304 is ampicillin resistant and its selective marker is the β-lactamase gene. The β-lactamase secreted into the culture broth caused rapid degradation of ampicillin and overgrowth of plasmid-free cells. Secondly, malonyl-CoA reductase catalyzes a two-step reaction: malonyl-CoA → malonate semialdehyde → 3HP (Hugler *et al.*, 2002). Malonate semialdehyde and 3HP are both toxic to *E. coli* cells (Poelarends *et al.*, 2003; Warnecke *et al.*, 2008), and aggravated pHP304 instability. To achieve a more stable strain, chromosomal insertion may be used in the future.

Low activity of malonyl-CoA reductase was also responsible for the low P3HP content. Fukui *et al.* (2009) suggested that activity of 3HP-CoA synthetase lower than that of malonyl-CoA reductase limited production of P3HP. In our study, 5-fold higher activity of 3HP-CoA synthetase over malonyl-CoA reductase was achieved, but the P3HP production still remained at a similar level. The 3HP monomer production in a recombinant *E. coli* strain using malonyl-CoA reductase was reported recently, and it also faced the same problem of low productivity (Rathnasingh *et al.*, 2012). These facts indicated that malonyl-CoA reductase was the real rate-limiting step. MCR is most active at 57°C (Kroeger *et al.*, 2011), and the remained activity at 37°C is only about 60% of maximal-activity (Fig. 3B). Furthermore, MCR activity is strictly regulated by its postulated physiological requirement. It was reported that only about 37% of the maximal rate could be measured in heterotrophically grown *C. aurantiacus* cells, although these cells grew five times faster than cells under autotrophic conditions (Hugler *et al.*, 2002). When expressed in *E. coli*, MCR enzymatic activity could be further down-regulated due to the change of physiological environment through some unknown mechanism.

In this study, we focused on microbial synthesis of P3HP without any supplement of related precursors. An artificial pathway for P3HP production was constructed by applying several enzymes including malonyl-CoA reductase, propionyl-CoA synthetase and PHA synthase, and the engineered *E. coli* strain showed ability to synthesize the P3HP polyester from glucose. Although the P3HP content is low, this pathway was expected to have some advantages over other reported P3HP biosynthetic pathways. It is necessary to carry out further development to achieve a high P3HP yield for large-scale P3HP production.

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References

Andreeßen, B., Lange, A.B., Robenek, H., and Steinbüchel, A. 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **76**, 622–626.

Andreeßen, B. and Steinbüchel, A. 2010. Biosynthesis and biodegradation of 3-hydroxypropionate-containing polyesters. *Appl. Environ. Microbiol.* **76**, 4919–4925.

Arai, Y., Cao, A., Yoshie, N., and Inoue, Y. 1999. Studies on co-monomer compositional distribution and its effect on some physical properties of bacterial poly(3-hydroxybutyric acid-*co*-3-hydroxypropionic acid). *Polym. Int.* **48**, 1219–1228.

Brandl, H., Gross, R.A., Lenz, R.W., and Fuller, R.C. 1988. *Pseudomonas oleovorans* as a source of poly(b-Hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl. Environ. Microbiol.* **54**, 1977–1982.

Cao, Y.J., Jiang, X.L., Zhang, R.B., and Xian, M. 2011. Improved phloroglucinol production by metabolically engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **91**, 1545–1552.

Chen, G.Q. 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem. Soc. Rev.* **38**, 2434–2446.

Clarke, D.M. and Bragg, P.D. 1985. Cloning and expression of the transhydrogenase gene of *Escherichia coli*. *J. Bacteriol.* **162**, 367–373.

Friedrich, B., Pohlmann, A., Fricke, W.F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Potter, M., and *et al.* 2006. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. *Nat. Biotechnol.* **24**, 1257–1262.

Fukui, T., Suzuki, M., Tsuge, T., and Nakamura, S. 2009. Microbial synthesis of poly((*R*)-3-hydroxybutyrate-*co*-3-hydroxypropionate) from unrelated carbon sources by engineered *Cupriavidus necator*. *Biomacromolecules* **10**, 700–706.

Green, P.R., Kemper, J., Schechtman, L., Guo, L., Satkowski, M., Fiedler, S., Steinbüchel, A., and Rehm, B.H. 2002. Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid beta-oxidation inhibited *Ralstonia eutropha*. *Biomacromolecules* **3**, 208–213.

Gresham, T.L., Jansen, J.E., and Shaver, F.W. 1948. β-Proponlactone. I. Polymerization reactions. *J. Am. Chem. Soc.* **70**, 998–999.

Horswill, A.R. and Escalante-Semerena, J.C. 1999. The *prpE* gene of *Salmonella typhimurium* LT2 encodes propionyl-CoA synthetase. *Microbiology* **145(Pt 6)**, 1381–1388.

Hugler, M., Menendez, C., Schagger, H., and Fuchs, G. 2002. Malonyl-coenzyme A reductase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO_2 fixation. *J. Bacteriol.* **184**, 2404–2410.

Kang, C.K., Lee, H.S., and Kim, J.H. 1993. Accumulation of PHA and its copolyesters by *Methylobacterium* sp. KCTC 0048. *Biotechnol. Lett.* **15**, 1017–1020.

Kroeger, J.K., Zarzycki, J., and Fuchs, G. 2011. A spectrophotometric assay for measuring acetyl-coenzyme A carboxylase. *Anal. Biochem.* **411**, 100–105.

Lageveen, R.G., Huisman, G.W., Preusting, H., Ketelaar, P., Eggink, G., and Witholt, B. 1988. Formation of polyesters by *Pseudomonas oleovorans* - Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* **54**, 2924–2932.

Liu, Q., Luo, G., Zhou, X.R., and Chen, G.Q. 2011. Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by β-oxidation pathway inhibited *Pseudomonas putida*. *Metab. Eng.* **13**, 11–17.

Nakamura, S., Kunioka, M., and Doi, Y. 1991. Biosynthesis and characterization of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxypropionate). *J. Macromol. Sci. Chem.* **A28**, 15–24.

Ostle, A.G. and Holt, J.G. 1982. Nile blue A as a fluorescent stain for poly-β-hydroxybutyrate. *Appl. Environ. Microbiol.* **44**, 238–241.

Poelarends, G.J., Johnson, W.H., Jr., Murzin, A.G., and Whitman, C.P. 2003. Mechanistic characterization of a bacterial malonate semialdehyde decarboxylase: identification of a new activity on the tautomerase superfamily. *J. Biol. Chem.* **278**, 48674–48683.

Rathnasingh, C., Raj, S.M., Lee, Y., Catherine, C., Ashok, S., and Park, S. 2012. Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. *J. Biotechnol.* **157**, 633–640.

Steinbüchel, A. and Lutke-Eversloh, T. 2003. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochem. Eng. J.* **16**, 81–96.

Valentin, H.E., Mitsky, T.A., Mahadeo, D.A., Tran, M., and Gruys, K.J. 2000. Application of a propionyl coenzyme A synthetase for poly(3-hydroxypropionate-*co*-3-hydroxybutyrate) accumulation in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **66**, 5253–5258.

Wang, H.H., Li, X.T., and Chen, G.Q. 2009. Production and characterization of homopolymer polyhydroxyheptanoate (P3HHp) by a *fadBA* knockout mutant *Pseudomonas putida* KTOY06 derived from *P. putida* KT2442. *Proc. Biochem.* **44**, 106–111.

Warnecke, T.E., Lynch, M.D., Karimpour-Fard, A., Sandoval, N., and Gill, R.T. 2008. A genomics approach to improve the analysis and design of strain selections. *Metab. Eng.* **10**, 154–165.

Yamashita, M., Takemoto, Y., Ihara, E., and Yasuda, H. 1996. Organolanthanide-initiated living polymerizations of ε-caprolactone, δ-valerolactone, and β-propiolactone. *Macromolecules* **29**, 1798–1806.

Zha, W., Rubin-Pitel, S.B., Shao, Z., and Zhao, H. 2009. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. *Metab. Eng.* **11**, 192–198.