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Metabolic engineering of *Escherichia coli* for the synthesis of the quadripolymer poly(glycolate-*co*-lactate-*co*-3-hydroxybutyrate-*co*-4-hydroxybutyrate) from glucose

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ABSTRACT

Escherichia coli was metabolically engineered to effectively produce a series of biopolymers consisted of four types of monomers including glycolate, lactate, 3-hydroxybutyrate and 4-hydroxybutyrate from glucose as the carbon source. The biosynthetic route of novel quadripolymers was achieved by the overexpression of a range of homologous and heterologous enzymes including isocitrate lyase, isocitrate dehydrogenase kinase/phosphatase, glyoxylate/hydroxypyruvate reductase, propionyl-CoA transferase, β-ketothiolase, acetoacetyl-CoA reductase, succinate semialdehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase, CoA transferase and PHA synthase. In shake flask cultures using Luria-Bertani medium supplemented with glucose, the recombinant *E. coli* reached 7.10 g/l cell dry weight with 52.60 wt% biopolymer content. In bioreactor study, the final cell dry weight was 19.61 g/l, containing 14.29 g/l biopolymer. The structure of the produced polymer was chemically characterized by proton NMR analysis. Assessment of thermal and mechanical properties demonstrated that the quadripolymer possessed decreased crystallinity and improved toughness, in comparison to poly-3-hydroxybutyrate homopolymer. This is the first study reporting efficient microbial production of the quadripolymer poly(glycolate-*co*-lactate-*co*-3-hydroxybutyrate-*co*-4-hydroxybutyrate) from glucose.

1. Introduction

Manufacturing chemicals and materials from renewable feedstocks using microbial biocatalysts has the potential to alleviate environmental concerns and volatility of oil market prices (Dugar and Stephanopoulos, 2011). The primary challenge for microbial fermentation production routes is achieving high titer, yield and productivity with low-cost substrates to make the overall process economically feasible (Shaw et al., 2016). Recent developments of metabolic engineering strategies have achieved great successes in constructing biosynthetic routes for valuable bulk commodity chemicals, such as ethylene glycol (Chen et al., 2016b; Pereira et al., 2016), glycolate (Deng et al., 2015; Pereira et al., 2016), succinate (Ahn et al., 2016), 1-butanol (Lan and Liao, 2012) and 1,4-butanediol (Yim et al., 2011). Polyhydroxyalkanoates (PHA) are microbially produced thermoplastic polymers with versatile material properties. The applications of PHA in packaging industry and biomedical materials have been extensively studied (Chen et al., 2015). Currently, the commercialization of PHA still depends on research aiming at lowering production cost, exploring high-value applications and achieving better material properties (Heinrich et al., 2016; Wang et al., 2014b).

Poly-3-hydroxybutyrate (P3HB) is most well-studied member of PHA family. Starting from acetyl-CoA, the P3HB producing pathway in *Ralstonia eutropha* involved three enzymes, β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase (Schubert et al., 1988; Slater et al., 1988). In terms of material properties, P3HB is rather brittle and rigid with low flexibility because of its high crystallinity. The incorporation of a variety of secondary monomers such as 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HHx), 4-hydroxybutyrate (4HB) and 3-hydroxypropionate (3HP) has been studied to improve the material properties of the polymers (Anderson and Dawes, 1990; Sudesh et al., 2000). For example, supplementation of structurally related carbon sources such as propionic acid or 4-hydroxybutyric acid as auxiliary substrate led to the accumulation of poly(3HB-*co*-3HV) (PHBV) or poly(3HB-*co*-4HB) (P3HB4HB) (Choi and Lee, 1999; Doi et al., 1988). Both PHBV and P3HB4HB copolymers showed decreased crystallinity and improved toughness in comparison to P3HB homopolymer. Furthermore, the alterations of monomer ratios generated a

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series of copolymers with diverse material properties, which could be achieved by changing the ratio of the supply of the different carbon sources (Doi et al., 1995; Sudesh et al., 2000).

Although the employment of structurally related precursors for PHA production enriched the diversity of PHA family, the use of multiple carbon substrates has several disadvantages in the fermentation scale-up process, including high feedstock costs and complexities in large-scale fermentation control. Therefore, considerable efforts have been devoted to engineering the microbial production of PHA with tailor-made monomer compositions from a single and abundant carbon source (Chen et al., 2011; Heinrich et al., 2015; Hokamura et al., 2015; Meng et al., 2015; Wang et al., 2014a, 2015). In this case, *de novo* pathway design and metabolic engineering allowed the reconstruction of complex pathways *via* heterologous and/or combinatorial expression of genes from multiple hosts in chassis cells such as *Escherichia coli* to fulfil the task. For example, the combination of succinate degradation pathway of *Clostridium kluyveri* and P3HB biosynthesis pathway of *R. eutropha* in *E. coli*, together with the inactivation of the *E. coli* native succinate semialdehyde dehydrogenase genes led to the accumulation of P3HB4HB copolymer from glucose as single carbon source (Li et al., 2010; Valentin and Dennis, 1997). This study provided proof of the concept that copolymers could be effectively produced by metabolic engineering *E. coli*.

Moreover, the identification and engineering of key biosynthesis enzymes (for example, PHA synthase possessing broad substrate specificity) led to the production of new and tailor-made polymers, such as lactate-based polyesters (Taguchi et al., 2008). Recently, the crystal structure of the catalytic C-terminal domain of PHA synthase from *R. eutropha* have been resolved (Kim et al., 2017; Wittenborn et al., 2016). These findings will facilitate further biochemical and structural characterization of PHA synthase, which could assist in engineering efforts to produce new tailor-made materials. Great diversity of PHA, as described by PHAome, can be achieved by changing monomers, monomer ratios, monomer arrangements and molecular weights (Chen and Hajnal, 2015). Novel PHA are continuously synthesized by researchers, leading to the discovery of new material properties and applications. Advances in metabolic engineering provided a technological and conceptual framework to speed up the creation of new metabolic pathways for novel PHA synthesis (Chen et al., 2015, 2016a). It has been suggested that efficient production of PHA with desirable structures by a single bacterial platform using a simple substrate such as glucose will help to build platforms to supply sufficient quantities of advanced materials for industrial development. Previously, we have engineered recombinant *E. coli* to produce poly(glycolate-*co*-lactate-*co*-3-hydroxybutyrate) [poly(GA-LA-3HB)] by overexpressing a combinatorial synthetic metabolic pathway consisting of seven genes (Li et al., 2016). In this study, we propose to overexpress more complicated metabolic pathways, including the *E. coli* native glyoxylate bypass pathway, the anaerobic succinate degradation pathway of *C. kluyveri* and the P3HB biosynthesis pathway of *R. eutropha*, to produce the quadripolymer poly (glycolate-*co*-lactate-*co*-3-hydroxybutyrate-*co*-4-hydroxybutyrate) [poly(GA-LA-3HB-4HB)] (Fig. 1). By combinatorial pathway assembling, we firstly demonstrated that *E. coli* could be engineered to effectively produce biopolymers with four types of monomers from glucose as single carbon source. In addition, nuclear magnetic resonance (NMR), molecular weight assay, differential scanning calorimetry (DSC) and mechanical tests were performed to assess the material properties of the produced biopolymer.

2. Materials and methods

2.1. Strains, plasmids and culture medium

Bacterial strains and plasmids used in this study are listed in Table 1. E. coli JM109 was used as the host for plasmid construction and PHA producing experiments. Gene deletions in E. coli JM109 were performed according to the protocols reported previously (Datsenko and Wanner, 2000; Li et al., 2010). The PCR product containing the kanamycin selection marker used for λ-Red recombination was amplified from the chromosome of E. coli JW2946-1 using primers glcDF/glcDR (for glcD knockout) (Table 2).

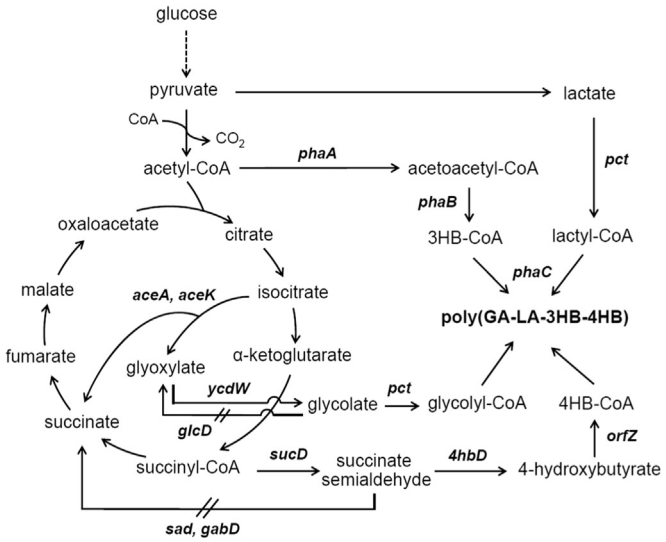

Fig. 1. Metabolic network for the synthesis of poly(GA-LA-3HB-4HB) from glucose by *Escherichia coli*. Genes: *phaA*, β-ketothiolase; *phaB*, NADPH-dependent acetoacetyl-CoA reductase; *phaC*, PHA synthase; *pct*, propionyl-CoA transferase; *aceA*, isocitrate lyase; *aceK*, isocitrate dehydrogenase kinase/phosphatase; *ycdW*, glyoxylate/hydroxypyruvate reductase; *sucD*, succinate semialdehyde dehydrogenase; *4hbD*, 4-hydroxybutyrate dehydrogenase; *orfZ*, CoA transferase; *glcD*, glycolate oxidase subunit, FAD-linked; *sad* and *gabD*, succinate semialdehyde dehydrogenase of *E. coli*.

All *E. coli* strains were cultivated in Luria-Bertani (LB) medium or minimal medium (MM). LB medium contained 5 g/l yeast extract, 10 g/l Bacto tryptone and 10 g/l NaCl. MM medium consisted of (g/l): NH_4Cl 2.0, $(NH_4)_2SO_4$ 5.0, KH_2PO_4 6.0, MOPS 8.4, NaCl 0.5, $MgSO_4$ 0.24, Na_2MoO_4 0.002 and trace elements solution 1 ml/l. The trace elements solution contained (g/l): $FeCl_2{\cdot}4H_2O$ 3.6, $CaCl_2{\cdot}2H_2O$ 5.0, $MnCl_2{\cdot}2H_2O$ 1.3, $CuCl_2{\cdot}2H_2O$ 0.38, $CoCl_2{\cdot}6H_2O$ 0.5, $ZnCl_2$ 0.94, H_3BO_3 0.0311, $Na_2EDTA{\cdot}2H_2O$ 0.4 and thiamine-HCl 1.01.

2.2. Plasmid construction

Standard molecular cloning procedures or manufacturers' instructions were followed for plasmid construction. Oligonucleotides were purchased from Sangon Biotech (Shanghai, China) and are listed in Table 2. In all cases, Q5® High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA, US) was employed for PCR reactions. Plasmid isolation and DNA purification kits were purchased from Biomed (Beijing, China). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs.

The CoA transferase gene *orfZ* of *C. kluyveri* DSM555 was amplified from the genomic DNA by primers orfZF/orfZR and cloned into expression vector pEn *via* *Bam*HI/*Kpn*I digestion and ligation to construct pEn-orfZ. Subsequently, *orfZ* gene along with the constitutive promoter was excised from pEn-orfZ using *Avr*II/*Xho*I and cloned into pMCSH5 digested with *Nhe*I/*Xho*I to generate pMCSH5Z. The *sucD-4hbD-orfZ* fragment was isolated from pMCSH5Z by *Eco*RI/*Xho*I digestion and cloned into *phaCAB* expression plasmid pBHR68 to construct p68H5Z (Fig. S1). To construct p68pcH5Z, the 3.4-kb *pct-phaC*$_{ps}$ fragment amplified from pCDF-pct-C using primers Re_pct_F/phaA_C_R and the 10.9-kb *phaA-phaB*-vector fragment amplified from p68H5Z using primers C_phaA_F/pct_Re_R were ligated together with Gibson assembly kit (New England Biolabs), which allowed the constitutive expression of *pct*, *phaC*$_{ps}$, *phaA*, *phaB*, *sucD*, *4hbD* and *orfZ* (Fig. S2).