Metabolic Engineering of *Escherichia coli* for the Production of Polylactic Acid and Its Copolymers

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**ABSTRACT:** Polylactic acid (PLA) is a promising biomass-derived polymer, but is currently synthesized by a two-step process: fermentative production of lactic acid followed by chemical polymerization. Here we report production of PLA homopolymer and its copolymer, poly(3-hydroxybutyrate-co-lactate), P(3HB-co-LA), by direct fermentation of metabolically engineered *Escherichia coli*. As shown in an accompanying paper, introduction of the heterologous metabolic pathways involving engineered propionate CoA-transferase and polyhydroxyalkanoate (PHA) synthase for the efficient generation of lactyl-CoA and incorporation of lactyl-CoA into the polymer, respectively, allowed synthesis of PLA and P(3HB-co-LA) in *E. coli*, but at relatively low efficiency. In this study, the metabolic pathways of *E. coli* were further engineered by knocking out the *ackA*, *ppc*, and *adhE* genes and by replacing the promoters of the *ldhA* and *acs* genes with the *trc* promoter based on in silico genome-scale metabolic flux analysis in addition to rational approach. Using this engineered strain, PLA homopolymer could be produced up to 11 wt% from glucose. Also, P(3HB-co-LA) copolymers containing 55–86 mol% lactate could be produced up to 56 wt% from glucose and 3HB. P(3HB-co-LA) copolymers containing up to 70 mol% lactate could be produced up to 46 wt% from glucose alone by introducing the *Cupriavidus necator* β-ketothiolase and acetoacetyl-CoA reductase genes. Thus, the strategy of combined metabolic engineering and enzyme engineering allowed efficient bio-based one-step production of PLA and its copolymers. This strategy should be generally useful for developing other engineered organisms capable of producing various unnatural polymers by direct fermentation from renewable resources.


**KEYWORDS:** polylactic acid; PLA; lactate-based copolymers; metabolic engineering; metabolic flux analysis

**Introduction**

PLA has been considered as a good alternative to petroleum-based plastic as it possesses several desirable properties such as biodegradability, biocompatibility, compostability, and low toxicity to humans (Drumright et al., 2000; Mehta et al., 2005; Södergård and Stolt, 2002; Vink et al., 2003). However, the current process for PLA synthesis is not simple as ring opening polymerization of lactide, a cyclic dimer resulting from the dehydration of lactic acid, or solvent-based azeotropic dehydrative condensation has to be performed (Drumright et al., 2000; Mehta et al., 2005; Södergård and Stolt, 2002; Vink et al., 2003). Improvement of the material properties of PLA, which are quite stiff and brittle, has been attempted by copolymerization or blending with other polymers including polyhydroxyalkanoates (PHAs) (Chen and Wu, 2005; Haynes et al., 2007; Noda et al., 2004; Schreck and Hillmyer, 2007). Despite of these efforts, existing
chemical methods are not effective considering the availability of lactonized monomers used in copolymerization and their cost.

Differently from PLA which requires chemical polymerization of lactic acid or lactide, PHAs are synthesized in vivo by PHA synthase (PhaC) which polymerizes various (D) hydroxacyl-CoA s generated through diverse metabolic pathways in the cell (Lee, 1996). Although lactate has also been suggested as a possible monomer unit of natural PHAs, until recently, there has been no report on the production of PHAs containing lactate as a monomer, which is most likely due to the limitation of substrate specificity of the existing PHA synthases (Steinbüchel and Valentin, 1995; Valentin and Steinbüchel, 1994; Yuan et al., 2001; Zhang et al., 2001). Several years ago, we were able to show that P(3HB-co-LA) could be synthesized in recombinant Cupriavidus necator H16 (formerly,Ralstonia eutropha H16) expressing the Clostridium propionicum propionate CoA-transferase (Pct) gene, which allows generation of (D)-lactyl-CoA in the cell (Cho et al., 2006; Selmer et al., 2002). Also, four representative PHA synthases (type I-IV), including Pseudomonas sp. MBEL 6-19 PHA synthase (PhaC1p6_19) as a type II PHA synthase, were examined for their ability to synthesize lactate-containing polymers, but they allowed production of only minute amounts of P(3HB-co-LA) (Cho et al., 2006). Therefore, Pct and PhaC1p6_19 were engineered by in vitro mutagenesis to efficiently generate lactyl-CoA and incorporate lactyl-CoA into the polymer, respectively, which resulted in the enhanced production of P(3HB-co-LA) (Park et al., 2008b; accompanying paper Yang et al., 2010). Recently, a similar strategy concerned with engineered PHA synthase was employed for the production of P(94 mol% 3HB-co-6 mol% LA) and P(53 mol% 3HB-co-47 mol% LA) in recombinant E. coli (Taguchi et al., 2008; Yamada et al., 2009). The former polymer having quite low lactate fraction was produced with the polymer content of 19 wt% of dry cell weight (DCW) by recombinant E. coli expressing the Megasphaera elsdenii propionate CoA-transferase, the engineered Pseudomonas sp. 61-3 PHA synthase, and C. necator β-ketothiolase and acetacetyl-CoA reductase in a complex medium (Taguchi et al., 2008). To increase the lactate fraction in P(3HB-co-LA), two stage cultivation process of aerobic cell growth phase followed by anaerobic phase for lactate synthesis was employed. Even though lactate fraction increased up to 47 mol%, the polymer content obtained was only 2 wt% of DCW in a complex medium (Yamada et al., 2009). Recombinant E. coli strains possessing our evolved enzymes, Pct and PhaC1p6_19, allowed production of PLA and its various copolymers (accompanying paper Yang et al., 2010). But, their contents were also rather low. Thus, it was necessary to increase the metabolic fluxes at the systems level in order to enhance biosynthesis of these polymers.

In this article, we report development of engineered E. coli strains capable of efficiently producing PLA and its copolymers having lactate as a major monomer by employing systems-level metabolic engineering combined with enzyme engineering (Fig. 1). This strategy not only provides a versatile platform for the one-step production of lactate-based polymers, but also opens up the possibility of producing other unnatural polymers from renewable resources.

**Materials and Methods**

**Materials**

The strains, plasmids and primers used are listed in Table I and Supplementary Table I. Detailed procedures for the construction of strains are described below. All DNA manipulations were performed according to standard procedures (Sambrook and Russell, 2001). All oligonucleotides were synthesized at Bioneer (Daejeon, Korea). Preparation of plasmids and DNA fragments were performed with Qiagen kits (Qiagen, Chatsworth, CA). All other chemicals used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO). DNA sequencing was carried out using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) on an ABI Prism 377 DNA sequencer (Perkin Elmer).

**Genome Engineering**

Deletion of the ackA (acetate kinase), ppc (phosphoenolpyruvate carboxylase), and adhE (acetaldehyde/alcohol dehydrogenase) genes was performed using one-step inactivation method (Datsenko and Wanner, 2000). Replacement of the native promoters of the ldhA (D-lactate dehydrogenase) and acs (acyt-CoA synthetase) genes with the trc promoter was performed by PCR-mediated λ-Red recombination (Yuan et al., 2006). Detailed procedures for genome engineering are provided below (Supplementary Fig. 1).

Deletion of the ackA, ppc, and adhE genes from the chromosome was performed by one-step inactivation method using the λ-Red recombinase expression plasmid, pKD46 (Datsenko and Wanner, 2000). Recombinant E. coli harboring pKD46 was cultivated at 30°C, and the expression of λ-Red recombinase was induced by adding 10 mM l-arabinose. Then electrocompetent cells were prepared by the standard protocol (Sambrook and Russell, 2001). For the deletion of the ackA gene, the PCR fragment for the homologous recombination was prepared in two steps. A 1,234 bp DNA fragment containing the lox71 site, the chloramphenicol resistance gene, and the lox66 site fused together was obtained by PCR using the primers FDackA1 and RDackA1. Plasmid pMloxC (Lee et al., 2007), which contains the chloramphenicol resistance gene flanked by lox sequences (Cre recognition target), was used as a template. Then, PCR was performed with the primers FDackA2 and RDackA2 using the PCR product (1,234 bp) obtained above as a template. The final PCR product was introduced by electroporation into E. coli harboring pKD46, expressing the λ-Red recombinase. The mutants in which gene inactivation occurred by double homologous recombination were selected on the LB agar plate containing 34 µg/mL chloramphenicol, and subsequently screened by direct colony PCR. Deletion of
the **ppc** gene was performed with the primers FDppc1, RDppc1, FDppc2, and RDppc2, while deletion of the **adhE** gene was performed using the primers FDadhE1, RDadhE1, FDadhE2, and RDadhE2, in the same manner as described for the **ackA** gene deletion. To construct marker-free mutant strains, the antibiotic selection marker was eliminated by using a helper plasmid, pJW168 (Palmeros et al., 2000), expressing the Cre recombinase and harboring ampicillin resistance gene and a temperature-sensitive replication origin. The chloramphenicol resistant mutants were transformed with the pJW168, and ampicillin-resistant transformants were selected on LB agar plates containing 100 μg/mL ampicillin and 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma–Aldrich) (for the expression of Cre recombinase) at 30°C. Those colonies that lost the chloramphenicol resistance were selected. Among them, positive colonies were cultivated in LB medium without antibiotic markers at 42°C, and then were examined for the loss of all antibiotic resistance markers by colony PCR.

To relieve the native regulation of the **ldhA** gene expression, the native promoter of the **ldhA** gene was replaced with the **trc** promoter. Substitution of the native promoter of the **ldhA** gene by the **trc** promoter was performed by PCR-mediated λ-Red recombination (Yuan et al., 2006). The PCR fragment for the homologous recombination was prepared in three steps. A 1,189 bp DNA fragment containing the lox71 site, the chloramphenicol resistance gene, and the lox66 site fused together was obtained by PCR using the primers FPldhA1 and RPldhA1. Plasmid pMloxC was used as a template. Then, PCR was performed with the primers FPldhA2 and RPldhA2 using the PCR product (1,189 bp) obtained above as a template. To introduce the **trc** promoter, two primers, RPldhA1 and RPldhA2, which contain the **trc** promoter sequence, were synthesized. The final PCR was performed with the primers FPldhA3 and RPldhA3 using the second PCR product as a template. The final PCR product was introduced by electroporation into **E. coli** harboring pKD46 (Datsenko and Wanner, 2000), expressing

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**Figure 1.** Metabolic engineering of **E. coli** for the production of PLA homopolymer and P(3HB-co-LA) copolymers. The overall metabolic network is shown together with the introduced metabolic pathways for the production of PLA homopolymer and P(3HB-co-LA) copolymer in **E. coli** by combining enzyme engineering and metabolic engineering strategies. The genes shown in orange represent those that were initially manipulated by rational engineering, and the genes shown in purple are those that were manipulated based on in silico gene knockout simulation and flux response analysis. The colored arrows (reactions) represent the results of in silico flux response analysis (see below). The gray arrows next to 3HB and SUC indicate the extracellular feeding of (D,L)-3-hydroxybutyrate and succinate, respectively. The graph in the right top panel represents the results of in silico single gene knockout simulation showing the change in in silico PLA production rate versus cell growth rate; the three best candidate genes (**adhE**, **gdhA**, and **udhA**) allowing high PLA production rate upon their knockout are shown. The control JLX7 strain expressing PhaC1310PsPs6-19 and Pct540Cp (green diamond; JLX7*) is also shown for comparison. The graphs in the right bottom panel represent the results of in silico flux response analysis. The x-axis denotes the normalized in silico cell growth rate, and the y-axis denotes the normalized in silico PLA production rate. There are four types of responses of the in silico PLA production and cell growth rates to varying the individual flux in the central metabolic pathway. Abbreviations are: G6P, glucose-6-phosphate; RL5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; F1,6dP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; ICT, isocitrate; α-KG, α-ketoglutarate; SUC, succinate; MAL, malate; OAA, oxaloacetate.
the λ-Red recombinase. The mutants in which promoter replacement occurred by double homologous recombination were selected on the LB agar plate containing 34 μg/mL chloramphenicol, and subsequently screened by direct colony PCR. Replacement of the promoter of the ldhA gene with the trc promoter was confirmed by DNA sequence analysis. Substitution of the native promoter of the acs gene by the trc promoter was performed using the primers FPacs1, RPacs1, FPacs2, RPacs, FPacs3, and RPacs3 in the same manner as described for the ldhA promoter replacement. To construct marker-free mutant strains, removal of antibiotic marker was performed in the same manner as described for the chromosomal gene inactivation above.

### Medium and Cultivation

Luria-Bertani (LB) medium (containing per liter: 10 g tryptone, 5 g yeast extract and 5 g NaCl) supplemented with appropriate antibiotics was used for gene cloning from *E. coli* XL1-Blue. For the production of PLA and its metabolites, a chemically defined MR medium was used. The MR medium (pH 7.0) contains per liter: 6.67 g KH₂PO₄, 4 g (NH₄)₂HPO₄, 0.8 g MgSO₄·7H₂O, 0.8 g citric acid, and 5 mL trace metal solution. The trace metal solution contains per liter of 0.5 M HCl: 10 g FeSO₄·7H₂O, 2 g CaCl₂, 2.2 g ZnSO₄·7H₂O, 0.5 g MnSO₄·4H₂O, 1 g CuSO₄·5H₂O, 0.1 g (NH₄)₆Mo₇O₂₄·4H₂O, and 0.02 g Na₂B₄O₇·10H₂O. Carbon source, MgSO₄·7H₂O, and 3HB were sterilized separately. Seed cultures were prepared in 15 mL test tubes containing 3 mL LB medium at 30°C overnight in a rotary shaker at 250 rpm. One milliliter of overnight culture was used to inoculate 250 mL flask containing 100 mL MR medium supplemented with 20 g/L glucose and 2 g/L 3HB (Acros organics, Geel, Belgium), unless otherwise specified. Flask cultures were carried out at 30°C in a rotary shaker at 250 rpm for 72 h. For the cultivation of strains lacking the ppc gene, 4 g/L sodium succinate (Sigma–Aldrich) was provided to overcome growth limitation. For the expression of the ldhA and the acs genes under trc promoter, 1 mM IPTG (Sigma–Aldrich) was added at the OD₆₀₀ of 0.5. When necessary, 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, 10 μg/mL tetracycline, and 10 μg/mL thiamine were added to the medium.

### In Silico Analysis

The in silico knockout simulation studies were performed by using the genome-scale metabolic model *E. coli* EcoM-BEL979 consisting of 979 metabolic reactions and 814 metabolites (144 extracellular metabolites and 670 intermediates), which is a slightly modified network of iJRR04 reported by Reed et al. (Lee et al., 2005; Reed et al., 2003) (Fig. 1, right top panel). Under the broad range of oxygen uptake rate, the gene knockout targets were identified by employing the minimization of metabolic adjustment (MOMA) algorithm (Segrè et al., 2002) and by using the linear and quadratic programming methods by using a GAMS script. The base steady-state solution required for the quadratic programming in MOMA was calculated for each simulation by first solving the linear programming problem.
of maximizing the growth rate. The effects of varying the surplus lactate and acetyl-CoA fluxes on the biosynthesis of PLA polymers were also examined by including two reactions, acetyl-CoA → acetyl-CoA (surplus) and D-lactate → D-lactate (surplus), in the genome-scale model of the wild-type E. coli K12 EcoMBEL979 (Fig. 2A). Changes in the cell growth rate were examined while the two fluxes were varied (Fig. 2B and Supplementary Fig. 2). To predict an effective target gene for amplification, the individual flux of the central metabolic pathway was altered to examine the in silico flux response (Moon et al., 2008) between the cell growth rate and the PLA production rate (Fig. 1, right bottom panel).

All flux analyses were performed on the rationally engineered JLX7 strain expressing PhaC1310Ps6-19 and Pct540Cp using the genome-scale model EcoMBEL979 containing additional surplus fluxes. For the flux analysis of the recombinant JLX7 strain, the glucose uptake rate of 4.46 mmol/g DCW/h was used as a constraint, which was the experimentally measured value at the growth phase of 0–25 h. The uptake rate of succinate, which was supplemented to the medium, was set to 0.46 mmol/g DCW/h. Also, the PLA production rate of 0.037 mmol/g DCW/h, which was the experimentally measured value obtained with the recombinant JLX7 strain, was used as an initial point for gene knockout simulation. Because PLA can have varying M_w’s, the PLA production rate is formulated in the genome-scale metabolic model as the rate of lactate monomer incorporated into the polymer during the simulation.

Transmission Electron Microscopy Analysis

Cells were fixed in 2.5% paraformaldehyde-glutaraldehyde mixture in 0.1 M phosphate buffer (pH 7.2) for 2 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812. Ultra-thin sections, made by ULTRACUT E (Leica, Vienna, Austria) ultramicrotome, were stained with uranyl acetate and lead citrate and examined under the CM 20 electron microscope (Philips, Eindhoven, Netherlands).

Polymer Analysis

The content and monomer composition of the synthesized polymer were determined by gas chromatography (GC) (Braunegg et al., 1978). Polymers were purified from the cells by the solvent extraction method (Jacquel et al., 2008). The structure and molecular weight of the polymer were determined by nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC), respectively. Detailed conditions for the purification and analyses of polymers are described below.

The content and monomer composition of polymers accumulated in the cells were determined by GC. Liquid cultures were centrifuged at 4,000g for 20 min, then the cells were washed twice with distilled water, and dried overnight at 100°C. About 30 mg of dried cell pellet was subjected to methanolysis with benzoic acid as an internal standard in the presence of 15% sulfuric acid. The resulting methyl esters of constituent lactate and 3HB were assayed by GC according to the method of previous report (Braunegg et al., 1978). GC analysis was performed by injecting 1 μL of sample into a Agilent 6890N GC system (Agilent Technologies, Palo Alto, CA) equipped with Agilent 7683 automatic injector, flame ionization detector, and a fused silica capillary column (AT™-Wax, 30 m, ID 0.53 mm, film thickness 1.20 μm, Alltech, Deerfield II). GC oven temperature was initially maintained at 80°C for 5 min and ramped to 230°C at 7.5°C/min. And then it was increased with a gradient 10°C/min until 260°C and held for 5 min. Helium was used as a carrier gas. The injector and detector were maintained at 250 and 300°C, respectively.

To determine the structure and molecular weights, the synthesized polymers were purified from the cells by the solvent extraction method. Cells accumulating polymers

Figure 2. The effects of varying the surplus lactate and acetyl-CoA fluxes on the biosynthesis of PLA polymers. A: Schematic illustration of surplus lactate and acetyl-CoA fluxes for the biosynthesis of LA-containing polymers, and (B) the response of cell growth to the presence of surplus lactate and acetyl-CoA fluxes in ackA, ppc and adhE triple knockout mutant.
were harvested by centrifugation at 2,500g at 4°C, washed twice with distilled water, and lyophilized in a freeze-dryer overnight. Polymer was extracted from the lyophilized cells (ca. 1 g of cells) with 200 volumes of hot chloroform refluxed in a Soxhlet apparatus (Corning, Lowell, MA) for 14 h. Cell debris was removed by filtration (number 2 filter, Whatman, Piscataway, NJ) and solvent was evaporated. The polymer concentrate was then precipitated using 10 volumes of ice-cold methanol. Finally, the isolated polymers were dried overnight at 55°C.

All of the 1D (1H, 13C) and 2D COSY NMR spectra of polymers were recorded on a Bruker AVANCE DMX 600MHz spectrometer (Bruker, Rheinstetten, Germany) using a BBO probe in CDCl3, with tetramethylsilane (TMS) as an internal chemical shift standard at 298K. In 1H NMR experiment, the optimized 90° pulse width was 7.6 µs at the delay time of 5 s. In quantitative 13C NMR experiment, the 30° pulse width was 3.3 µs at the delay time of 2 s for PLA and 10 s for P(3HB-co-LA). The conditions for 2D COSY NMR experiments were as follows: 1.3 s delay time, 128 ms acquisition time, processing size of 2k × 1k for PLA and 1k × 1k for P(3HB-co-LA), number of increments, 256 for PLA and 128 for P(3HB-co-LA), number of scans per increment, 16 for PLA and 8 for P(3HB-co-LA), and sinebell processing function were used (Lim et al., 2008).

Molecular weights of polymers were determined by GPC at 40°C using a Waters Alliance 2695 Separation Module (Waters, Milford, MA) equipped with Waters 2414 RI detector and two PL Gel columns (Mixed C, 5 µm particles, 30 cm, ID 7.5 mm, Polymer Laboratories, Amherst, MA). Chloroform was used as the eluent at a flow rate of 0.8 mL/min. Polystyrene molecular weight standards (weight average molar masses of 3,900,000, 316,500, 52,200, 31,400, 9,860, 3,940, 381 Da, Polymer Standard Service-C, washed 6-19, and Pct540 mutant having V193A mutation, and four silent nucleotide mutations of T78C, T669C, A1125G, T1158C) among various mutants of PhaC1Pc_6-19 and Pct540 cop, which led to the increase of both polymer content and lactate mole fraction in the copolymer among various mutants of Pctcp, were selected as the enzymes to be introduced into E. coli for the production of PLA polymers (accompanying paper Yang et al., 2010). The PldhA::Ptrc mutation (JLX2) and the Δppc mutation (JLX3) showed positive effect on increasing the lactate fraction, while ΔackA mutation (JLX1) resulted in lower lactate fraction compared with the wild-type (Fig. 3A). JLX7 which harbors all three mutations resulted in the highest lactate fraction even though the polymer content was slightly lower than JLX2 (Fig. 3A).

As lactyl-CoA is generated endogenously, PLA homopolymer can be theoretically synthesized in recombinant E. coli from glucose if 3HB is not fed. To examine the effect of 3HB concentration on copolymer biosynthesis, the JLX7 strain expressing PhaC1310Pc_6-19 and Pct540 cop was cultured in a medium containing 20 g/L glucose and varying concentrations of 3HB (0–2 g/L). As the 3HB concentration increased, the lactate fraction decreased (Supplementary Fig. 3A). When no 3HB was fed, PLA homopolymer could be produced with the polymer content of 3 wt% of DCW (Supplementary Fig. 3A).

Metabolic Engineering of E. coli Based on In Silico Genome-Scale Metabolic Flux Analysis

In Silico Gene Knockout Simulation

In order to possibly increase the polymer content, JLX7 was further engineered based on in silico genome-scale gene knockout simulation and flux response analysis. Figure 1 shows the results of the in silico gene knockout simulation with respect to the PLA production rate versus cell growth rate. The in silico PLA production rate of a control strain, JLX7 expressing PhaC1310Pc_6-19 and Pct540 cop, was 0.037 mmol/g DCW/h, which was rather low. The genes to be knocked out to increase the PLA production rate predicted by simulation are shown in Figure 1 (right top panel). Knocking out the adhE gene encoding acetaldehyde/ alcohol dehydrogenase resulted in the greatest increase of the PLA production rate. Knocking out the gdhA and udhA genes encoding glutamate dehydrogenase and pyridine nucleotide transhydrogenase, respectively, also increased the native regulation of the ldhA gene expression (Jiang et al., 2001). Thirdly, the ppc gene encoding phosphoenolpyruvate carboxylase was deleted to make more pyruvate become available for lactate synthesis. To investigate the effects of the above manipulations on P(3HB-co-LA) synthesis in detail, strains with single and all three mutations were constructed (Table I). These strains were transformed with the same plasmid expressing PhaC1310Pc_6-19 (PhaC1Pc_6-19 mutant having E130D, S477F, and Q481K mutations) and Pct540 cop (Pctcp, mutant having V193A mutation, and four silent nucleotide mutations of T78C, T669C, A1125G, T1158C) (Table I). PhaC1310Pc_6-19, which resulted in both relatively high polymer content and mutant among various mutants of PhaC1Pc_6-19 and Pct540 cop, which led to the increase of both polymer content and lactate mole fraction in the copolymer among various mutants of Pctcp, were selected as the enzymes to be introduced into E. coli for the production of PLA polymers (accompanying paper Yang et al., 2010). The PldhA::Ptrc mutation (JLX2) and the Δppc mutation (JLX3) showed positive effect on increasing the lactate fraction, while ΔackA mutation (JLX1) resulted in lower lactate fraction compared with the wild-type (Fig. 3A). JLX7 which harbors all three mutations resulted in the highest lactate fraction even though the polymer content was slightly lower than JLX2 (Fig. 3A).

As lactyl-CoA is generated endogenously, PLA homopolymer can be theoretically synthesized in recombinant E. coli from glucose if 3HB is not fed. To examine the effect of 3HB concentration on copolymer biosynthesis, the JLX7 strain expressing PhaC1310Pc_6-19 and Pct540 cop was cultured in a medium containing 20 g/L glucose and varying concentrations of 3HB (0–2 g/L). As the 3HB concentration increased, the lactate fraction decreased (Supplementary Fig. 3A). When no 3HB was fed, PLA homopolymer could be produced with the polymer content of 3 wt% of DCW (Supplementary Fig. 3A).

Results and Discussion

Production of PLA and P(3HB-co-LA) Having High Lactate Fraction by Metabolic Engineering of E. coli

Recombinant E. coli harboring a new metabolic pathway involving engineered propionate CoA-transferase and PHA synthase allowed production of P(3HB-co-LA) containing 20–49 mol% lactate from glucose and 3HB (accompanying paper Yang et al., 2010). To further increase the lactate fraction in P(3HB-co-LA) and possibly to produce PLA homopolymer, E. coli XL1-Blue was metabolically engineered (Fig. 1). First, the ackA gene encoding acetate kinase was deleted to block a major acetate forming pathway, and consequently to increase the level of acetyl-CoA, a major CoA donor for the generation of (D)-lactyl-CoA and (D)-3HB-CoA. Secondly, to enhance lactate synthesis, the native promoter of the ldhA gene encoding (D)-lactate dehydrogenase was replaced with the trc promoter, relieving the
expressing PhaC1310 of higher than 50 mol% is possible by varying the 3HB concentration in JLX10 of PLA homopolymer and P(3HB-co-LA) copolymers with different lactate fractions. Synthesis of PLA homopolymer and P(3HB-co-LA) copolymers having controlled lactate fraction of higher than 50 mol% is possible by varying the 3HB concentration in JLX10 expressing PhaC1310 and Pct540. Black and gray bars represent the P(3HB-co-LA) content and the lactate fraction in P(3HB-co-LA), respectively. Results are presented as mean ± standard deviation (SD). Error bars indicate SD. The genotypes of the host strains employed are: XB, wild-type XL1-Blue; JLX1, XB ΔackA; JLX2, XB ΔPhac:A::Ptrc; JLX3, XB Δppc ΔadhE; JLX7, XB ΔackA Phac:A::Ptrc Δppc; JLX10, XB ΔackA Phac:A::Ptrc Δppc ΔadhE. A: Production of PLA homopolymer and P(3HB-co-LA) copolymers by metabolically engineered E. coli strains under various conditions. A: Polymer content and the lactate fraction of P(3HB-co-LA) copolymers obtained by employing different engineered E. coli strains expressing PhaC1310 and Pct540. Black and gray bars represent the P(3HB-co-LA) content and the lactate fraction in P(3HB-co-LA), respectively. Results are presented as mean ± standard deviation (SD). Error bars indicate SD. The genotypes of the host strains employed are: XB, wild-type XL1-Blue; JLX1, XB ΔackA; JLX2, XB ΔPhac:A::Ptrc; JLX3, XB Δppc; JLX7, XB ΔackA Phac:A::Ptrc Δppc; JLX10, XB ΔackA Phac:A::Ptrc Δppc ΔadhE. B: Production of PLA homopolymer and P(3HB-co-LA) copolymers with different lactate fractions. Synthesis of PLA homopolymer and P(3HB-co-LA) copolymers having controlled lactate fraction of higher than 50 mol% is possible by varying the 3HB concentration in JLX10 expressing PhaC1310 and Pct540. Black and gray bars represent the polymer content and the lactate fraction in the polymer, respectively. The dashed line represents the M_w of the polymer. Results are presented as mean ± SD. Error bars indicate SD. Two reactions, acetyl-CoA → acetyl-CoA (surplus) and D-lactate → D-lactate (surplus), were added to the genome-scale metabolic model in order to investigate the effects of surplus lactate and acetyl-CoA fluxes on the biosynthesis of PLA polymers in silico (Fig. 2A) (Lee et al., 2005; Varma and Palsson, 1994). This allowed prediction of the surplus lactate and acetyl-CoA fluxes after their use in the formation of biomass. More specifically, the changes in the cell growth rate were examined while the two fluxes were varied in various knockout mutants. The effects of surplus lactate and acetyl-CoA fluxes on cell growth rate were examined, and the results are shown in Figure 2B and Supplementary Figure 2. Flux balance analysis (FBA) (Varma and Palsson, 1994) showed that the cell growth rate decreased as the surplus lactate and acetyl-CoA fluxes for the biosynthesis of PLA polymers increased in the wild-type strain. This implies that excessive use of lactate and acetyl-CoA pools for the biosynthesis of polymers lowers the cell growth rate. When the FBA results were compared among the ackA single knockout strain, adhE single knockout strain, and the ackA and adhE double knockout strain, the synergetic effect of the ackA adhE double knockout on increasing the surplus lactate flux for PLA synthesis was observed with the maximum cell growth rate (Supplementary Fig. 2) (Gupta and Clark, 1989). The ackA, ppc and adhE triple knockout strain allowed the excess lactate pool to be available for the biosynthesis of PLA and lactate-containing polymers without negatively affecting the cell growth rate. This metabolic alteration positively affected synthesis of PLA homopolymer and copolymers containing high lactate fraction (Fig. 2B and Supplementary Fig. 2). Thus, the adhE gene was additionally knocked out in JLX7, and further engineering was performed as described below.

Flux Response Analysis for the Gene Amplification Target

Next, flux response analysis was performed to predict the gene amplification target to increase the PLA production rate. The responses of the PLA production rate and cell growth rate to the varying individual fluxes of the central metabolic reactions were examined. There were four types of responses as shown in Figure 1 (right bottom panel). Those reactions that upon increasing their flux result in increased PLA production flux and cell growth rate are shown in green, while those result in decreased PLA production flux and cell growth rate are shown in blue. Those reactions that upon increasing their flux result in increased PLA
production flux and decreased cell growth rate are shown in red. Those reactions that upon increasing their flux result in decreased PLA production flux and increased cell growth rate are shown in yellow. The reactions belonging to these four types are indicated by the arrows of respective colors in the metabolic network shown in Figure 1 (left panel). It was predicted that the PLA production rate can be increased by amplifying genes in the glycolytic pathway or the acs gene encoding acetyl-CoA synthetase (green and red arrows, respectively, as shown in Figure 1, left panel and right bottom panel). Instead of amplifying multiple enzymes in glycolysis, which can be a burden to the cell, the acs gene was amplified to increase the PLA production rate; again, to avoid the metabolic burden caused by plasmid-based overexpression, the acs promoter in the chromosome was replaced with the strong trc promoter (red arrow in Fig. 1, left panel and right bottom panel). Consequently, JLX10 strain (ΔackA PldhA::Ptrc Δppc ΔadhE Pacs::Ptrc) was constructed. As detailed below, the JLX10 strain allowed the most efficient production of PLA homopolymer and P(3HB-co-LA) copolymers having the lactate fraction of greater than 50 mol%, which agrees well with the in silico simulation results (Figs. 2–4).

Enhanced Production of PLA and P(3HB-co-LA) Having High Lactate Fraction in Finally Engineered E. coli JLX10 Strain

The JLX10 strain expressing PhaC1310pse.19 and Pct540cp was able to synthesize P(3HB-co-55 mol% LA) with the content of 48 wt% in a medium containing 20 g/L glucose and 2 g/L 3HB; both polymer content and lactate fraction were ca. 1.6-fold higher than those obtained with the wild-type strain harboring the same plasmid (Fig. 3A). Also, this engineered strain was able to produce PLA homopolymer to a content of 4 wt%, which is ca. 1.3-fold higher than that obtained with recombinant JLX7 strain (Fig. 3B and Supplementary Fig. 3A). Using JLX10 as a parent strain, polymer content and lactate fraction could be increased up to ca. 3.7-fold (in case of expressing PhaC1310pse.19, Pct540cp and PhaBCA) and 2.6-fold (in case of expressing PhaC1400pse.19, Pct532cp and PhaBCA), compared with those obtained with the wild-type strain expressing the same enzymes, respectively (Fig. 3C). This enhancement demonstrates that JLX10 has a superior metabolic ability to synthesize PLA polymers.

As reported in our previous reports on the production of PHAs (Lee, 1996), the pH-stat fed-batch culture was effective for the production of lactate-containing copolymers for characterizing their material properties (see the accompanying paper; Yang et al., 2010). Even though the detailed production studies were not carried out in this work, it is believed that the lactate-containing polymers can be produced to the similar levels to PHAs, for example, greater than 100 g/L (Lee, 1996). However, further studies are needed before high cell density cultivation of JLX strains can be carried out. The metabolic pathways need to be further engineered so that succinate feeding can be avoided. Also, the promoter system used in this study requires IPTG induction, which can be changed to more industrially preferred system such as constitutive one.

Characterization of PLA and P(3HB-co-LA) Synthesized in Recombinant E. coli

PLA and P(3HB-co-LA) polymers were accumulated as distinct inclusion granules in engineered E. coli cells...
(Fig. 5A), which were similar to P(3HB) granules (Lee et al., 1994). The composition and the monomer sequence distribution of P(3HB-co-LA) were investigated by 1D ($^1$H and $^{13}$C) NMR and 2D ($^1$H-$^1$H) COSY NMR spectroscopy, representing that P(3HB-co-LA) synthesized in this study has the 3HB and lactate unit in the (D) configuration with a random sequence (accompanying paper Yang et al., 2010). PLA homopolymer produced by fermentation showed the same structure as the chemically synthesized PLA (Fig. 5B). In the 600 MHz $^1$H NMR spectrum of PLA, the oxymethylene proton (–OCH–) and methyl protons (–CH$_3$) of PLA are assigned at 5.18 and 1.59 ppm, respectively. The $^1$H–$^1$H COSY spectrum highlights the intra-three bond coupling of protons, and thereby represents the configurational structure of PLA. The coupling between the oxymethylene proton (–OCH–) and...
the methylene protons (–CH₂) next to the oxymethylene proton can be seen as a cross peak at 5.18 ppm/1.59 ppm. In the 150 MHz ¹³C NMR spectrum of PLA, the carbonyl carbon (−C=O−), oxymethylene carbon (−OCH−), and methyl carbon (−CH₃) of PLA are assigned at 170, 69.4, and 16.9 ppm, respectively.

The molecular weight of P(3HB-co-LA) was inversely proportional to the mole fraction of lactate monomer (Fig. 3B and C). Furthermore, PLA production had rather low molecular weight (Fig. 3B). This suggests that (D)-lactyl-CoA is still an unfavorable substrate even for the engineered PHA synthases for the prolonged elongation to reach a high Mn. This phenomenon was also observed in the previous study involved in microbial polythioesters; the total polymer yield and molecular weight of poly(3-hydroxybutyrate-co-3-mercaptopropionate), P(3HB-co-3MP), decreased simultaneously according to higher MP fraction of the polymer, indicating that 3HB-CoA is the preferred substrate of the PHA synthase used (Lütke-Eversloh et al., 2002; Lütke-Eversloh and Steinbüchel, 2003). The molecular weight of the polymers is influenced by several factors, such as the type of PHA synthase, the expression level of active PHA synthase, the concentration of substrate for PHA synthase, the presence and activities of PHA depolymerase, esterase, and/or lipase, chain transfer reaction during polymerization, and frequent re-starting of the polymerization (Jurasek and Marchessault, 2004; Rehm, 2003). Further studies need to be performed to understand the detailed enzyme characteristics of evolved PHA synthases in using (D)-lactyl-CoA as a monomer substrate.

Conclusions

In this article, we reported the development of E. coli strains that are capable of efficiently producing unnatural polymers, PLA homopolymer and its copolymers containing various lactate fractions, by one-step fermentation process. The metabolic pathways in E. coli were rationally engineered based on the in silico genome-scale simulation so that more precursors become available for the polymer synthesis. This strategy is not restricted to the production of P(3HB-co-LA) as 3HB monomer can be replaced with various hydroxyalkanoates (Lee, 1996). The mole fraction of each monomer in the copolymers can be modulated through further fine-tuned metabolic engineering as well as cultivation strategies (Hazer and Steinbüchel, 2007; Park et al., 2008a). The combined approaches of systems-level metabolic engineering and enzyme engineering described here should be useful for the improved production of lactate-based polyesters by direct microbial fermentation of renewable resources, and for the development of new strategies for the production of other unnatural polymers.

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References


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