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Engineered *Corynebacterium glutamicum* as an endotoxin-free platform strain for lactate-based polyester production

Yuyang Song · Ken'ichiro Matsumoto · Miwa Yamada · Aoi Gohda · Christopher J. Brigham · Anthony J. Sinskey · Seiichi Taguchi

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Abstract The first biosynthetic system for lactate (LA)-based polyesters was previously created in recombinant *Escherichia coli* (Taguchi et al. 2008). Here, we have begun efforts to upgrade the prototype polymer production system to a practical stage by using metabolically engineered Gram-positive bacterium *Corynebacterium glutamicum* as an endotoxin-free platform. We designed metabolic pathways in *C. glutamicum* to generate monomer substrates, lactyl-CoA (LA-CoA), and 3-hydroxybutyryl-CoA (3HB-CoA), for the copolymerization catalyzed by the LA-polymerizing enzyme (LPE). LA-CoA was synthesized by D-lactate dehydrogenase and propionyl-CoA transferase, while 3HB-CoA was supplied by β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB). The functional expression of these enzymes led to a production of P(LA-*co*-3HB) with high LA fractions (96.8 mol%). The omission of PhaA and PhaB from this pathway led to a further increase in LA fraction up to 99.3 mol%. The newly engineered *C. glutamicum* potentially serves as a food-grade and biomedically applicable platform for the production of poly(lactic acid)-like polyester.

Keywords Polylactide · Biobased plastic · PHA synthase · Polyhydroxyalkanoate · Polyhydroxybutyrate

Y. Song · K. Matsumoto · M. Yamada · A. Gohda · S. Taguchi (✉)
Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan
e-mail: staguchi@eng.hokudai.ac.jp

M. Yamada
Department of Biological Chemistry and Food Science, Iwate University, Ueda-3, Morioka 020-8550, Japan

C. J. Brigham · A. J. Sinskey
Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Introduction

The severe problem of the dwindling petroleum resources and an increasing emission of carbon dioxide have increased demand for the development of bio-based plastics as a means of reducing the environmental impact of petroleum-derived polymers. Poly(lactic acid) is a representative bio-based plastic that is used in packaging, stationery, containers, etc. (Madhavan Nampoothiri et al. 2010). In addition, the utilization of the polyester has been expanded to the medical field for drug delivery, resorbable sutures, and as material for medical implants and other related applications (Auras et al. 2004). PLA is chemically synthesized by heavy metal-catalyzed ring-opening polymerization of lactide, which in turn is derived from fermentative lactate (LA) (Auras et al. 2004). However, the chemo-process often leaves harmful chemical residues that are a cause of health and safety concerns. The switch from the multistep chemo-bio process to a complete bio-process for LA-based polyester production is thus preferable to overcome this problem.

Recently, a whole-cell biosynthesis system for LA-based polyester production without heavy metal catalyst has been constructed using engineered *Escherichia coli* (Matsumoto and Taguchi 2009; Taguchi et al. 2008). The discovery of LA-polymerizing enzyme (LPE) (Taguchi et al. 2008), which was an engineered polyhydroxyalkanoate (PHA)

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synthase (Taguchi and Doi 2004), was a key to develop the first microbial system. To date, the *E. coli* platform has been used to produce various LA-based polymers incorporating 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) (Shozui et al. 2009; Yamada et al. 2009). Most recently, we have successfully incorporated new 2-hydroxy acids such as 2-hydroxybutyrate and glycolate using LPE (Han et al. 2011; Matsumoto et al. 2011). However, Gram-negative bacteria, such as *E. coli*, are known to produce potentially harmful substances, (i.e., endotoxin and lipopolysaccharide) (Furrer et al. 2007; Lee et al. 1999; Valappil et al. 2007). In considering practical applications of LA-based polyester, especially for food-grade and biomedical demands, the use of endotoxin-free Gram-positive bacteria is preferable.

Corynebacterium glutamicum is an aerobic, Gram-positive, non-sporulating, bacterium with GRAS status that has been extensively employed for the industrial production of several food-grade amino acids, feed, and pharmaceutical products for several decades based on classical metabolic engineering (Leuchtenberger et al. 2005). In addition, *C. glutamicum* has extensive ability in assimilating crude sugar, for example the agricultural by-product, molasses (Schneider et al. 2010; Wittmann et al. 2005). These advantages make *C. glutamicum* an attractive candidate as a host for biopolymer production.

Therefore, the aim of this study is to construct an endotoxin-free production system for LA-based polyester using *C. glutamicum*. We previously reported productions of P(3HB) and P(3HB-*co*-3HV) in engineered *C. glutamicum* harboring PHA biosynthetic genes, indicating the capability of polyester synthesis of this organism (Jo et al. 2009; Jo et al. 2006; Jo et al. 2007; Matsumoto et al. 2010). In this study, we designed a new metabolic pathway in this organism for the production of LA-based polyester, P(LA-*co*-3HB). The copolymer is synthesized from glucose as a sole carbon source through successive enzymatic reactions including (1) generation of D-LA-CoA by D-lactate dehydrogenase (D-LDH) and propionyl-CoA transferase (PCT), (2) 3HB-CoA generation catalyzed by β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), and (3) copolymerization of LA-CoA and 3HB-CoA catalyzed by LPE (Fig. 1). The 3HB-CoA pathway was shown to be essential to the LPE-catalyzed synthesis of LA-based polyester in *E. coli* system, presumably because 3HB units act as a primer to activate LPE (Taguchi et al. 2008). For the synthesis of P(LA-*co*-3HB), these three steps were needed to be functional. Thus, we confirmed the expression of each enzyme involved in the pathway in *C. glutamicum* and then combined the pathways to synthesize P(LA-*co*-3HB). This is the first report of the production of LA-based polyesters in Gram-positive bacteria.

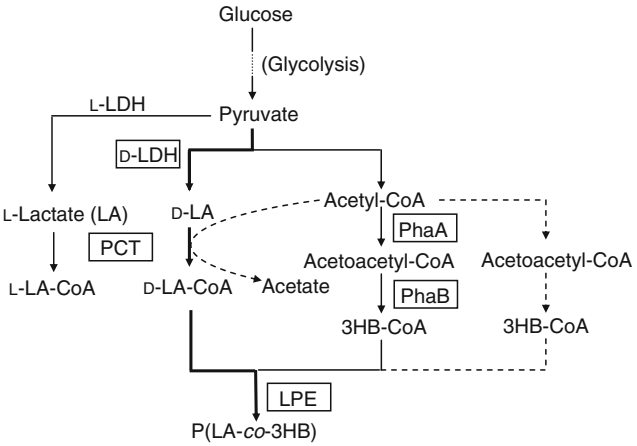


Fig. 1 Metabolic pathways relevant to production of P(lactate-*co*-3-hydroxybutyrate) in engineered *Corynebacterium glutamicum*. *L-LDH* L-lactate dehydrogenase, *D-LDH* D-lactate dehydrogenase, *PCT* propionyl-CoA transferase, *PhaA* β-ketothiolase, *PhaB* acetoacetyl-CoA reductase, *LPE* lactate-polymerizing enzyme. *Boxed* enzymes indicate exogenous enzymes. *Thick lines* indicate reinforced pathways. *Dashed lines* indicate proposed pathways; acetyl-CoA could act as a CoA donor for CoA transferring reaction using PCT enzyme, and a small amount of 3HB-CoA was supplied by intrinsic, but uncharacterized, pathway

Materials and methods

Strain, culture conditions, and LA analysis in the medium

C. glutamicum ATCC13803, used as the host strain for P(LA-*co*-3HB) production throughout this study, was transformed by electroporation as described previously (Liebl et al. 1989). For polymer production, the engineered strains were grown in 100 ml nutrient-rich CM2G medium (Kikuchi et al. 2003) at 30°C for 24 h with reciprocal shaking at 130 strokes/min. Cells were harvested, washed with distilled water, transferred into 100 ml minimal MMTG medium containing 6% glucose (Kikuchi et al. 2003) and 0.45 mg/l of biotin, and further cultivated for 72 h at 30°C. *C. glutamicum* does not produce glutamate under the presence of high concentration of biotin (Shiio et al. 1962). When needed, kanamycin (25 μg/ml) and/or chloramphenicol (5 μg/ml) was added to the medium. After the cultivation, cells were lyophilized for polymer extraction. The concentration of LA in the MMTG medium after 72 h cultivation was determined using a D-/L-lactic acid assay kit (R-Biopharm, Roche, Germany).

Plasmid constructions

The kanamycin-resistant shuttle vector for *C. glutamicum* pPSPTG1 (Kikuchi et al. 2003) was digested with *Xba*I and self-ligated after T4 DNA polymerase blunting to eliminate *Xba*I site. The resulting plasmid was digested with *BstE*II and *Cpo*I, and ligated with a synthetic *BstE*II-*Xho*I-GC-

*Xba*I-*Cpo*I linker (Table 1) to yield a new vector, pPS. Then, a 3.9-kb *Xba*I/*Bam*HI fragment from the pGEM*C1* (STQK)*AB* plasmid (Takase et al. 2003), containing the S325T/Q481K mutated PHA synthase gene from *Pseudomonas* sp. 61-3 [PhaC1$_{Ps}$(ST/QK)], also termed LPE (Takase et al. 2003), and *phaA* and *phaB* genes from *Ralstonia eutropha* (Peoples and Sinskey 1989) was inserted into *Xba*I/*Bam*HI sites of pPS to yield pPS*C1* (STQK)*AB* (Fig. 2). Similarly, a 1.7-kb *Pst*I/*Xba*I fragment of pGEM*C1*(STQK)*AB* containing *phaC1(ST/QK)* gene was inserted into *Bam*HI/*Xba*I site of pPS after T4 DNA polymerase blunting to yield pPS*C1*(STQK).

The chloramphenicol-resistant shuttle vector pVC7 (Kikuchi et al. 2003) was digested with *Hin*dIII and *Eco*RI, and ligated with a synthetic *Hin*dIII-*Kpn*I-*Bam*HI-*Sac*I-*Bgl*II-*Pst*I-*Eco*RI linker (Table 1) to yield the new plasmid pVC7-L. The P_{csp} promoter, which is constitutively expressed in *C. glutamicum*, was amplified from pPSPTG1 using primers P_{csp} F and P_{csp} R (Table 1). The *Kpn*I/*Bam*HI digested PCR product was inserted into *Kpn*I/*Bam*HI sites of pVC7-L to yield pVC7-LP. D-lactate dehydrogenase (D-LDH) gene (*ldhA*) from *E. coli* was amplified using the primer pair, Ecoli ldhA F and Ecoli ldhA R (Table 1). The *Bam*HI/*Sac*I digested PCR product was inserted into *Bam*HI/*Sac*I sites of pVC7-LP to construct pVC7*ldhA*. Propionyl-CoA transferase (PCT) gene (Elsden et al. 1956) was amplified from *Megasphaera elsdenii* genomic DNA using a pair of primers, PCT F and PCT R (Table 1). The *Bgl*II/*Pst*I digested PCR product was inserted into *Bgl*II/*Pst*I sites of pVC7*ldhA* to yield pVC7*ldhApct*.

Immunoblot analysis

C. glutamicum transformants were cultivated at 30°C for 20 h in CM2G medium. Cells were harvested by centrifugation and re-suspended in 25 mM Tris–HCl buffer (pH 7.5). The soluble fraction of cell lysate was prepared by sonication and centrifugation (12,000×*g*, 4°C, 10 min).

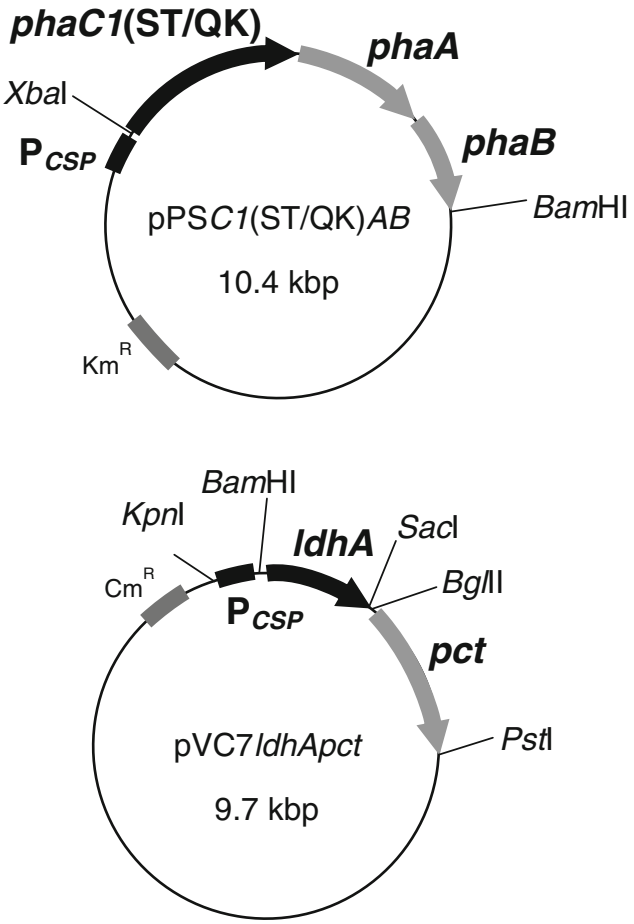


Fig. 2 Map of the plasmids used in this study. P_{csp} denotes the promoter region. The *pct* gene encodes propionyl-CoA transferase from *Megasphaera elsdenii*. The *ldhA* gene encodes D-lactate dehydrogenase from *Escherichia coli*. The *phaC1*(STQK) gene encodes the Ser325Thr/Gln481Lys mutant of PHA synthase from *Pseudomonas* sp. 61-3 (lactate-polymerizing enzyme, LPE). The *phaA* and *phaB* genes encode β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB) derived from *R. eutropha*, respectively

Table 1 Oligonucleotide sequences

Oligonucleotide linkers and primers	Sequences
*BstE*II-*Xho*I-GC-*Xba*I-*Cpo*I linker	5′-GTCACCTCGAGCGTCTAGACG-3′
	5′-GACCGTCTAGACGCTCGAG-3′
*Hin*dIII-*Kpn*I-*Bam*HI-*Sac*I-*Bgl*II-*Pst*I-*Eco*RI linker	5′-AGCTTGGGGTACCCGGGATCCATGAGCTCGAAGATCTAACTGCAGAAG-3′
	5′-AATTCTTCTGCAGTTAGATCTTCGAGCTCATGGATCCCGGGTACCCCA-3′
P_{csp} F	5′-CTCGGTACCCAAATTCCTGTGA-3′
P_{csp} R	5′-ATGGATCCCTCCTTGAATAGGTATCGAAAGAC-3′
Ecoli ldhA F	5′-GGATCCGCCACCATGAAACTCGCCGTTTATAG-3′
Ecoli ldhA R	5′-GAGCTCAAGATTAAACCAGTTCGTTCG-3′
PCT F	5′-AGATCTAGGAGGTAAACAATGAGAAAAGTAGAAATCA-3′
PCT R	5′-GAGCTCTGCAGGTTATTTTTTCAGTC-3′

Immunoblotting was performed using antisera to PCT and PhaC1, and Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate (BIO-RAD) as previously described (Jo et al. 2007). Rabbit antiserum to PCT was developed using purified PCT expressed in *E. coli*. The N-terminal His-tag fusion of *pct* gene was constructed using pQE31 (Qiagen), and PCT was purified using affinity chromatography with Ni^{+} NTA resin using a standard protocol.

Polymer extraction from *C. glutamicum* cells

The lyophilized cells (~1 g) were washed with 10 ml methanol three times at room temperature prior to the polymer extraction. Polymer was extracted by incubation with 10 ml chloroform at 60°C for 48 h. Cell debris was removed by passing through a PTFE filter. A tenfold volume of hexane was then added in order to precipitate the polymer. The polymer was dried in vacuo at room temperature to determine cellular polymer content based on cell dry weight and the weights of polymer. The extracted polymer was used for further analyses.

Analysis of LA-based polyesters

The monomer composition of the polymers was determined by gas chromatography/mass spectroscopy (GC/MS) as described previously (Arai et al. 2002). The molecular weights of the polymers were determined by gel permeation chromatography (GPC) (Shimadzu, Japan) using a TSKgel Super HZM-H (Tosoh, Japan) with polystyrene standards (Waters, USA) for calibration as described (Taguchi et al. 2008). The ^{1}H and ^{13}C NMR spectra of the polymers dissolved in $CDCl_3$ were obtained using a Bruker MSL400 spectrometer (400 MHz for ^{1}H NMR) using tetramethylsilane as an internal reference.

Results

Overproduction of D-lactic acid in *C. glutamicum* by introduction of a heterologous D-lactate dehydrogenase

A key factor in the construction of a metabolic pathway to produce LA-based polyesters in *C. glutamicum* was the stereochemistry of LA, because it has been demonstrated that LPE has strict stereospecificity toward D-LA-CoA (Taguchi et al. 2008; Tajima et al. 2009; Yamada et al. 2009). However, the *C. glutamicum* strain used here is known to produce mainly L-LA, as reported in several previous studies (Inui et al. 2004; Okino et al. 2008; Toyoda et al. 2009), whereas *E. coli* can produce D-LA (Bunch et al. 1997; Chang et al. 1999). Therefore, *C. glutamicum* needed to be remodeled as a D-LA overproducer by introduction of D-LDH. In order to confirm the activity of the heterologously expressed D-LDH, the engineered *C. glutamicum* harboring pVC7*ldhA* was cultured, and D- and L-LA concentrations in the culture medium were measured (Table 2). In the parent strain, L-LA concentration was determined to be sevenfold higher than that of D-LA, which was consistent with the previous reports mentioned above. In contrast, the engineered strain exhibited an enhancement of D-LA production that was 14-fold higher than L-LA, indicating the functional expression of D-LDH in *C. glutamicum*. In this engineered strain, the production of L-LA was decreased by the expression of D-LDH, probably due to the consumption of pyruvate by D-LDH competing with that of the intrinsic L-LDH. This result demonstrated that the engineered strain of *C. glutamicum* was suitable for production of LA-based polyesters.

Expression of PCT gene in *C. glutamicum*

The next essential step toward synthesis of LA-based polyesters was activation of D-LA to produce D-LA-CoA. To achieve this, pVC7*ldhApct* bearing propionyl-CoA transferase (PCT) gene from *M. elsdenii,* as well as the *ldhA* gene, was introduced into *C. glutamicum*. The expression of the *pct* gene in *C. glutamicum* was confirmed by immunoblot analysis. As shown in Fig. 3, the translated product for the *pct* gene was detected with the same molecular mass compared with PCT expressed in *E. coli*, but was absent in the wild-type strain. This result suggested that the *pct* gene was successfully expressed in recombinant *C. glutamicum*. Together with functional expression of D-LDH, LA-CoA supplying pathway should be constructed in the strain.

Functional expression of LPE

The functions of the enzymes of the 3HB-CoA supplying pathway and LPE were investigated based on P(3HB) production in *C. glutamicum* harboring pPS*C1*(STQK)*AB*. The engineered strain accumulated 1.0 wt.% of P(3HB), suggesting the functional expressions of the three enzymes, PhaA, PhaB, and LPE. However, the intracellular polymer content was lower than the previous result of engineered *C.*

Table 2 Concentration of lactate (LA) isomers in culture supernatants

Strain	D-LA (mg/l)	L-LA (mg/l)
Wild-type	3.0±0.2	22±1
Engineered strain[a]	209±2	14±2

[a] Cells harboring pVC7*ldhA* were grown on MMTG containing 6% glucose for 72 h at 30°C. Data is average of three independent trials

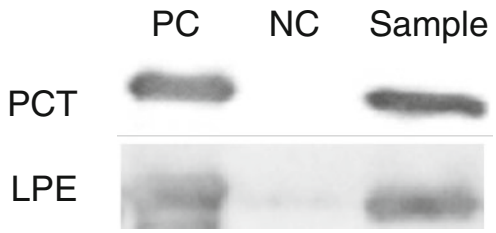


Fig. 3 Immunoblot analysis of PCT and LPE expressed in *C. glutamicum*. *PC* (positive controls), recombinant *E. coli* expressing PCT and LPE [PhaC1(ST/QK)], respectively; *NC* (negative control) wild-type *C. glutamicum*; *Sample C. glutamicum* expressing PCT and LPE, respectively

glutamicum harboring the *phaC*, *phaA*, and *phaB* genes from *R. eutropha* (22.5 wt.%) (Jo et al. 2006). Thus, the expression of LPE was further analyzed by immunoblotting. The translated product of the LPE [*phaC1(STOK)*] gene in *C. glutamicum* was observed as a clear band having the same size of LPE expressed in *E. coli* (Fig. 3). Thus, the low P(3HB) content in the engineered strain might be partly due to the relatively low activity of LPE toward 3HB-CoA compared to PhaC from *R. eutropha* (Matsumoto et al. 2005). Regardless from these results, LPE, PhaA, and PhaB were shown to be functionally expressed in *C. glutamicum*.

Construction of metabolic pathway for production of P(LA-*co*-3HB) in *C. glutamicum*

With the expression of the five genes relevant to the biosynthesis of P(LA-*co*-3HB) confirmed, the plasmids pVC7*ldhApct* (for LA-CoA supply) and pPS*C1*(STQK)*AB* (for 3HB-CoA supply and polymerization) were co-introduced into *C. glutamicum* for construction of the metabolic pathway illustrated in Fig. 1. The engineered strain harboring the dual plasmids produced 2.4 wt.% polymer. GC/MS analysis of the extracted polymer revealed that the polymer was P(LA-*co*-3HB) containing a surprisingly high LA fraction (96.8 mol%) (Table 3). The weight-average molecular weight of the polymer was 7,400, indicating that LA units were incorporated into the polymer chain.

The result of the incorporation of small quantities of 3HB units into the polymer prompted us to evaluate the essentiality of the 3HB-supplying pathway. To examine this, we introduced plasmids pVC7*ldhApct* and pPS*C1* (STQK) encoding D-LDH, PCT, and LPE into *C. glutamicum*. The *phaAB* genes were omitted in this experiment. The new engineered strain accumulated P(LA-*co*-3HB) with even higher LA fraction (99.3 mol%). Thus, the introduction of *phaAB* genes was shown to be not essential to the production of P(LA-*co*-3HB). However, it should be noted that a small amount of 3HB units were incorporated into the polymer without the introduction of *phaAB* genes. Thus, this result cannot be a counterexample excluding the essentiality of 3HB-CoA to biosynthesis of P(LA-*co*-3HB). The polymer content (1.4 wt.%) and its molecular weight (5,700) were decreased compared to those produced by the strain expressing the five relevant LA-copolymer producing genes, including *phaAB*. These inverse relationships between LA fraction and polymer content or molecular weight were consistent with the trends observed in the previously examined *E. coli* system (Yamada et al. 2011).

NMR analysis of LA-based polyesters produced in *C. glutamicum*

In order to analyze polymer structure, the samples were subjected to NMR analyses. The ^{13}C NMR of the copolymer with 96.8 mol% LA exhibited strong resonance of LA units (δ 16.6, 69.0, and 169.6) as well as slight signal for the 3HB units (δ 19.7, 40.1, and 67.6) (Fig. 4a). The carbonyl carbon of LA was observed as a single peak (δ 169.6), indicating that the polymer was enantiopure. Based on the previous reports that LPE had strict stereospecificity toward D-LA-CoA as demonstrated by in vivo (Yamada et al. 2009) and in vitro (Tajima et al. 2009)

Table 3 Production of polyesters in engineered *C. glutamicum* strains

Relevant genes	Cell dry weight (g/l)	Polymer content[a] (wt%)	Monomer composition (mol%)[b]		Yield (mg/l)		Molecular weight [c]		
			LA	3HB	LA	3HB	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n
phaC1(STQK), phaAB	7.0±1.0	1.0±0.8	0	100	0	74±58	8.4	15.1	1.8
phaC1(STQK), phaAB, *ldhA*, *pct*	12.7±1.6	2.4±0.5	96.8±0.7	3.2±0.7	295±78	12±4	5.2	7.4	1.4
phaC1(STQK), *ldhA*, *pct*	13.6±1.7	1.4±0.1	99.3±0.7	0.7±0.7	182±16	1.5±1.3	4.3	5.7	1.3

[a] Cells were grown on MMTG medium containing 6% glucose for 72 h at 30°C. Polymer content was determined based on cell dry weight and weight of extracted polymer

[b] Monomer composition was determined by GC/MS. *LA* lactate, *3HB* 3-hydroxybutyrate

[c] M_n number-average molecular weight, M_w weight-average molecular weight, M_w/M_n polydispersity index. Data is average of three independent trials

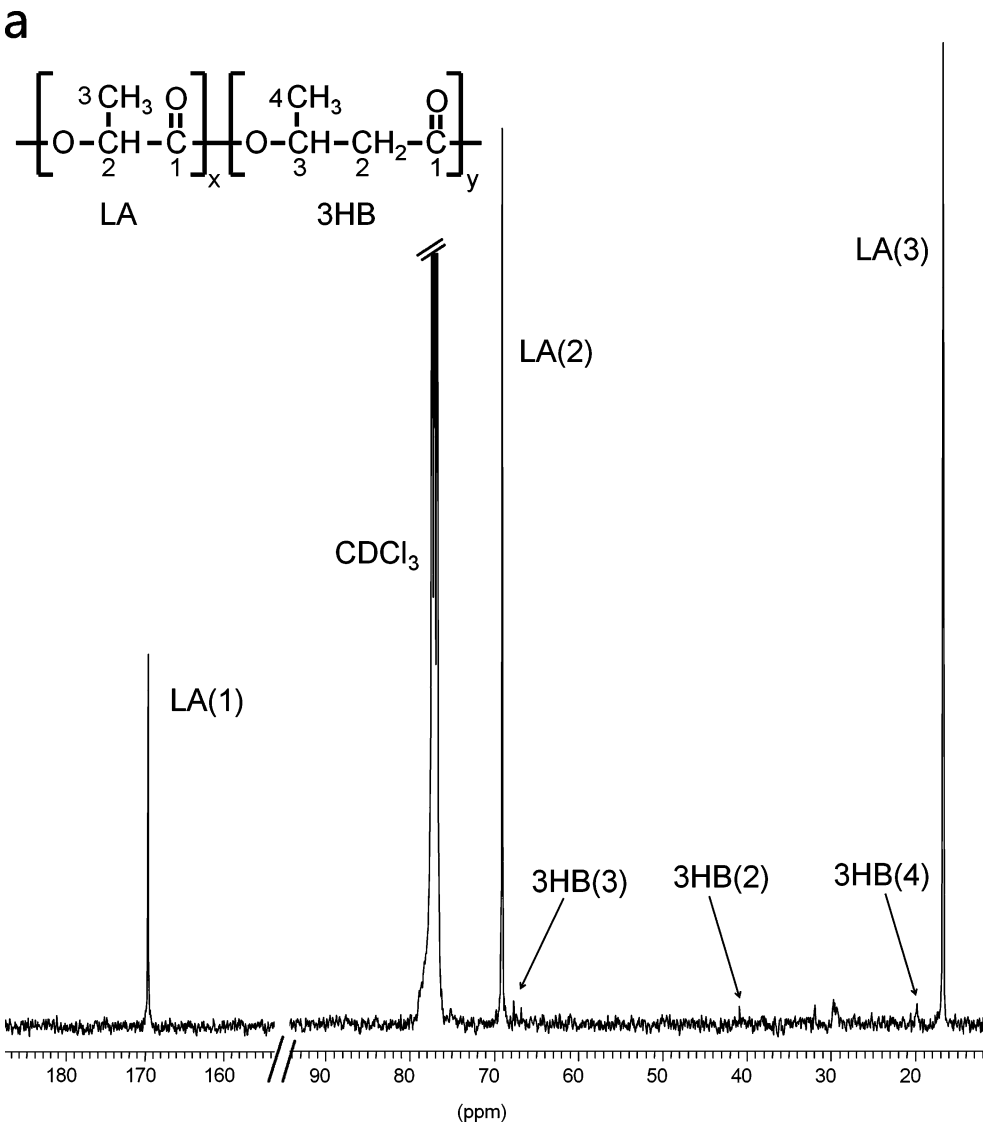


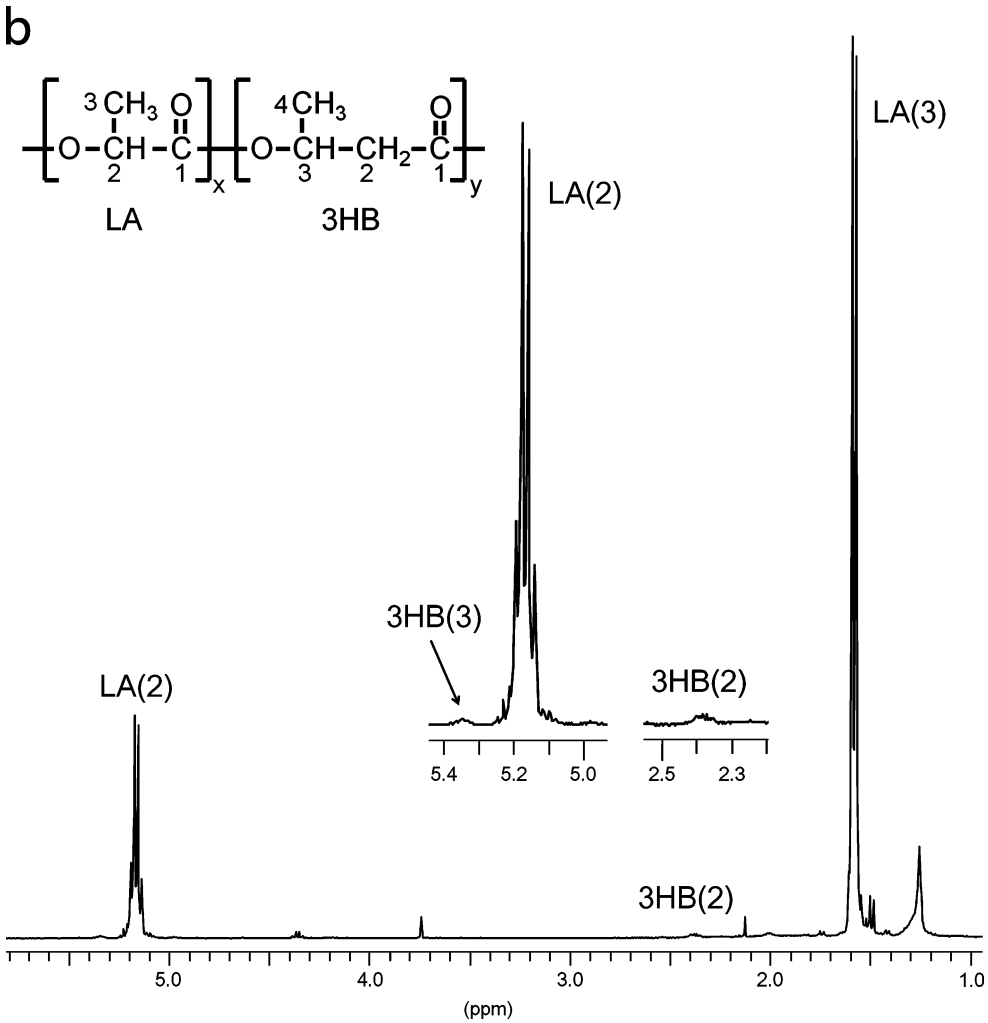
Fig. 4 Analyses of a lactate-based copolymer produced in *C. glutamicum*. **a** ^{13}C NMR spectrum, **b** ^{1}H NMR spectrum. *LA* lactate, *3HB* 3-hydroxybutyrate

experiments, the obtained polymer should be composed of D-LA. The signal of carbonyl carbon of 3HB (supposed to be δ 169.1) was not detected, probably because the peak was overlapped with the neighboring peak of LA carbonyl carbon. In addition, the ^{1}H NMR spectrum of the polymer (Fig. 4b) showed strong resonances, which were identical to those of chemically synthesized PLA and weak resonances of 3HB units. The signal of methyl proton of 3HB unit (4) was not clear because of impurity, presumably lipid or fatty acids. The 3HB fraction was determined to be 3.2 mol%, which was consistent with GC/MS result (Table 3). Previous ^{1}H NMR analyses of P(LA-*co*-3HB)s demonstrated that the resonance of methine proton of LA units exhibited high-field shift (δ 5.0–5.2) compared to that of PLA that was due to the copolymerization of the LA units and 3HB units (Taguchi et al. 2008; Yamada et al. 2009). The shifted signal of LA units was not detected in the copolymer with 96.8 mol% LA probably because of a very small 3HB fraction. NMR analyses of P(99.3 mol% LA-*co*-3HB) exhibited similar spectra while peaks for 3HB units were beyond the detectable level (data not shown). These results supported that the engineered *C. glutamicum* produced PLA-like polyesters.

Discussion

P(LA-*co*-3HB)s produced in *C. glutamicum* exhibited extremely high LA fractions, approaching 100 mol%. This result had a striking contrast to the results obtained by the *E. coli* system using the same set of genes and same carbon source, in which 47 mol% was the maximum LA fraction (Shozui et al. 2009; Yamada et al. 2009; Yamada et al. 2010). The high LA fraction could be partly due to the weak 3HB monomer supply in *C. glutamicum*, which was suggested by the low P(3HB) content (1.0 wt.%, Table 3). It

Fig. 4 (continued)



is worth noting that LPE accumulated 40 wt.% P(3HB) in *E. coli* (Takase et al. 2003), suggesting that the flux toward 3HB-CoA supplying pathway in *C. glutamicum* was relatively low compared to that in *E. coli*. Furthermore, under copolymer-producing conditions, the flux toward 3HB monomer was further decreased by overexpression of D-LDH (Table 3), probably because of D-LDH out-competing pyruvate dehydrogenase for consumption of pyruvate. In addition, acetyl-CoA is presumably used by PCT for CoA transferring reaction that led to a reduction in 3HB pathway (Fig. 1). These factors could potentially further increase LA fraction in the copolymers by decreasing the 3HB-CoA precursor pool. In terms of polymer properties, it would be of interest to compare LA-based polyesters with the high LA fractions, ranging from 70 to nearly 100 mol%, with PLA homopolymer. Polymer with LA fractions of close to 100% has been hardly prepared in *E. coli* system.

In the engineered strain, LA fraction in the copolymer should be determined by two major factors; monomer fluxes and activity of LPE. Since the first P(LA-*co*-3HB) with 6 mol% LA was synthesized (Taguchi et al. 2008), LA-reinforcing approaches using anaerobic culture conditions and an LA-overproducing mutant produced a variety of higher LA fractions, up to 47 mol% (Yamada et al. 2009). As a result, the D-LA concentration in the supernatant of *E. coli* culture medium (up to 5.7 g/L) was much higher than the amount of LA units in the copolymer, indicating that sufficient amount of LA was produced in the engineered *E. coli*. In order to further increase the LA fraction in the copolymer, evolution of LPE toward enhanced LA-polymerizing activity and/or reduction in 3HB monomer flux is necessary. In fact, the engineering of LPE to increase its activity that led to an increase in LA fraction up to 62 mol% (Yamada et al. 2010). Here, we demonstrated that the latter approach achieved the synthesis of copolymer with very high LA fractions. This result suggested that regulation of 3HB monomer flux could also be effective to cover a wide range (from 6 mol% to nearly 100 mol% of LA fraction) of LA fractions in a produced copolymer. The methodology would also be useful for production of LA-based polyesters, with tailor-made

monomer composition, conducted using various microbial platforms.

The engineered *C. glutamicum* harboring D-LDH, PCT, and LPE produced copolymer contained small amount of 3HB units, indicative of an intrinsic 3HB-CoA supply in *C. glutamicum*. This result is in good accordance with the fact that 3HB-CoA should serve as a priming unit for incorporation of LA unit in the initial step of polymerization (Taguchi et al. 2008 and Tajima et al. 2009). A potential 3HB-CoA supplying pathway was proposed in the Biocyc databases (Caspi et al. 2010), which might be catalyzed by homologous enzymes to PhaA and PhaB (YP_226966.1 and YP_226913.1). Further analysis will be necessary to clarify the roles of these genes in LA-based polyester biosynthesis.

At present, overcoming the low intracellular content of LA-based polyesters is a promising challenge toward practical polymer production using the engineered *C. glutamicum*. It should be noted that the *C. glutamicum* system achieved sixfold higher polymer content (2.4 wt.%) compared to *E. coli* system that accumulated only 0.4 wt.% LA-based polyesters containing 96 mol% LA (Shozui et al. 2011), indicative of superior capacity of synthesizing LA-based polyesters of this organism. To further increase the polymer content, the activity of LPE should be an effective factor, because there is an inverse relationship between cellular content of LA-based polyesters and their LA fraction as observed in *E. coli* experiments. We have reported that additional beneficial mutations in LPE enhanced its LA-incorporating ability and increased cellular content of LA-based polyesters (Yamada et al. 2010). A combination of this strategy with pathway engineering for monomer supply would improve the productivity of the LA-based polyesters in *C. glutamicum*. For example, the LA synthesis in *C. glutamicum* will be increased by cultivating the engineered strain under anaerobic conditions. We can enhance the amount of LA produced by eliminating or decreasing expression of cellular pathways that compete with LA-based polyester production, such as succinate or pyruvate production (Okino et al. 2005; Okino et al. 2008). Furthermore, the use of tunable gene expression systems in *C. glutamicum* (Plassmeier et al. 2011) could help increase the production of D-LA-CoA and 3HB-CoA precursors, under inducing conditions.

In this study, we succeeded in establishing an engineered *C. glutamicum* that can produce LA-based polyesters. This new endotoxin-free platform should be suitable for wider range of applications, especially food and medical related uses. The P(LA-*co*-3HB)-producing pathway should be applicable to other sugars that give pyruvate as a metabolized product. Thus, this process could be more cost effective by using low grade, low cost sugars, such as molasses, as a carbon source.

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