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

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

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3 **1 Abstract**
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6 2 The first biosynthetic system for lactate (LA)-based polyesters was previously created
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9 3 in recombinant *Escherichia coli*. Here, we have begun efforts to upgrade the prototype
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12 4 polymer production system to a practical stage by using metabolically engineered
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15 5 Gram-positive bacterium *Corynebacterium glutamicum* as an endotoxin-free platform.
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18 6 We designed metabolic pathways in *C. glutamicum* to generate monomer substrates,
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22 7 lactyl-CoA (LA-CoA) and 3-hydroxybutyryl-CoA (3HB-CoA), for the
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25 8 copolymerization catalyzed by the LA-polymerizing enzyme (LPE). LA-CoA was
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28 9 synthesized by D-lactate dehydrogenase and propionyl-CoA transferase, while 3HB-
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32 10 CoA was supplied by β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA
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35 11 reductase (PhaB). The functional expression of these enzymes led to a production of
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38 12 P(LA-co-3HB) with high LA fractions (96.8 mol%). The omission of PhaA and PhaB
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41 13 from this pathway led to a further increase in LA fraction up to 99.3 mol%. The newly
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44 14 engineered *C. glutamicum* potentially serves as a food-grade and biomedically
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47 15 applicable platform for the production of poly(lactic acid)-like polyester.
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54 17 **Key words:** polylactide, biobased plastic, PHA synthase, polyhydroxyalkanoate,
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57 18 polyhydroxybutyrate
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3 **1 Introduction**
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9 3 The severe problem of the dwindling petroleum resources and an increasing emission of
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11 4 carbon dioxide have increased demand for the development of bio-based plastic as a
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13 5 means of reducing environmental impact. Poly(lactic acid) (PLA) is a representative
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15 6 bio-based plastic that is used in packaging, stationery, containers, etc. (Madhavan
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17 7 Nampoothiri et al., 2010). In addition, the utilization of the polyester has been expanded
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19 8 to the medical field for drug delivery, resorbable sutures, and as material for medical
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21 9 implants and other related applications (Auras et al., 2004). PLA is chemically
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23 10 synthesized by heavy metal-catalyzed ring-opening polymerization of lactide, which in
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25 11 turn are derived from fermentative lactate (LA) (Auras et al., 2004). However, the
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27 12 chemo-process often leaves harmful chemical residues that are a cause of health and
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29 13 safety concerns. The switch from the multistep chemo-bio process to a complete bio-
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31 14 process for LA-based polyester production is thus preferable to overcome this problem.
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47 15 Recently, a whole-cell biosynthesis system for LA-based polyester production
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50 16 without heavy metal catalyst has been constructed using engineered *Escherichia coli*
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53 17 (Matsumoto and Taguchi, 2009; Taguchi et al., 2008). The discovery of LA-
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56 18 polymerizing enzyme (LPE), which was an engineered polyhydroxyalkanoate (PHA)
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1 synthase (Taguchi and Doi, 2004), was a key to develop the microbial system. To date,
2 the *E. coli* platform has been used to produce various LA-based polymers incorporating
3 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx)
4 (Shozui et al., 2009; Yamada et al., 2009; Yamada et al., 2010). Most recently, we have
5 successfully incorporated new 2-hydroxy acids such as 2-hydroxybutyrate and glycolate
6 using LPE (Han et al., 2011; Matsumoto et al., 2011). However, Gram-negative bacteria,
7 such as *E. coli*, are known to produce potentially harmful substances, (*i.e.*, endotoxin
8 and lipopolysaccharide) (Furrer et al., 2007; Lee et al., 1999; Valappil et al., 2007). In
9 considering practical applications of LA-based polyester, especially for food grade and
10 biomedical demands, the use of endotoxin-free Gram-positive bacteria is preferable.

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

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

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2 Materials and methods

4 Strain, culture conditions and LA analysis in the medium

6 *C. glutamicum* ATCC13803, used as the host strain for P(LA-co-3HB) production
7 throughout this study, was transformed by electroporation as described previously
8 (Liebl et al., 1989). For polymer production, the engineered strains were grown in 100
9 ml nutrient-rich CM2G medium (Kikuchi et al., 2003) at 30°C for 24 h with reciprocal
10 shaking at 130 rpm. Cells were harvested, washed with distilled water, transferred into
11 100 ml minimal MMTG medium containing 6% glucose (Kikuchi et al., 2003) and 0.45
12 mg/l of biotin, and further cultivated for 72 h at 30°C. *C. glutamicum* does not produce
13 glutamate under the presence of high concentration of biotin (Shiio and OtSuka, 1962).
14 When needed, kanamycin (25 µg/ml) and/or chloramphenicol (5 µg/ml) was added to
15 the medium. After the cultivation, cells were lyophilized for polymer extraction. The
16 concentration of LA in the MMTG medium after 72 h cultivation was determined using
17 a D-/L-lactic acid assay kit (R-Biopharm, Roche, Germany).

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3 1 Plasmid constructions
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9 3 The kanamycin-resistant shuttle vector for *C. glutamicum* pPSPTG1 (Kikuchi et al.,
10 4 2003) was digested with *Xba*I, and self-ligated after T4 DNA polymerase blunting to
11 5 eliminate *Xba*I site. The resulting plasmid was digested with *Bst*EII and *Cpo*I, and
12 6 ligated with a synthetic *Bst*EII-*Xho*I-GC-*Xba*I-*Cpo*I linker (Table 1) to yield a new
13 7 vector, pPS. Then, a 3.9 kb *Xba*I/*Bam*HI fragment from the pGEMC1(STQK)AB
14 8 plasmid (Takase et al., 2003), containing the S325T/Q481K mutated PHA synthase
15 9 gene from *Pseudomonas* sp. 61-3 [PhaC1_{Ps}(ST/QK)], also termed LPE (Takase et al.,
16 10 2003), and *phaA* and *phaB* genes from *Ralstonia eutropha* (Peoples and Sinskey, 1989)
17 11 was inserted into *Xba*I/*Bam*HI sites of pPS to yield pPSCI(STQK)AB (Fig. 2). Similarly,
18 12 a 1.7 kb *Pst*I/*Xba*I fragment of pGEMC1(STQK)AB containing *phaC1*(ST/QK) gene
19 13 was inserted into *Bam*HI/*Xba*I site of pPS after T4 DNA polymerase blunting to yield
20 14 pPSCI(STQK).
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47 15 The chloramphenicol-resistant shuttle vector pVC7 (Kikuchi et al., 2003) was
48 16 digested with *Hind*III and *Eco*RI, and ligated with a synthetic *Hind*III-*Kpn*I-*Bam*HI-
49 17 *Sac*I-*Bgl*III-*Pst*I-*Eco*RI linker (Table 1) to yield the new plasmid pVC7-L. The P_{csp}
50 18 promoter, which is constitutively expressed in *C. glutamicum*, was amplified from
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1 pPSPTG1 using primers P_{csp} F and P_{csp} R (Table 1). The *KpnI/BamHI* digested PCR
2 product was inserted into *KpnI/BamHI* sites of pVC7-L to yield pVC7-LP. D-lactate
3 dehydrogenase (D-LDH) gene (*ldhA*) from *E. coli* was amplified using the primer pair,
4 Ecoli *ldhA* F and Ecoli *ldhA* R (Table 1). The *BamHI/SacI* digested PCR product was
5 inserted into *BamHI/SacI* sites of pVC7-LP to construct pVC7*ldhA*. Propionyl-CoA
6 transferase (PCT) gene (Elsden et al., 1956) was amplified from *Megasphaera elsdenii*
7 genomic DNA using a pair of primers, PCT F and PCT R (Table 1). The *BglII/PstI*
8 digested PCR product was inserted into *BglII/PstI* sites of pVC7*ldhA* to yield
9 pVC7*ldhApct*.

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11 Immunoblot analysis

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13 *C. glutamicum* transformants were cultivated at 30°C for 20 h in CM2G medium. Cells
14 were harvested by centrifugation and re-suspended in 25 mM Tris-HCl buffer (PH 7.5).
15 The soluble fraction of cell lysate was prepared by sonication and centrifugation
16 (12,000×g, 4°C, 10 min). Immunoblotting was performed using antisera to PCT and
17 PhaC1, and Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate (BIO-RAD) as
18 previously described (Jo et al., 2007). Rabbit antiserum to PCT was developed using

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1 purified PCT expressed in *E. coli*. The N-terminal His-tag fusion of *pct* gene was
2 constructed using pQE31 (Qiagen), and PCT was purified using affinity
3 chromatography with Ni⁺ NTA resin using a standard protocol.

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5 Polymer extraction from *C. glutamicum* cells

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7 The lyophilized cells (~1 g) were washed with 10 ml methanol three times at room
8 temperature prior to the polymer extraction. Polymer was extracted by incubation with
9 10 ml chloroform at 60 °C for 48 h. Cell debris was removed by passing through a
10 PTFE filter. A 10-fold volume of hexane was then added in order to precipitate the
11 polymer. The polymer was dried *in vacuo* at room temperature to determine cellular
12 polymer content based on cell dry weight and the weights of polymer. The extracted
13 polymer was used for further analyses.

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15 Analysis of LA-based polyesters

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17 The monomer composition of the polymers was determined by gas
18 chromatography/mass spectroscopy (GC/MS) as described previously (Arai et al., 2002).

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3 1 The molecular weights of the polymers were determined by gel permeation
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6 2 chromatography (GPC) (Shimadzu, Japan) using a TSKgel Super HZM-H (Tosoh,
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9 3 Japan) with polystyrene standards (Waters, USA) for calibration as described (Taguchi
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12 4 et al., 2008). The ^1H and ^{13}C NMR spectra of the polymers dissolved in CDCl_3 were
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16 5 obtained using a Bruker MSL400 spectrometer (400 MHz for ^1H NMR) using
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19 6 tetramethylsilane as an internal reference.
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25 8 **Results**

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32 10 Overproduction of D-lactic acid in *C. glutamicum* by introduction of a heterologous D-
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41 13 A key factor in the construction of a metabolic pathway to produce LA-based polyesters
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44 14 in *C. glutamicum* was the stereochemistry of LA, because it has been demonstrated that
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47 15 LPE has strict enantiospecificity toward D-LA-CoA (Tajima et al., 2009; Yamada et al.,
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51 16 2009). However, the *C. glutamicum* strain used here is known to produce mainly L-LA,
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54 17 as reported in several previous studies (Inui et al., 2004; Okino et al., 2008; Toyoda et
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57 18 al., 2009), whereas *E. coli* can produce D-LA (Bunch et al., 1997; Chang et al., 1999).
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1 Therefore, *C. glutamicum* needed to be remodeled as a D-LA over-producer by
2 introduction of D-LDH. In order to confirm the activity of the heterologously expressed
3 D-LDH, the engineered *C. glutamicum* harboring pVC7ldhA was cultured, and D- and L-
4 LA concentrations in the culture medium were measured (Table 2). In the parent strain,
5 L-LA concentration was determined to be 7-fold higher than that of D-LA, which was
6 consistent with the previous reports mentioned above. In contrast, the engineered strain
7 exhibited an enhancement of D-LA production that was 14-fold higher than L-LA,
8 indicating the functional expression of D-LDH in *C. glutamicum*. In this engineered
9 strain, the production of L-LA was decreased by the expression of D-LDH, probably due
10 to the consumption of pyruvate by D-LDH competing with that of the intrinsic L-LDH.
11 This result demonstrated that the engineered strain of *C. glutamicum* was suitable for
12 production of LA-based polyesters.

13 14 Expression of PCT gene in *C. glutamicum*

15
16 The next essential step toward synthesis of LA-based polyesters was activation of D-LA
17 to produce D-LA-CoA. To achieve this, pVC7ldhApct bearing propionyl-CoA
18 transferase (PCT) gene from *M. elsdenii*, as well as the *ldhA* gene, was introduced into

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1 *C. glutamicum*. The expression of the *pct* gene in *C. glutamicum* was confirmed by
2 immunoblot analysis. As shown in Fig. 3, the translated product for the *pct* gene was
3 detected with the same molecular mass compared with PCT expressed in *E. coli*, but
4 was absent in the wild-type strain. This result suggested that the *pct* gene was
5 successfully expressed in recombinant *C. glutamicum*. Together with functional
6 expression of D-LDH, LA-CoA supplying pathway should be constructed in the strain.

7
8 Functional expression of LPE

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10 The functions of the enzymes of the 3HB-CoA supplying pathway and LPE were
11 investigated based on P(3HB) production in *C. glutamicum* harboring pPSC1(STQK)AB.
12 The engineered strain accumulated 1.4 wt% of P(3HB), suggesting the functional
13 expressions of the three enzymes, PhaA, PhaB and LPE. However, the intracellular
14 polymer content was lower than the previous result of engineered *C. glutamicum*
15 harboring the *phaC*, *phaA* and *phaB* genes from *R. eutropha* (22.5 wt%) (Jo et al., 2006).
16 Thus, the expression of LPE was further analyzed by immunoblotting. The translated
17 product of the LPE [*phaC1*(STOK)] gene in *C. glutamicum* was observed as a clear
18 band having the same size of LPE expressed in *E. coli* (Fig. 3). Thus, the low P(3HB)

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1 content in the engineered strain might be partly due to the relatively low activity of LPE
2 toward 3HB-CoA compared to PhaC from *R. eutropha* (Matsumoto et al., 2005).
3 Regardless, from these results, LPE, PhaA and PhaB were shown to be functionally
4 expressed in *C. glutamicum*.

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

8 With the expression of the five genes relevant to the biosynthesis of P(LA-co-3HB)
9 confirmed, the plasmids pVC71dhApct (for LA-CoA supply) and pPSC1(STQK)AB (for
10 3HB-CoA supply and polymerization) were co-introduced into *C. glutamicum* for
11 construction of the metabolic pathway illustrated in Fig. 1. The engineered strain
12 harboring the dual plasmids produced 2.4 wt% polymer. GC/MS analysis of the
13 extracted polymer revealed that the polymer was P(LA-co-3HB) containing a
14 surprisingly high LA fraction (96.8 mol%) (Table 3). The weight-average molecular
15 weight of the polymer was 7400, indicating that LA units were incorporated into the
16 polymer chain.

17 The result of the incorporation of small quantities of 3HB units into the
18 polymer prompted us to evaluate the essentiality of the 3HB-supplying pathway. To

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1 examine this, we introduced plasmids pVC7ldhApct and pPSC1(STQK) encoding D-
2 LDH, PCT, and LPE into *C. glutamicum*. The *phaAB* genes were omitted in this
3 experiment. The new engineered strain accumulated P(LA-co-3HB) with even higher
4 LA fraction (99.3 mol%). Thus, the introduction of *phaAB* genes was shown to be not
5 essential to the production of P(LA-co-3HB). However, it should be noted that a small
6 amount of 3HB units were incorporated into the polymer without the introduction of
7 *phaAB* genes. Thus, this result cannot be a counterexample excluding the essentiality of
8 3HB-CoA to biosynthesis of P(LA-co-3HB). The polymer content (1.4 wt%) and its
9 molecular weight (5700) were decreased compared to those produced by the strain
10 expressing the five relevant LA-copolymer producing genes, including *phaAB*. These
11 inverse relationships between LA fraction and polymer content or molecular weight
12 were consistent with the trends observed in the previously examined *E. coli* system
13 (Yamada et al., 2011).

14
15 NMR analysis of LA-based polyesters produced in *C. glutamicum*

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17 In order to analyze polymer structure, the samples were subjected to NMR analyses.
18 The ¹³C NMR of the copolymer with 96.8 mol% LA exhibited strong resonance of LA

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1 units (δ 16.6, 69.0 and 169.6) as well as slight signal for the 3HB units (δ 19.7, 40.1 and
2 67.6) (Fig. 4A). The signal of carbonyl carbon of 3HB (supposed to be δ 169.1) was not
3 detected, probably because the peak was overlapped with the neighboring peak of LA
4 carbonyl carbon. In addition, the ^1H NMR spectrum of the polymer (Fig. 4B) showed
5 strong resonances, which were identical to those of chemically synthesized PLA, and
6 weak resonances of 3HB units. The signal of methyl proton of 3HB unit (4) was not
7 clear because of impurity, presumably lipid or fatty acids. The 3HB fraction was
8 determined to be 3.2 mol%, which was consistent with GC/MS result (Table 3).
9 Previous ^1H NMR analyses of P(LA-*co*-3HB)s demonstrated that the resonance of
10 methine proton of LA units exhibited high-field shift (δ 5.0-5.2) compared to that of
11 PLA that was due to the copolymerization of the LA units and 3HB units (Taguchi et al.,
12 2008; Yamada et al., 2009). The shifted signal of LA units was not detected in the
13 copolymer with 96.8 mol% LA probably because of a very small 3HB fraction. NMR
14 analyses of P(99.3 mol% LA-*co*-3HB) exhibited similar spectra while peaks for 3HB
15 units were beyond the detectable level (data not shown). These results supported that
16 the engineered *C. glutamicum* produced PLA-like polyesters.

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18 **Discussion**

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2 P(LA-co-3HB)s produced in *C. glutamicum* exhibited extremely high LA fractions,
3 approaching 100 mol%. This result had a striking contrast to the results obtained by the
4 *E. coli* system using the same set of genes and same carbon source, in which 47 mol%
5 was the maximum LA fraction (Shozui et al., 2009; Yamada et al., 2009; Yamada et al.,
6 2010). The high LA fraction could be partly due to the weak 3HB monomer supply in *C.*
7 *glutamicum*, which was suggested by the low P(3HB) content (1.4 wt%, Table 3). It is
8 worth noting that LPE accumulated 40 wt% P(3HB) in *E. coli* (Takase et al., 2003),
9 suggesting that the flux toward 3HB-CoA supplying pathway in *C. glutamicum* was
10 relatively low compared to that in *E. coli*. Furthermore, under copolymer-producing
11 conditions, the flux toward 3HB monomer was further decreased by overexpression of
12 D-LDH (from 40 to 9.7 mg/L, Table 3), probably because of D-LDH out-competing
13 pyruvate dehydrogenase for consumption of pyruvate. In addition, acetyl-CoA is
14 presumably used by PCT for CoA transferring reaction that led to a reduction in 3HB
15 pathway (Fig. 1). These factors could potentially further increase LA fraction in the
16 copolymers by decreasing the 3HB-CoA precursor pool. In terms of polymer properties,
17 it would be of interest to compare LA-based polyesters with the high LA fractions,
18 ranging from 70 mol% to nearly 100 mol%, with PLA homopolymer. Polymer with LA

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3 1 fractions of close to 100% has been hardly prepared in *E. coli* system.
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6 2 In the engineered strain, LA fraction in the copolymer should be determined by
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9 3 two major factors; monomer fluxes and activity of LPE. Since the first P(LA-co-3HB)
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12 4 with 6 mol% LA was synthesized (Taguchi et al., 2008), LA-reinforcing approaches
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15 5 using anaerobic culture conditions and an LA-overproducing mutant produced a variety
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18 6 of higher LA fractions, up to 47 mol% (Yamada et al., 2009). As a result, the LA
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21 7 concentration in the supernatant of *E. coli* culture medium (up to 5.7 g/L) was much
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24 8 higher than the amount of LA units in the copolymer, indicating that sufficient amount
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27 9 of LA was produced in the engineered *E. coli*. In order to further increase the LA
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30 10 fraction in the copolymer, evolution of LPE toward enhanced LA-polymerizing activity
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33 11 and/or reduction in 3HB monomer flux is necessary. In fact, the engineering of LPE to
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36 12 increase its activity that led to an increase in LA fraction up to 62 mol% (Yamada et al.,
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39 13 2010). Here, we demonstrated that the latter approach achieved the synthesis of
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42 14 copolymer with very high LA fractions. This result suggested that regulation of 3HB
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45 15 monomer flux could also be effective to cover a wide range (from 6 mol% to nearly 100
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48 16 mol% of LA fraction) of LA fractions in a produced copolymer. The methodology
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51 17 would also be useful for production of LA-based polyesters, with tailor-made monomer
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54 18 composition, conducted using various microbial platforms.
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3 1 The engineered *C. glutamicum* harboring D-LDH, PCT and LPE produced
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6 2 copolymer contained small amount of 3HB units, indicative of an intrinsic 3HB-CoA
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9 3 supply in *C. glutamicum*. This result is in good accordance with the fact that 3HB-CoA
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12 4 should serve as a priming unit for incorporation of LA unit in the initial step of
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15 5 polymerization (Taguchi et al., 2008 and Tajima et al., 2009). A potential 3HB-CoA
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18 6 supplying pathway was proposed in the Biocyc databases (Caspi et al., 2009), which
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22 7 might be catalyzed by homologous enzymes to PhaA and PhaB (YP_226966.1 and
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25 8 YP_226913.1). Further analysis will be necessary to clarify the roles of these genes in
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28 9 LA-based polyester biosynthesis.

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32 10 In this study, we succeeded in establishing an engineered *C. glutamicum* that
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35 11 can produce LA-based polyesters. This new endotoxin-free platform should be suitable
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38 12 for wider range of applications, especially food and medical related uses. The P(LA-co-
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41 13 3HB)-producing pathway should be applicable to other sugars that give pyruvate as a
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44 14 metabolized product. Thus this process could be more cost effective by using low grade,
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47 15 low cost sugars, such as molasses, as a carbon source.

51 52 16 53 54 55 17 **Acknowledgments**

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3 **1 Figure Captions**
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6 **3 Figure 1.** Metabolic pathways relevant to production of P(lactate-co-3-
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10 4 hydroxybutyrate) in engineered *Corynebacterium glutamicum*. L-LDH, L-lactate
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13 5 dehydrogenase; D-LDH, D-lactate dehydrogenase; PCT, propionyl-CoA transferase;
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16 6 PhaA, β -ketothiolase; PhaB, acetoacetyl-CoA reductase; LPE, lactate-polymerizing
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19 7 enzyme. Boxed enzymes indicate exogenous enzymes. Thick lines indicate reinforced
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22 8 pathways. Dashed lines indicate proposed pathways; acetyl-CoA could act as a CoA
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25 9 donor for CoA transferring reaction using PCT enzyme, and a small amount of 3HB-
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29 10 CoA was supplied by intrinsic, but uncharacterized, pathway.
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35 **12 Figure 2.** Map of the plasmids used in this study. P_{csp} denotes the promoter region. The
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38 13 *pct* gene encodes propionyl-CoA transferase from *Megasphaera elsdenii*. The *ldhA* gene
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41 14 encodes D-lactate dehydrogenase from *Escherichia coli*. The *phaC1*(STQK) gene
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44 15 encodes the Ser325Thr/Gln481Lys mutant of PHA synthase from *Pseudomonas* sp. 61–
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48 16 3 (Lactate-polymerizing enzyme, LPE). The *phaA* and *phaB* genes encode β -
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51 17 ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB) derived
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54 18 from *R. eutropha*, respectively.
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1 **Figure 3.** Immunoblot analysis of PCT and LPE expressed in *C. glutamicum*. PC
2 (positive controls), recombinant *E. coli* expressing PCT and LPE [PhaC1(ST/QK)],
3 respectively; NC (negative control), wild-type *C. glutamicum*; Sample, *C. glutamicum*
4 expressing PCT and LPE, respectively.

6 **Figure 4.** Analyses of a lactate-based copolymer produced in *C. glutamicum*. **A:** ¹³C
7 NMR spectrum, **B:** ¹H NMR spectrum. LA, lactate; 3HB, 3-hydroxybutyrate.

Table 1. Oligonucleotide sequences

Oligonucleotide linkers and primers	Sequences
<i>BstEII-XhoI-GC-XbaI-</i>	5'-GTCACCTCGAGCGTCTAGACG-3'
<i>CpoI</i> linker	5'-GACCGTCTAGACGCTCGAG-3'
<i>HindIII-KpnI-BamHI-</i>	5'-AGCTTGGGGTACCCGGGATCCATGAGCTCGAAGATCTAACTGCAGAAG-3'
<i>SacI-BglIII-PstI-EcoRI</i> linker	5'-AATTCTTCTGCAGTTAGATCTTCGAGCTCATGGATCCCGGGTACCCCA-3'
<i>Pcsp</i> F	5'-CTCGGTACCCAAATTCCTGTGA-3'
<i>Pcsp</i> R	5'-ATGGATCCCTCCTTGAATAGGTATCGAAAGAC-3'
<i>Ecoli ldhA</i> F	5'- GGATCCGCCACCATGAAACTCGCCGTTTATAG-3'
<i>Ecoli ldhA</i> R	5'- GAGCTCAAGATTAAACCAGTTCGTTTCG-3'
PCT F	5'- AGATCTAGGAGGTAAACAATGAGAAAAGTAGAAATCA-3'
PCT R	5'- GAGCTCTGCAGGTTATTTTTTCAGTC-3'

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

strain	D-LA (mg/l)	L-LA (mg/l)
Wild-type	3.0	21.7
Engineered strain ^a	209.0	14.3

^a Cells harboring pVC71*ldhA* were grown on MMTG containing 6% glucose for 72 h at 30°C.

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

Relevant genes	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
<i>phaCI(STQK), phaAB</i>	1.4 ± 0.1	0	100	0	40 ± 1	8.4	15.1	1.8
<i>phaCI(STQK), phaAB, ldhA, pct</i>	2.4 ± 0.1	96.8 ± 0.7	3.2 ± 0.6	277 ± 1	9.7 ± 0.4	5.2	7.4	1.4
<i>phaCI(STQK), ldhA, pct</i>	1.4 ± 0.1	99.3 ± 0.6	0.7 ± 0.3	205 ± 1	1.4 ± 0	4.3	5.7	1.3

^a Cells were grown on MMTG 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.

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Fig. 1

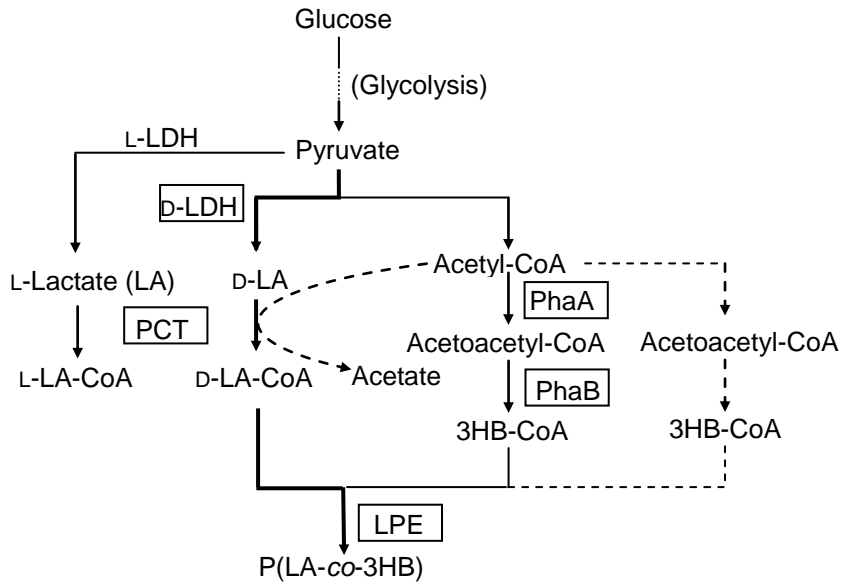
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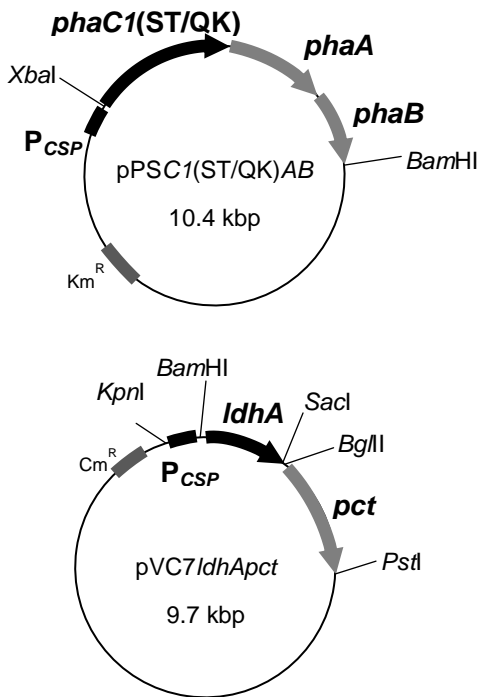
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10 **Fig. 2**

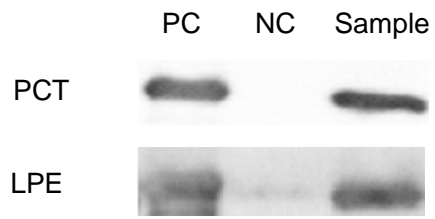
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6 **Fig. 3**

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