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Enhanced production of poly(lactate-*co*-3-hydroxybutyrate) from xylose in engineered *Escherichia coli* overexpressing a galactitol transporter

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Abstract Poly(lactate-*co*-3-hydroxybutyrate) (P(LA-*co*-3HB)) was previously produced from xylose in engineered *Escherichia coli*. The aim of this study was to increase the polymer productivity and LA fraction in P(LA-*co*-3HB) using two metabolic engineering approaches: (1) deletions of competing pathways to lactate production and (2) overexpression of a galactitol transporter (GatC), which contributes to the ATP-independent xylose uptake. Engineered *E. coli* mutants (Δ*pflA*, Δ*pta*, Δ*ackA*, Δ*poxB*, Δ*dld*, and a dual mutant; Δ*pflA* + Δ*dld*) and their parent strain, BW25113, were grown on 20 g l^{-1} xylose for P(LA-*co*-3HB) production. The single deletions of Δ*pflA*, Δ*pta*, and Δ*dld* increased the LA fraction (58–66 mol%) compared to BW25113 (56 mol%). In particular, the Δ*pflA* + Δ*dld* strain produced P(LA-*co*-3HB) containing 73 mol% LA. Furthermore, GatC overexpression increased both polymer yields and LA fractions in Δ*pflA*, Δ*pta*, and Δ*dld* mutants, and BW25113. The Δ*pflA* + *gatC* strain achieved a productivity of 8.3 g l^{-1}, which was 72 % of the theoretical maximum yield. Thus, to eliminate limitation of the carbon source, higher concentration of xylose was fed. As a result, BW25113 harboring *gatC* grown on 40 g l^{-1} xylose reached the highest P(LA-*co*-3HB) productivity of 14.4 g l^{-1}. On the other hand, the Δ*pflA* + Δ*dld* strain grown on 30 g l^{-1} xylose synthesized 6.4 g l^{-1} P(LA-*co*-3HB) while maintaining the highest LA fraction (73 mol%). The results indicated the usefulness of GatC for enhanced production of P(LA-*co*-3HB) from xylose, and the gene deletions to upregulate the LA fraction in P(LA-*co*-3HB). The polymers obtained had weight-averaged molecular weights in the range of 34,000–114,000.

Keywords Lactate-based polymer · Polyhydroxyalkanoate · Biobased plastic · Lignocellulosic biomass

Introduction

Polylactic acid (PLA) is a biodegradable polymer (Drumright et al. 2000), which offers great promises in a wide range of applications, such as for biomedical uses, as a packaging material, and in the agriculture fields (Auras et al. 2004; Nampoothiri et al. 2010; Lasprilla et al. 2012). PLA thus can serve as an alternative to the conventional petroleum-derived plastics such as polyethylene, propylene, and polystyrene (Lim et al. 2008). PLA is produced via ring-opening polymerization (Kricheldorf 2001), which employs heavy metal catalysts such as tin that might contaminate the polymer and reduce its applications especially in the biomedical field and food handling (Chanfreau et al. 2010; Taguchi 2010). Moreover, the requirement for high-purity lactic acid for polymerization makes PLA less cost competitive compared with the conventional polymers (Corma et al. 2007).

To circumvent these challenges, the lactate-based polymer, poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] has been produced in engineered microorganisms expressing LA-polymerizing enzyme (LPE) (Taguchi et al. 2008), and

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for reviews, see Matsumoto and Taguchi (2010, 2013). The copolymer was wholly biosynthesized by one-pot fermentation without the chemoprocess. In addition, an interesting feature of the copolymer over PLA is that the variation of the LA/3HB ratio in P(LA-*co*-3HB) has demonstrated the generation of polymers with different properties (Yamada et al. 2011). Therefore, there have been concerted efforts to regulate the monomer composition in the copolymers using enzyme and metabolic engineering, fermentation technology, and culture media manipulation techniques (Yamada et al. 2009; Shozui et al. 2011; Song et al. 2012; Nduko et al. 2013). During the course of these studies, it has been found that the productivity of P(LA-*co*-3HB) having >50 mol% LA fraction is relatively low, limiting the exploration of polymer properties of the LA-enriched copolymers (Shozui et al. 2011; Song et al. 2012).

In this context, we have recently demonstrated that the utilization of xylose and a further evolved LPE, PhaC1$_{Ps}$ST/FS/QK could achieve P(60 mol% LA-*co*-3HB) production with a productivity of 7.3 g l^{-1}, which was a critical breakthrough toward efficient production of LA-enriched polymers (Nduko et al. 2013). In addition, since xylose can be readily extracted from the lignocellulosic biomass (Liu et al. 2010), it is a suitable substrate for cost-effective production of P(LA-*co*-3HB)s. Pursuant to this, in the present study, we embarked on the construction of a platform for high-yield synthesis of LA-enriched P(LA-*co*-3HB) from xylose.

To meet this goal, we investigated two distinct strategies. We previously used Δ*pflA* mutant of *Escherichia coli* as a host strain that strengthened the supply of lactic acid (Fig. 1), which could eventually be incorporated into P(LA-*co*-3HB) (Yamada et al. 2010; Shozui et al. 2010). However, other gene disruptants, which have been reported to increase lactic acid production, as well as their parent strain *E. coli* BW25113 have not been evaluated for P(LA-*co*-3HB) synthesis from xylose. We thus first recruited *E. coli* BW25113 and four mutants (Δ*pta*, Δ*ackA*, Δ*poxB*, and Δ*dld*), which reduces the competition for carbon flux at the pyruvate node and redirect it into lactic acid (Zhou et al. 2011). Second, we focused on the enhancement of xylose uptake by *E. coli*. In this organism, there are two different D-xylose-specific transport systems: the XylE, a member of the major facilitator superfamily of transporters and the ATP-binding cassette (ABC) transporter encoded by the *xylFGH* operon (Hasona et al. 2004). The ATP-dependent transporter is the major xylose transporter and could reduce the amount of ATP available to the cells. Therefore, to eliminate the possibility of ATP limitation, we overexpressed a non-ATP consuming galactitol permease, GatC, that has been demonstrated to transport xylose efficiently for lactic acid production (Utrilla et al. 2012). Herein, we describe the efforts that led to the synthesis of P(LA-*co*-3HB)s with high productivities and varied LA/3HB ratios.

Materials and methods

Bacterial strains and plasmids

The expression vector pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB*, which harbors the *pct*, *phaC1*$_{Ps}$(ST/FS/QK), *phaA*, and *phaB* genes was constructed previously (Yamada et al. 2009). *E. coli* BW25113, the single-gene knockout mutants (Keio collection strains) (Table 1) (Baba et al. 2006), and pCA24N*gatC* plasmid harboring *gatC* (Kitagawa et al. 2005) were purchased from the National BioResource Project, Japan. The dual-gene knockout mutant (Δ*pflA* and Δ*dld*) was created using the quick and easy *E. coli* gene deletion kit (Funakoshi Co. Ltd, Tokyo, Japan) as follows: to construct marker-free mutant strain of *E. coli* JW0885 (Δ*pflA*), the kanamycin selection marker was excised, and successful elimination of the marker was examined by PCR as described previously (Datsenko and Wanner 2000). The deletion of *dld* in the chromosome of the marker-free *E. coli* JW0885 was conducted via a single-step inactivation method previously reported (Datsenko and Wanner 2000) using the pair of primers, 5′-CGCTATTCTAGTTTGTGATATTTTTCGCCACCACAAGGAGTGGAAAATGATTCCGGGGATCCGTCGACC-3′ and 5′-GGATGGCGATACTCTGCCATCCGTAATTTTACTCCACTTCCTGCCAGTTTGTAGGCTGGAGCTGCTTCG-3′. The pKD46 plasmid that contain the Red recombinase genes was used for gene deletion (Baba et al. 2006). Successful deletion of the *dld* gene was confirmed by PCR using the verification primers 5′-GCCTCTTCCATGACAACAACTGA-3′ and 5′-CCTTCCACTTCCTGCCAGTTTTT-3′. The obtained strain was designated as *E. coli* JWMB1.

Culture conditions

Cultivations for the production and extraction of P(LA-*co*-3HB) polymers, lactic acid, and sugar consumption measurements were carried out in 500-ml shake flasks. The precultures used to inoculate the shake flask cultivations were prepared as follows. A single colony of recombinant *E. coli* cells harboring pTV118N*pctphaC1*$_{Ps}$(ST/FS/QK)*AB* with or without pCA24N*gatC* was used to inoculate 10-ml test tubes having 2 ml of LB medium supplemented with the necessary antibiotic/s (ampicillin 100 μg l^{-1}, chrolamphenicol 30 μg l^{-1}). The tubes were cultivated at 30 °C for 12 h in a rotary shaker with reciprocal shaking at 180 rpm. One milliliter of the pre-culture was then transferred into 100-ml LB medium containing 100 μg l^{-1} ampicillin (and 30 μg l^{-1} chrolamphenicol and 1 mM IPTG for pCA24N*gatC*-harboring transformants), 20/30/40 g l^{-1} xylose and 10 mM calcium pantothenate, a CoA precursor which was demonstrated to be effective for increasing LA fractions in P(LA-*co*-3HB) (Table S1) in a 500-ml shake flask and cultured at 30 °C for

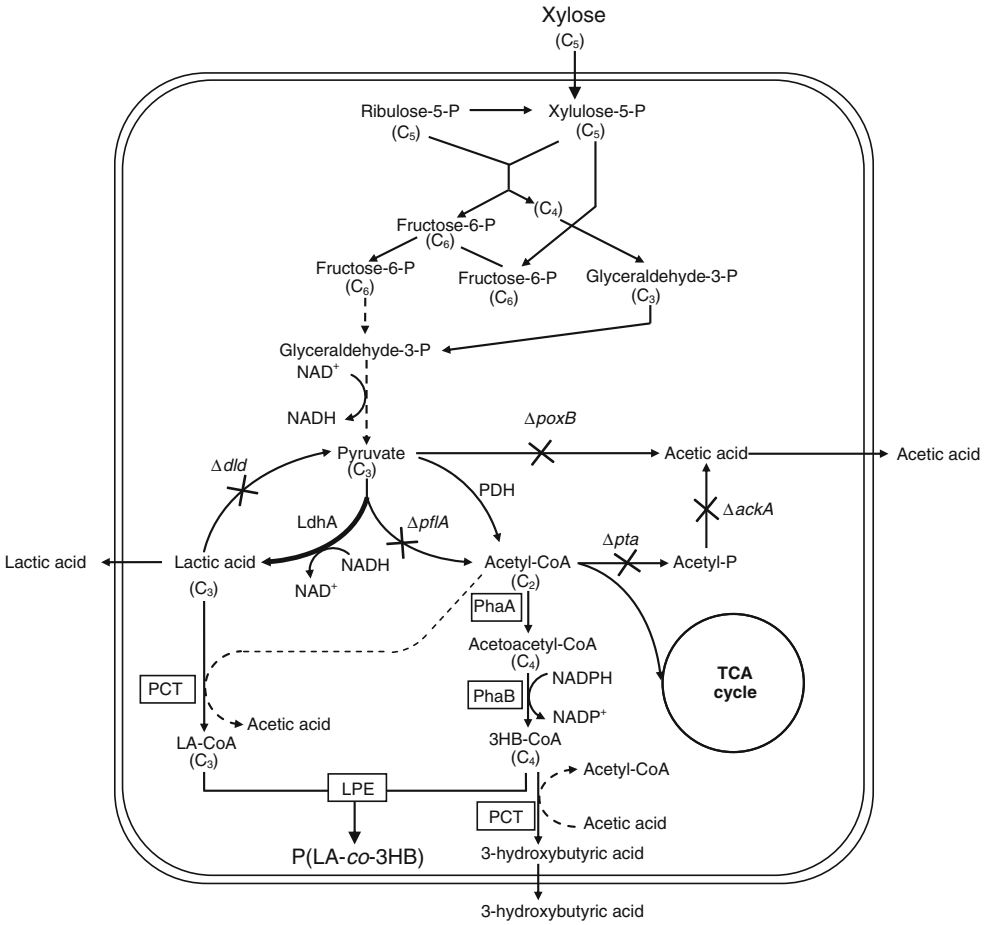

Fig. 1 Metabolic pathways in *E. coli* for xylose metabolism for the production of P(LA-*co*-3HB) and related metabolites. Deleted pathways are indicated by *crosses*, and the genes codes for: *pflA*, pyruvate formate lyase activating enzyme; *poxB*, pyruvate oxidase; *dld*, NAD^+-independent lactate dehydrogenase; *ackA*, acetate kinase; *pta*, phosphate acetyltransferase; PDH, pyruvate dehydrogenase complex; and LdhA, lactate dehydrogenase. The enzymes in the *boxes* were heterologously expressed and are involved in P(LA-*co*-3HB) synthesis. *LPE* lactate polymerizing enzyme [$PhaC1_{Ps}$(ST/FS/QK)], *PCT* propionyl-CoA transferase, *PhaA* β-kethothiolase, *PhaB* NADPH-dependent acetoacetyl-CoA reductase. The *dashed arrows* indicate the proposed pathways catalyzed by PCT

72 h with reciprocal shaking at 120 rpm. The samples from the *E. coli* cultures were taken at the end of cultivation and centrifuged at 12,000 rpm for 5 min to separate the cells and the supernatant. The concentrations of sugars, lactic acids, and other metabolites in the supernatant were determined by HPLC system using a refractive index detector as previously described (Matsumoto et al. 2013).

Table 1 *E. coli* strains used in this study

Strain	Relevant genotype	Reference
BW25113	*rrnB3 ΔlacZ4887 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	Baba et al. (2006)
JW0885	*ΔpflA::FRT-kan^R-FRT*	Baba et al. (2006)
JW2121	*Δdld::FRT-kan^R-FRT*	Baba et al. (2006)
JW2294	*ΔPTA::FRT-kan^R-FRT*	Baba et al. (2006)
JW2293	*ΔackA::FRT-kan^R-FRT*	Baba et al. (2006)
JW0855	*ΔpoxB::FRT-kan^R-FRT*	Baba et al. (2006)
JWMB1	*ΔpflA Δdld::FRT-FRT*	This study

Polymer extraction and analyses

The lyophilized cells were soaked in chloroform at room temperature for 2 days in glass flasks with constant stirring. The cell debris was separated by filtration using a 0.2-μm PTFE filter. A tenfold volume of methanol was then added into the filtrate to precipitate the polymer as described previously (Nduko et al. 2013). Polymer yields and monomer compositions of P(LA-*co*-3HB)s were determined by HPLC as described previously (Yamada et al. 2009). ^{1}H NMR resonances of the copolymer in $CDCl_3$ were observed as multiplet peaks at δ 1.4–1.6 (CH_3) and δ 4.9–5.2 (CH) for the LA unit and at δ 1.2–1.4 (CH_3), δ 2.4–2.8 (CH_2) and δ 5.2–5.4 (CH) for the 3HB unit, which is consistent with the previous reports (Yamada et al. 2009). The molecular weights of the extracted P(LA-*co*-3HB)s were estimated by gel permeation chromatography (GPC, JUSCO, Japan) equipped with a Shodex GPC KF-805 column (Showa Denko K. K., Japan) with polystyrene standards (Waters, USA) for calibration (Taguchi et al. 2008).

Results

Single-gene knockout increased the LA fraction in P(LA-*co*-3HB)

The P(LA-*co*-3HB) yields in the cells cultivated on 20 g l^{-1} xylose are shown in Table 2 (Nos. 1–7). For the knockout mutants, the Δ*pta*, Δ*ackA*, and Δ*dld* mutants had higher polymer productivities (6.5–7.4 g l^{-1}) than BW25113 (6.3 g l^{-1}). Focusing on the LA fractions in P(LA-*co*-3HB)s, the Δ*pta* and Δ*dld* mutants had higher LA fractions (58 and 66 mol%, respectively) compared to BW25113 (56 mol%). Thus, these mutations as well as Δ*pflA* were effective for improving both or either of the polymer productivity or the LA fractions in the polymers. On the contrary, the Δ*poxB* mutant had lower LA fractions and polymer productivity than BW25113 strain, suggesting an important role of its gene product in P(LA-*co*-3HB) production. Since the Δ*pflA* and Δ*dld* single-gene knockout mutants were found to effectively increase the P(LA-*co*-3HB) productivity and LA fraction (Table 2, Nos. 2 and 3), the two mutations were combined to create a dual mutant, *E. coli* JWMB1. The strain exhibited an additive effect on the increase of the LA fraction (73 mol% LA) in P(LA-*co*-3HB) (Table 2, No. 7), which was the highest value among the conditions tested in this study. However, the JWMB1 had lower polymer productivity than the other mutants.

To understand the carbon flux from xylose to metabolites, the amounts of residual xylose, polymer, lactic acid (the only

Table 2 P(LA-*co*-3HB) production from xylose in engineered *E. coli* harboring pTV118N*pctphaC*1(ST/FS/QK)AB

No.	Host genotype	Xylose concentration (g l^{-1})	Additional plasmid	Cell dry weight (g l^{-1})	Polymer yield (g l^{-1})	Polymer content (wt.%)	LA fraction (mol%)
1	Parent (BW25113)	20	–	9.5±0.2	6.3±0.2	67±4	56±5
2	Δ*pflA*	20	–	10.4±0.1	7.3±0.2	61±5	60±2
3	Δ*dld*	20	–	8.6±0.7	6.5±0.6	76±1	66±2
4	Δ*pta*	20	–	10.1±0.2	7.4±0.4	73±3	58±1
5	Δ*ackA*	20	–	9.7±0.8	7.3±0.4	75±2	54±2
6	Δ*poxB*	20	–	8.4±0.3	5.0±0.4	60±6	43±4
7	Δ*pflA*,Δ*dld*	20	–	4.8±0.3	2.8±0.6	58±7	*73*±1
8	Parent	20	pCA24N*gatC*	9.7±0.2	7.7±0.3	79±5	58±3
9	Δ*pflA*	20	pCA24N*gatC*	9.4±0.2	8.3±0.8	88±7	67±5
10	Δ*dld*	20	pCA24N*gatC*	8.1±0.1	6.6±0.5	81±6	66±3
11	Δ*pta*	20	pCA24N*gatC*	7.8±0.6	5.0±0.5	65±6	57±4
12	Δ*ackA*	20	pCA24N*gatC*	8.4±0.4	7.3±0.7	87±5	64±3
13	Δ*poxB*	20	pCA24N*gatC*	4.3±0.3	0.6±0.0	15±1	8±2
14	Δ*pflA*,Δ*dld*	20	pCA24N*gatC*	7.9±0.5	4.4±0.3	55±5	70±2
15	Parent	30	–	13.7±0.6	10.7±1.0	78±5	64±4
16	Δ*pflA*	30	–	10.9±0.9	9.1±0.5	83±0	62±1
17	Δ*dld*	30	–	11.5±0.4	9.2±0.9	79±8	62±5
18	Δ*pta*	30	–	10.9±0.4	7.8±0.7	71±4	63±1
19	Δ*ackA*	30	–	9.8±0.4	7.7±0.2	79±3	69±2
20	Δ*poxB*	30	–	8.3±0.6	5.9±0.9	71±4	63±5
21	Δ*pflA*,Δ*dld*	30	–	6.7±0.5	6.4±0.2	96±4	*73±1*
22	Parent	30	pCA24N*gatC*	12.9±0.4	12.0±0.5	93±2	63±3
23	Δ*pflA*	30	pCA24N*gatC*	11.4±0.5	9.7±0.5	85±6	66±6
24	Δ*dld*	30	pCA24N*gatC*	8.9±0.5	7.5±0.9	84±3	69±4
25	Δ*pta*	30	pCA24N*gatC*	10.0±0.1	7.0±0.5	70±4	66±1
26	Δ*ackA*	30	pCA24N*gatC*	11.2±0.1	9.2±0.6	82±6	67±3
27	Δ*poxB*	30	pCA24N*gatC*	5.6±0.6	1.2±0.4	22±4	23±1
28	Δ*pflA*,Δ*dld*	30	pCA24N*gatC*	8.4±0.4	7.4±0.7	88±10	69±5
29	Parent	40	–	15.8±0.7	11.1±0.8	70±5	61±3
30	Parent	40	pCA24N*gatC*	15.9±0.1	*14.4±0.4*	91±3	66±1

The data shown are means±standard deviations of triplicate cultures

Italicized values mean the highest score among tested

metabolite detected in the medium), and CO_2 are shown as the molar amount of the carbon atom, e.g., 20 g l^{-1} xylose (C_5) corresponds to 667 mM carbon in molar amount (Fig. 2a). The CO_2 amount was theoretically estimated based on the release of two CO_2 molecules upon the formation of one 3HB molecule (Matsumoto and Taguchi 2013). Acetic acid was below the detection limit under the conditions employed. As shown in Fig. 2a, at 20 g l^{-1} xylose feeding, 121–428 mM carbon was captured, and the remaining part probably went into cell mass and/or energy. The total lactate carbon yield (LA units in the polymer and the lactic acid secreted into the medium) for the Δ*dld*, Δ*pta*, and Δ*ackA* mutants was 1.1–1.4-fold higher compared to that of BW25113, suggesting that elimination of lactic acid formation-competing (Δ*dld*) and by-product formation (Δ*pta* and Δ*ackA*) pathways is effective for enhancing the carbon flux toward lactic acid that is eventually incorporated into P(LA-*co*-3HB). For the JWMB1 mutant, the flux toward the 3HB units was remarkably reduced, resulting in the synthesis of LA-enriched polymers. From these results, the potency of improved lactic acid production in the enhancement of the LA fractions in the copolymers and improvement of polymer productivities was highlighted by select single-gene knockout mutants. Italicized numbers are the highest values among examined.

The weight-averaged molecular weights (M_w) of the polymers were in the range of 34,000–114,000 and varied with the LA/3HB ratio (Table 3), consistent with earlier observations that increased LA fractions reduces the molecular weights of the P(LA-*co*-3HB)s (Yamada et al. 2011).

Overexpression of the xylose transporter, GatC, enhanced LA fraction and productivity of P(LA-*co*-3HB)

To enhance xylose consumption and potentially improve polymer productivity, we sought to facilitate xylose uptake by *E. coli*. The *E. coli* strains harboring pCA24N*gatC* and

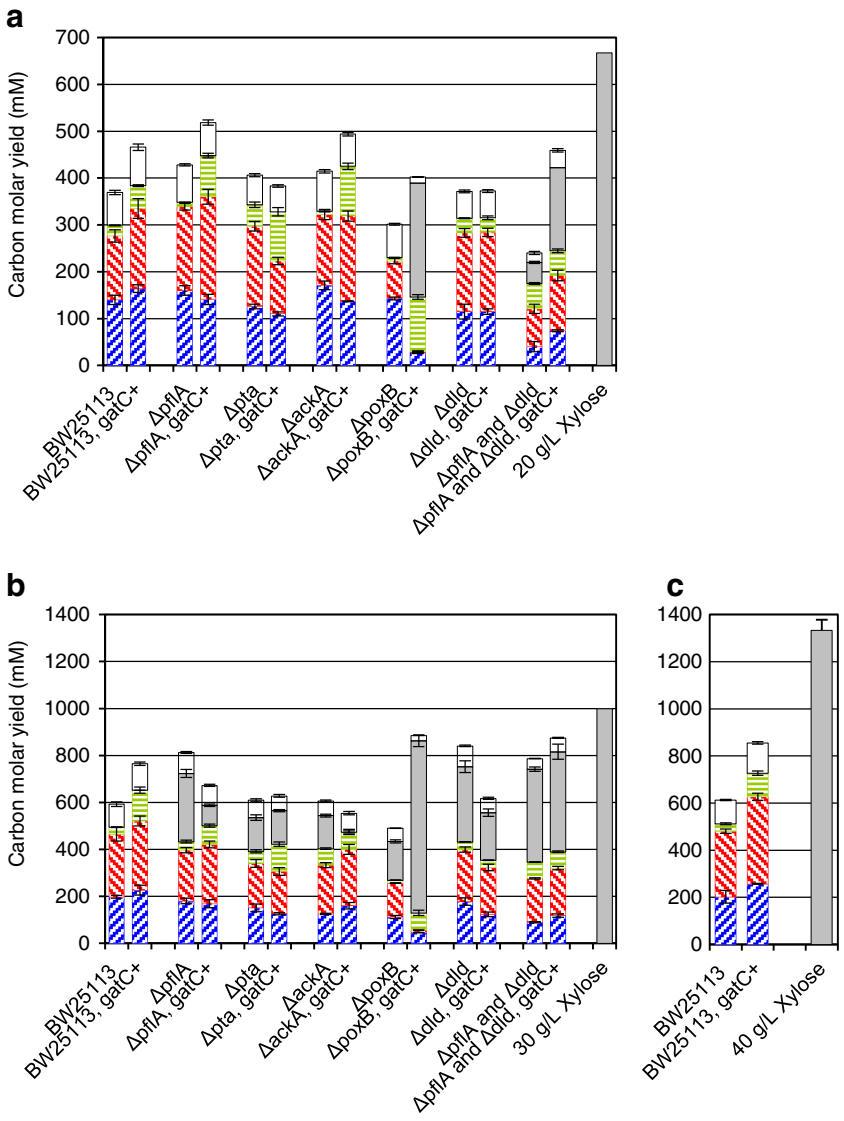

Fig. 2 Molar carbon amount of LA (*red up-down diagonal strips*) and 3HB units (*blue down-up diagonal strips*) in P(LA-*co*-3HB), lactic acid secreted into the medium (*green horizontal strips*), residual xylose (*gray bars*), and theoretical CO_2 (*white bars*) by *E. coli* BW25113 and its derivatives. The xylose *bar* at the end of each figure shows the initial carbon molar amount of xylose used for polymer production. The strains with "*gatC*+" harbored pTV118N*pctphaC*1(ST/FS/QK)*AB* and pCA24N*gatC*. The values shown are means± standard deviations of triplicate experiments. The CO_2 indicated is an estimate based on the generation of two CO_2 molecules for every 3HB unit formed. The xylose supplied were **a** 20 g l^{-1} (667 mM carbon amount), **b** 30 g l^{-1} (1,000 mM carbon amount), and **c** 40 g l^{-1} (1333 mM carbon amount)

Table 3 Molecular weights of P(LA-*co*-3HB) synthesized in engineered *E. coli*

No.	Monomer composition (mol%)		Molecular weights		
	LA	3HB	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n
1	56	44	44	89	2.0
2	60	40	15	34	2.3
3	66	34	13	36	2.8
4	58	42	22	50	2.3
5	54	46	42	114	2.7
6	43	57	46	102	2.2
7	73	27	17	38	2.2

The sample numbers in this table correspond to sample Nos. 1–7 in Table 2

pTV118N*pctphaC*1(ST/FS/QK)AB were cultivated for P(LA-*co*-3HB) production from 20 g l^{-1} xylose. As can be seen in Table 2 (Nos. 8–14) and Fig. 2a, GatC overexpression in BW25113 increased the polymer productivity (7.7 g l^{-1}) without significant change in the LA/3HB ratio. For the Δ*pflA*, Δ*ackA*, and JWMB1 mutants, the overexpression of GatC enhanced the LA fractions in P(LA-*co*-3HB) together with the carbon yield of the polymers (72 % of theoretical maximum for the best mutant) and lactic acid from xylose. Therefore, GatC is a dual-useful toolkit for increasing the productivity of P(LA-*co*-3HB) and/or LA fraction in the polymer for BW25113 and mutants of Δ*pflA*, Δ*ackA*, and JWMB1. However, the beneficial effect was not observed for Δ*dld* mutant and rather reduced the polymer yield and/or LA fraction for Δ*pta* and Δ*poxB* mutants (Fig. 2a).

Effects of additional xylose on P(LA-*co*-3HB) synthesis

The results above indicates that Δ*pflA*+*gatC* achieved the production of 8.3 g l^{-1} P(LA-*co*-3HB), which was 72 % relative to the theoretical limit, suggesting that the amount of xylose fed (20 g l^{-1}) could be limiting the strains from expressing their true phenotypes, i.e., high P(LA-*co*-3HB) yields and LA fractions. Thus, to evaluate the effect of xylose concentrations on polymers, recombinant cells were cultivated on 30 g l^{-1} xylose for P(LA-*co*-3HB) production.

All the mutants and BW25113 had significant increases in the polymer productivities (1.1-fold to 2.3-fold) relative to polymer production at 20 g l^{-1} (Table 2, Nos. 15–21), demonstrating that sugar concentration had been the limiting factor to the polymer productivity in the aforementioned conditions. Among the tested conditions, BW25113 exhibited relatively high polymer productivity (10.7 g l^{-1}). On the other hand, JWMB1 produced P(LA-*co*-3HB) having the highest LA fraction (73 mol%) with the productivity of 6.4 g l^{-1} (Table 2, No. 21).

Subsequently, the potential of GatC was also investigated at 30 g l^{-1} xylose. Similar to 20 g l^{-1} xylose feeding, GatC overexpression increased the polymer productivity for BW25113 (12 %), Δ*pflA* (7 %), Δ*ackA* (20 %), and JWMB1 (16 %) strains (Table 2, Nos. 22–28 and Fig. 2b) relative to the corresponding cells not expressing GatC. On the contrary, GatC overexpression adversely affected the polymer productivities for the Δ*dld*, Δ*pta*, and Δ*poxB* mutants. Overall, the BW25113 had higher polymer productivity of 12.0 g l^{-1} with 63 mol% LA than the mutants (Table 2, Nos. 22–28). With the exception of Δ*poxB* mutant, all the other mutants had insignificant changes in the LA fractions in P(LA-*co*-3HB) (Fig. 2b); however, these LA fractions were slightly higher than those of BW25113. The *poxB* mutation adversely affected the polymer production. Currently, the reason for the negative effect of *poxB* mutant is not clear. It has been reported that *poxB* mutation reduces the growth rate of *E. coli* (Abdel-Hamid et al. 2001), suggesting that the direct conversion of pyruvate into acetate by PoxB plays an important role in the growth efficiency in *E. coli*.

Because BW25113 fully consumed 30 g l^{-1} xylose unlike the mutants (Fig.2b), the sugar concentration was increased to 40 g l^{-1} for this strain. The polymer productivity was increased along with the sugar concentration (Table 2, No. 29). In addition, the GatC overexpression further increased the polymer productivity, where the highest polymer productivity of 14.4 g l^{-1} with 66 mol% LA was obtained (Table 2, No. 30), the highest productivity for P(LA-*co*-3HB) ever reported. These results demonstrated that the overexpression of GatC was effective to the utilization of high concentration of xylose for high-yield polymer production in BW25113.

Discussion

Since the discovery of LPE for the synthesis of P(LA-*co*-3HB), our next goal has been to enrich the LA fraction in the copolymer. With success, copolymers having LA fractions greater than 90 mol% have been synthesized (Shozui et al. 2011; Song et al. 2012). However, copolymers with LA fractions >50 mol% have been characterized by low productivity, limiting the exploration of polymer properties. To address the issue of polymer productivity, we have recently demonstrated xylose to be effective for the synthesis of LA-enriched copolymers (60 mol%) with high productivity (7.3 g l^{-1}) (Nduko et al. 2013). In this study, we made efforts to exploit the advantages of xylose for the synthesis of LA-enriched P(LA-*co*-3HB). For the first time, by the combination of metabolic engineering (Δ*pflA*+Δ*dld*+*gatC*) and use of xylose, we successively produced P(LA-*co*-3HB) having LA fractions greater than 70 mol% with significantly high productivity (6.4 g l^{-1}) compared to our previous reports, for example, LA-based polymer having 96 mol% LA with

productivity of 14.8 mg l^{-1} (Shozui et al. 2011). The *pflA* mutation is known to eliminate formate formation and redirecting the flux toward lactic acid formation (Zhu and Shimizu 2004). On the other hand, the *dld* mutation hampers lactic acid reutilization (Zhu and Shimizu 2005). Therefore, the combination of the two mutations had a synergistic effect in lactic acid production.

Our previous study demonstrated that P(LA-*co*-3HB) was more efficiently produced than P(3HB) (Nduko et al. 2013). To interpret this result, it is worth to emphasize that one LA unit (C_3) is generated from one pyruvate molecule (C_3), while one 3HB unit (C_4) is synthesized using two pyruvate molecules ($C_3 \times 2$). This stoichiometry contributes to the high-yield production of LA-enriched P(LA-*co*-3HB), namely, the copolymer with higher LA fraction is supposed to be produced with higher carbon yield. However, it is also true that LPE requires 3HB-CoA for the incorporation of LA-CoA; thus, the inverse relationship between LA fraction and polymer productivity has been observed. The essentiality of 3HB-CoA for the synthesis of LA-based polymers has been interpreted as a role of 3HB-CoA as a priming unit of polymerization (Matsumoto and Taguchi 2010). Therefore, the productivity of P(LA-*co*-3HB) and LA fraction in the polymer is thought to be determined by the interactivity of the two factors.

The mutants examined in this study had been reported to increase the lactic acid productivity (Zhou et al. 2011). In fact, some of the mutants were shown to increase the accumulation of LA units and the LA fractions in the polymer, namely, the part of carbon flux for 3HB units was channeled toward LA units, suggesting that the mutations cause redistribution of the carbon flux. It should also be noted that the carbon yield (the carbon amount of polymer/the consumed xylose) of certain mutants was higher than that of BW25113, although the absolute productivity was lower, where limitation of polymer production was observed with high xylose feeding (Fig. 2b). This fact suggested that the blockage of the bypass pathway reduced the futile flux of carbon. All the strains had residual lactic acid in the supernatant not incorporated as LA units in the polymer (Fig. 2b), suggesting that the polymerization step would be a bottleneck, possibly due to the LA-incorporating capacity of LPE. The results demonstrated that the productivities of lactic acid and P(LA-*co*-3HB) were closely related, but not directly linked. Furthermore, although the *poxB* mutation has been shown to improve lactic acid production (Zhou et al. 2011), it had poor polymer yields. Therefore, the simple metabolic engineering toward enhanced lactic acid production would not always be useful for the production of LA-based polymers.

In terms of polymer productivity, therefore, BW25113 was shown to be the best among the strains tested. The BW25113 strain fully consumed xylose even with high concentration feeding, but the large portion of the consumed carbon was not distributed to cell mass formation (Fig. 2b,c and Table 2), suggesting that some carbon was lost in BW25113. However, although BW25113 seemed to waste a part of the carbon source, this weakness was improved by the introduction of the *gatC* gene. In fact, the beneficial effect of GatC on the overall carbon yield was particularly apparent in BW25113, supporting the hypothesis that some xylose was being lost in BW25113.

In conclusion, in this study, we have employed the metabolic engineering approaches to achieve high productivity of LA-enriched P(LA-*co*-3HB)s from xylose, which were further enhanced by the overexpression of a galactitol transporter, GatC. The deletions of some of the competing pathways (Δ*pflA*, Δ*dld*, Δ*pta*, Δ*ackA* and Δ*pflA*+Δ*dld*) increased the LA fraction in the copolymer, but at the expense of the productivity. Considering xylose as a constituent of lignocellulosic biomass that could be readily obtained, the platform established here could be a potent avenue for efficient utilization of xylose. Moreover, the results of this study facilitate the exploration of material properties and potential applications of LA-enriched polymers, which will be the next target.

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