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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)-mediated *de novo* synthesis of glycolate-based polyhydroxyalkanoate in *Escherichia coli*

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Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) generates 2-phosphoglycolate (2PG) as one of the metabolites from the Calvin-Benson-Bassham (CBB) cycle. In this study, we focused on the fact that glycolate (GL) derived from 2PG can be incorporated into the bacterial polyhydroxyalkanoate (PHA) as the monomeric constituent by using the evolved PHA synthase (PhaC1$_{Ps}$STQK). In this study, the function of the RuBisCO-mediated pathway for GL-based PHA synthesis was evaluated using *Escherichia coli* JW2946 with the deletion of glycolate oxidase gene (Δ*glcD*) as the model system. The genes encoding RuBisCO, phosphoribulokinase and 2PG phosphatase (PGPase) from several photosynthetic bacteria were introduced into *E. coli*, and the cells were grown on xylose as a sole carbon source. The functional expression of RuBisCO and relevant enzymes was confirmed based on the increases in the intracellular concentrations of RuBP and GL. Next, PHA biosynthetic genes encoding PhaC1$_{Ps}$STQK, propionyl-CoA transferase and 3-hydroxybutyryl(3HB)-CoA-supplying enzymes were introduced. The cells accumulated poly(GL-*co*-3HB)s with GL fractions of 7.8–15.1 mol%. Among the tested RuBisCOs, *Rhodosprium rubrum* and *Synechococcus elongatus* PCC7942 enzymes were effective for P(GL-*co*-3HB) production as well as higher GL fraction. The heterologous expression of PGPase from *Synechocystis* sp. PCC6803 and *R. rubrum* increased GL fraction in the polymer. These results demonstrated that the RuBisCO-mediated pathway is potentially used to produce GL-based PHA in not only *E. coli* but also in photosynthetic organisms.

[**Key words:** Polyhydroxybutyrate; Polyglycolic acid; Carbon fixation; Biodegradable plastic; Lactate-polymerizing enzyme]

Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBisCO) is a key enzyme in Calvin–Benson–Bassham (CBB) cycle and plays a central role in photosynthesis (1,2). RuBisCO cleaves RuBP into two molar equivalents of 3-phosphoglycerate (3PG) via carboxylation. 3PG is subsequently converted into glyceraldehyde-3-phosphate (GAP), which is withdrawn for the central metabolic pathway. In addition, RuBisCO catalyzes oxidation reaction of RuBP that generates 3PG and 2-phosphoglycolate (2PG), which is subsequently dephosphorylated into glycolate (GL) by phosphoglycolate phosphatase (PGPase) (3). The GL is traditionally thought to be recycled into the CBB cycle via photorespiration (4). However, recent studies proposed that the photorespiratory cycle will interact with many other pathways (5).

GL is the simplest organic acid that can be polymerized. GL-based polymers, such as polyglycolide, are known as a hydrolytically degradable polymer material (6). In 2011, GL was found to be incorporated into the bacterial polyester polyhydroxyalkanoates (PHAs) in the engineered *Escherichia coli* platform expressing the evolved PHA synthases with unusual activity toward 2-hydroxyacyl-CoAs (7). The artificial GL-based PHA, poly(GL-*co*-3-hydroxybutyrate) [P(GL-*co*-3HB)], exhibited higher hydrolytic degradability compared to its corresponding natural homopolymer P(3HB). In addition, P(GL-*co*-3HB) film possessed flexible and stretchy properties in contrast to rigid polyglycolide and P(3HB) (8). The incorporation of GL units decreased melting temperature of the polymer (8). Therefore, P(GL-*co*-3HB) has a potential to be used in wider range of applications.

In the GL-based PHA synthetic system, a key enzyme is the engineered PHA synthase that is a class II PHA synthase from *Pseudomonas* sp. 61-3 with a pairwise mutation S325T/Q481K (PhaC1$_{Ps}$STQK) (9,10). PhaC1$_{Ps}$STQK possesses extremely broad substrate specificity toward short-chain-length and medium-chain-length 3-hydroxyacyl-CoAs, and various 2-hydroxyacyl-CoAs, such as lactate, GL, 2-hydroxybutyrate, 2-hydroxy-4-methylvalerate (11–13). PhaC1$_{Ps}$STQK is also referred as lactate-polymerizing enzyme (LPE). As for the supply of monomer substrate glycolyl-CoA (GL-CoA) for PhaC1$_{Ps}$STQK, propionyl-CoA transferase (PCT) was useful (7). GL units in the polymer were derived from exogenous GL supplemented in the medium. In fact, this GL-based production system allowed us to investigate the polymer products in detail (8). In addition, it was demonstrated

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that GL can be supplied via de novo pathways. For example, the expression of glyoxylate reductase in *E. coli* resulted in the GL-containing PHA production from glucose (14).

Here we focused on the new de novo route to generate GL that can be directly channeled into the synthesis of GL-based polymers. In our design, GL as the CBB cycle product in photosynthetic system would serve as a monomer component of the polymer material. Toward the proof-of-concept, in this study, we attempted to utilize RuBisCO for de novo synthesis of P(GL-*co*-3HB) in *E. coli* (Fig. 1). Several combinations of RuBisCO and relevant enzymes were investigated for efficient polymer production. For the first time, the RuBisCO-mediated pathway served as a monomer-supplying route for production of GL-based PHA.

MATERIALS AND METHODS

Gene cloning and vector constructions The ribulose-1,5-bisphosphate carboxylase/oxygenase genes including large and small subunits from *Synechococcus elongatus* PCC7942 ($RuBisCO_{7942}$) and from *Synechocystis* sp. PCC6803 ($RuBisCO_{6803}$) were amplified from genomic DNA of each strain using two pairs of primers, 5′-GGGG**ACAACTTTGTATACAAAAGTTGTA**GACATATCTCTAGGGAGAGA-3′ and 5′-GGGG**ACAACTTTGTATAGAAAAGTTGGGTT**TTAGTAGCGGCCGGGACG-3′, and 5′-GGGG**ACAACTTTGTATACAAAAGTTGTA**GGGAGAAAGCTAGGCTATT-3′ and 5′-GGGG**ACAACTTTGTATAGAAAAGTTGGGTT**TTAGTAACGGCCTTGGTT-3′, respectively. The bold letters indicate the Gateway recognition sequence. The obtained gene fragments were integrated into pDONR221 using Gateway technology. The resulted plasmids were digested at *Apa*I and *Pst*I sites of pDONR221 and each fragment was inserted into pBBR1-MCS5 to yield $pBB5RuBisCO_{7942}$ and $pBB5RuBisCO_{6803}$, respectively. The phosphoribulokinase gene from *S. elongatus* PCC7942 (PRK_{7942}) was amplified using a pair of primers, 3′-CCGGCCGCTACTAACTGCAGCTTAAGGAGGGATCCTATG-5′ and 3′-TGGCGGCCGC TCTAGACTAGACGCTAGCGGCGAC-5′, and inserted into $pBB5RuBisCO_{7942}$ using In-Fusion HD Cloning Kit (Takara) to yield $pBB5RuBisCO_{7942}PRK_{7942}$. $pBB5PRK_{7942}$, which bore the PRK_{7942} gene only, was constructed by removing the $RuBisCO_{7942}$ fragment from $pBB5RuBisCO_{7942}PRK_{7942}$. The 2PG phosphatase gene from *S. elongatus* PCC7942 (PGP_{7942}) was amplified using a pair of primers 5′-TAGCGTCTAGTCTAGAAGTAGGGGCTGTGGAAAAT-3′ and 5′-TATAGGGCGAATTG GAGCTCCTACTGTCGCATCAGTTGC-3′, and the genomic DNA as a template. The obtained fragment was inserted into $pBB5RuBisCO_{7942}PRK_{7942}$ using In-Fusion to yield $pBB5RuBisCO_{7942}PRK_{7942}PGP_{7942}$. The $RuBisCO_{Rr}$ and 2PG phosphatase (PGP_{Rr}) genes from *Rhodosprium rubrum*, which were optimized with the codon-usage for the expression in *E. coli*, were obtained by gene synthesis (Eurofins). The $RuBisCO_{7942}$ gene in $pBB5RuBisCO_{7942}PRK_{7942}PGP_{7942}$ was replaced with the *Apa*I/*Afl*II fragments of $RuBisCO_{Rr}$ gene to yield $pBB5RuBisCO_{Rr}PRK_{7942}PGP_{7942}$. Subsequently, the *Xba*I/*Sac*I fragment of PGP_{Rr} gene replaced the PGP_{7942} gene to yield $pBB5RuBisCO_{Rr}PRK_{7942}PGP_{Rr}$. The 2PG phosphatase ($PGP_{6803}$, *cbbZp*) gene from *Synechocystis* sp. PCC6803 were amplified using a pair of primers, 5′-CTCTGTGTAAACTAGTGAAGGACTGACCAACTATTT-3′ and 5′-TATAGGGCCAAT TGGAGCTCCTAGGATTTTAATGGATACCA-3′, and the genomic DNA as a template. 2PG phosphatase from *E. coli* (PGP_{Ec}) was amplified using a pair of primers, 5′-CCCTCTAGACAAAGGTAAGTCATGAATAAGTTTGAAGAT-3′ and 5′-CCCGGATCCCC AGCCAGGACAGAAATGCCTCG-3′, and ASKA clone plasmid (*gph*, JW3348) as a template. The *Spe*I/*Sac*I fragment of PGP_{6803} and the *Xba*I/*Sac*I fragment of PGP_{Ec} replaced the PGP_{7942} gene in $pBB5RuBisCO_{Rr}PRK_{7942}PGP_{7942}$ to yield $pBB5RuBisCO_{Rr}PRK_{7942}PGP_{6803}$ and $pBB5RuBisCO_{Rr}PRK_{7942}PGP_{Ec}$, respectively. The $RuBisCO_{Rr}$ gene in these pBBR1MCS5-derived vectors was replaced with the *Apa*I/*Afl*II fragments of *pct* gene to yield $pBB5pctPRK_{7942}PGP_{7942}$, $pBB5pctPRK_{7942}PGP_{Rr}$, $pBB5pctPRK_{7942}PGP_{6803}$ and $pBB5pctPRK_{7942}PGP_{Ec}$, respectively. The *Sal*I/*Eco*RI fragment of $RuBisCO_{Rr}$ gene was inserted into *Sal*I/*Eco*RI sites of pUC19 to yield $pUCRuBisCO_{Rr}$. The $RuBisCO_{Rr}$ gene was inserted into the *Sma*I site of pTV118N*pct*C1STQKAB via T4 blunting to yield pTV118N*pct*$RuBisCO_{Rr}$*C1STQKAB*. The $RuBisCO_{7942}$ and $RuBisCO_{6803}$ genes replaced $RuBisCO_{Rr}$ to yield pTV118N*pct*$RuBisCO_{7942}$*C1STQKAB* and pTV118N*pct*$RuBisCO_{6803}$*C1STQKAB*, respectively.

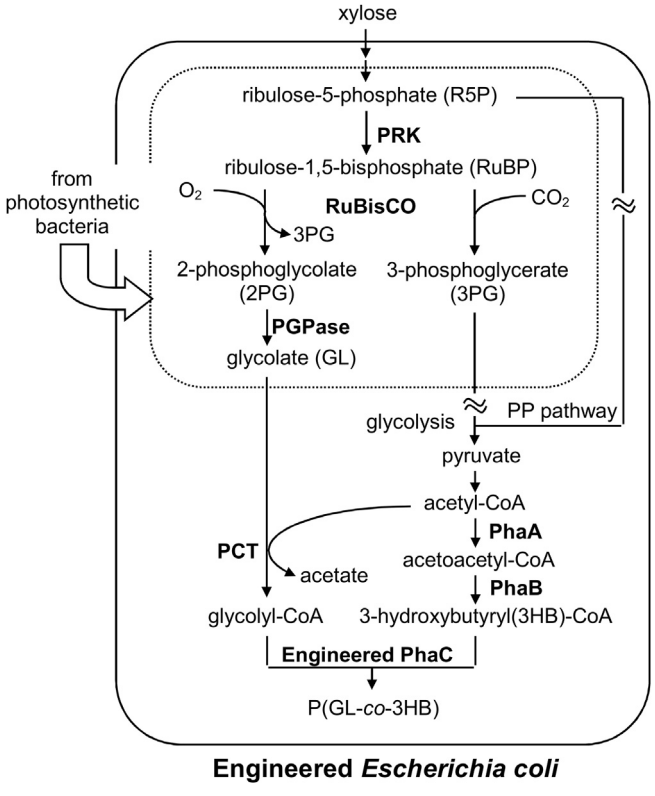

FIG. 1. Designed pathway for GL-based PHA synthesis from xylose via RuBisCO-mediated pathway in *E. coli*. PRK, phosphoribulokinase; PGPase, 2-phosphoglycolate phosphatase; PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PCT, propionyl-CoA transferase; Engineered PhaC, lactate-polymerizing enzyme (LPE, $PhaC1_{Ps}$STQK).

Strain and culture conditions The glycolate oxidase knockout mutant *E. coli* JW2946 (Δ*glcD*) (Keio collection) was used as the host strain. Preculture was prepared using 1.5 mL LB medium at 30°C for 24 h with reciprocal shaking of 180 rpm. The cells were entirely transferred to the main culture of 1.5 mL M9 medium supplemented with 20 g/L xylose, 1 mM IPTG and antibiotics when needed. The concentrations of antibiotics were 100 μg/mL ampicillin and 20 μg/mL gentamycin. The culture in the test tube was cultivated at 30°C for 48 h with reciprocal shaking of 180 rpm. One milliliter of aliquot was lyophilized to determine cell dry weight. For large scale polymer production, the cells were cultivated in 100 mL media in the 500 mL Sakaguchi flask at 30 °C for 48 h with reciprocal shaking of 120 rpm using the same preculture and culture media.

Intermediate analysis of RuBP and GL using liquid chromatography-mass spectrometry For intracellular intermediate analysis, the cells in two test tubes were cultivated for 18 h. Then, the cultures were combined to obtain 3 mL culture medium. The cells in 1.5 mL aliquot was harvested and re-suspended in 100 μL chilled water. The suspension was immediately combined with 500 μL chilled acetonitrile containing 0.1 M formic acid. The mixture in microtube was treated with sonication for 1 min × 7 times using a cup horn unit (CH-63, Tomy, Japan) with ice-cold water, then centrifuge at 4°C and 13,000 *g* for 10 min. The supernatant was concentrated using a centrifugal evaporator (CC-105, Tomy) and dried sample was dissolved in 400 μL ultrapure water. The sample was passed through polytetrafluoroethylene filter (0.2 μm pore size) and the filtrated fraction was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS analysis was performed using an LCMS-8030 (Shimadzu) equipped with a ZIC-cHILIC column (3 μm, 100 Å, PEEK 150 × 2.1 mm, Merck Millipore), electrospray ionization (ESI) and triple quadrupole mass spectroscopy with collision-induced dissociation and multiple reaction monitoring (MRM). GL was detected using the single ion mode. Argon was used as a collision gas. The ESI voltage was 3.5 kV in the negative mode. Nitrogen was used as a nebulizer (3.0 mL/min) and drying gas (15.0 mL/min). Carrier A: 10 mM ammonium acetate (pH 6.8) and carrier B: acetonitrile were used with a flow rate of 0.2 mL/min in gradient mode as follows: 0 min, 80% B; 1 min, 80% B; 14 min, 48% B; 21 min, 48% B; 21 min, 80% B; 30 min, 80% B. The column oven was set at 25°C. The injection volume was 5 μL. $[M-H]^-$ ions were selected as precursor ions and an abundant and specific product ion was chosen to quantify the metabolites. Ribulose-5-phosphate (Ru5P) [m/z = 229 → 97, retention time (rt): 9.8 min, collision energy (ce): 12 V], ribulose-1,5-bisphosphate (RuBP) (m/z = 309 → 97, rt: 10.0 min, ce: 19 V); glycolate (GL) (m/z = 75, rt: 6.8 min) were monitored. Standard Ru5P and RuBP were purchased from Sigma–Aldrich. GL was purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). Intermediate level was normalized based on cell dry weight.

Immunodetection of RuBisCO Cells in 5 mL culture were harvested at 24 h and disrupted in 4 mL of lysis buffer (50 mM sodium phosphate buffer pH 8; 300 mM sodium chloride; 10 mM imidazole) by sonication. The protein concentration was measured by the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) and appropriate amount of the crude proteins were applied to SDS-PAGE analysis (Bio-Rad). The proteins were transferred onto a polyvinylidene difluoride membrane. RuBisCO from *R. rubrum* was immunolabeled using a rabbit anti-

RuBisCO antibody developed using the peptide CGDADQIYPGWRKALGVEDT (Sigma Aldrich Japan, Tokyo, Japan), and detected by an Amersham ECL Select Western Blotting Detection Reagents kit (GE Healthcare Life Sciences, Marlborough, MA, USA). The chemiluminescence was visualized using the ChemiDoc XRS+ System (Bio-Rad). RuBisCO expression level was estimated based on the chemiluminescence intensity of the bands using ATTO CS Analyzer 4 ver. 2.3.1 software (ATTO Corp., Tokyo, Japan).

Gas chromatography The monomer composition of synthesized polymer was determined by gas chromatography (GC) analysis as described previously. In brief, the lyophilized cells were treated with sulfuric acid in ethanol/chloroform at 100°C for 120 min to convert polyester into corresponding ethyl esters, which were determined using GC.

Nuclear magnetic resonance The polymer in the lyophilized cells was extracted with chloroform at 60°C for 48 h. The chloroform solution was concentrated with air flow, and the polymer was precipitated by adding excess amount of hexane. The precipitant was dissolved in chloroform and precipitated again using methanol. The purified polymer was dried and used for further analysis. Nuclear magnetic resonance (NMR) spectra were acquired with a JEOL ECS400 spectrometer using tetramethylsilane (δ 0) as standard. Poly(glycolate-*co*-3-hydroxybutyrate): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.27 (d, 3H, J = 6.4), 2.47 (dd, H, J = 16.2 Hz and 6.0 Hz), 2.61 (dd, H, J = 15.6 Hz and 7.2 Hz), 4.60 (s, 2H), 5.26 (m, H).

RESULTS AND DISCUSSION

RuBP synthesis in the engineered *E. coli* The designed pathway for P(GL-*co*-3HB) production is illustrated in Fig. 1. Based on KEGG pathway database (http://www.genome.jp/kegg/), ribulose-5-phosphate (Ru5P) can be supplied from xylose via the pentose phosphate (PP) pathway. To evaluate the function of the pathway, the concentration of Ru5P, RuBP and intracellular/extracellular GL were measured using LC-MS. As the result, the intracellular Ru5P concentration was below the detection limit in *E. coli* harboring the control plasmid. However, the significant amount of RuBP was accumulated in cells of the recombinant *E. coli* harboring the PRK gene from *S. elongatus* PCC7942 (Table 1, No. 2). These results indicate that the metabolic pathway from xylose to RuBP was functional in *E. coli*. *E. coli* JW2946 harboring the control plasmid produced 75 mg/L GL in the medium that should be due to the intrinsic GL synthesis in *E. coli* (15). The introduction of PRK_{7942} alone also slightly increased GL production. Currently the reason of the result was unknown. *E. coli* might possess intrinsic RuBP oxygenase activity generating 2PG.

Effect of RuBisCO and PGPase expression on GL production As the next step, RuBisCO gene was introduced into *E. coli*. The RuBisCO gene from *R. rubrum* ($RuBisCO_{Rr}$) was initially investigated, because the S_{rel} value [(k_{catCO2}/k_{catO2}) (K_{mO2}/K_{mCO2})] of which is reportedly 15, while ones found in cyanobacteria are 35–40 (16). Given the designed the metabolic pathway (Fig. 1), RuBisCO enzyme with relatively low S_{rel} value seemed to be suitable for P(GL-*co*-3HB) production in *E. coli*. The introduction of the $RuBisCO_{Rr}$ gene slightly increased extracellular GL production (Table 1), suggesting that $RuBisCO_{Rr}$ was functionally expressed. However, the immunodetection of $RuBisCO_{Rr}$ revealed that the expression level of the enzyme was very low (Fig. 2A, lane 2). To increase the RuBisCO expression level, the $RuBisCO_{Rr}$ gene was expressed using a high-copy-number plasmid pUC19 (Table 1, No. 4). The cells harboring $pBB5RuBisCO_{Rr}PRK_{7942}$ and $pUCRuBisCO_{Rr}$ exhibited significantly increased expression of $RuBisCO_{Rr}$ (Fig. 2A, lane 3). In addition, intracellular/extracellular concentrations of GL were considerably increased (Table 1). Therefore, RuBisCO activity was a dominant factor determining the efficiency of the constructed pathway. This is consistent with the previous knowledge that RuBisCO is the rate-limiting step in the CBB cycle.

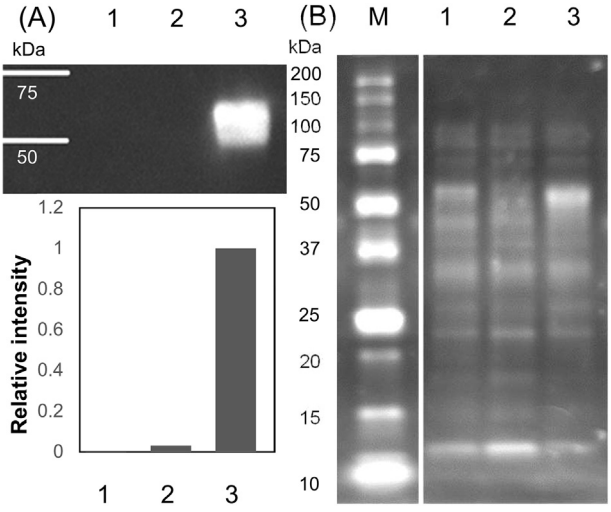

FIG. 2. Immunoblot analysis of $RuBisCO_{Rr}$ in recombinant *E. coli* JW2946 under non-polymer-producing conditions and the relative chemiluminescence intensities of RuBisCO (A). SDS-PAGE image of the same sample (B). Eight microgram of crude protein was loaded in each lane. Lane 1, $pBB5PRK_{7942}$; lane 2, $pBB5RuBisCO_{Rr}PRK_{7942}$; lane 3, $pBB5RuBisCO_{Rr}PRK_{7942}$ + $pUCRuBisCO_{Rr}$; M, size maker. Whole images are shown in Fig. S1.

TABLE 1. Glycolate production by recombinant *E. coli* JW2946 harboring RuBisCO and related genes without polymer biosynthetic genes[a].

No.	Plasmids	CDW (g/L)[b]	Concentration of intracellular intermediates (μmol/g-CDW)[c]		Concentration of extracellular GL (mg/L) [(mmol/g-CDW)]
			RuBP	GL	
1	pBBR1-MCS5	1.9 ± 0.3	ND[d]	8.9 ± 1.4	75 ± 19 (0.3 ± 0.1)
2	$pBB5PRK_{7942}$	1.7 ± 0.2	4.2 ± 1.9	20.1 ± 3.7	120 ± 7 (1.0 ± 0.2)
3	$pBB5RuBisCO_{Rr}PRK_{7942}$	1.7 ± 0.3	2.7 ± 0.7	20.4 ± 3.7	167 ± 56 (1.1 ± 0.4)
4	$pBB5RuBisCO_{Rr}PRK_{7942}$ + $pUCRuBisCO_{Rr}$	2.1 ± 0.1	1.2 ± 0.3	45.8 ± 5.8	405 ± 50 (1.8 ± 0.2)
5	$pBB5RuBisCO_{Rr}PRK_{7942}PGP_{Ec}$ + $pUCRuBisCO_{Rr}$	1.0 ± 0.3	7.3 ± 5.3	22.7 ± 6.4	324 ± 23 (2.1 ± 0.2)
6	$pBB5RuBisCO_{Rr}PRK_{7942}PGP_{6803}$ + $pUCRuBisCO_{Rr}$	2.2 ± 0.3	1.0 ± 0.5	42.1 ± 2.0	275 ± 43 (1.1 ± 0.2)
7	$pBB5RuBisCO_{Rr}PRK_{7942}PGP_{7942}$ + $pUCRuBisCO_{Rr}$	0.9 ± 0.1	3.0 ± 0.7	24.2 ± 2.6	227 ± 103 (1.3 ± 0.8)
8	$pBB5RuBisCO_{Rr}PRK_{7942}PGP_{Rr}$ + $pUCRuBisCO_{Rr}$	1.5 ± 0.2	11.8 ± 2.0	40.1 ± 6.2	290 ± 48 (1.2 ± 0.3)

[a] The cells were grown on M9 medium containing 2% xylose.
[b] CDW, cell dry weight.
[c] RuBP, ribulose-1,5-bisphosphate; GL, glycolate. Ribulose-5-phosphate concentration was below the detection limit all conditions tested.
[d] ND, not detected. Data are mean ± standard deviation of three independent trials.

The introduction of pBB5RuBisCO$_{Rr}$PRK$_{7942}$ increased intracellular/extracellular GL production (Table 1), probably because of the PGPase gene (*gph*) in the *E. coli* genome (17). In fact, the presence of PGPase activity in the *E. coli* crude extract was recently reported (18). In order to investigate whether PGPase is rate limiting in the GL production, the plasmid-encoded PGPase genes from *E. coli*, *S. elongatus* PCC7942, *Synechocystis* sp. PCC6803 and *R. rubrum* were introduced into *E. coli*. Unexpectedly, the introduction of heterogenous PGPase genes, as well as the self-cloning, rather decreased extracellular GL production (Table 1). Based on this result, PGPase seemed to be not a bottleneck of GL production.

P(GL-*co*-3HB) production via RuBisCO-mediated pathway The RuBisCO-mediated pathway was applied to the polymer production. At this stage, the effect of RuBisCOs on the polymer production was also investigated. To this end, the double plasmids of pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ and pTV118N*pct*RuBisCO$_X$*C1STQKAB* were used. The RuBisCO genes were inserted into the high-copy-number pTV118N-derived plasmid to increase its expression level. The *pct* gene was inserted into pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ to reduce the expression level of the PRK$_{7942}$ gene. The PGP$_{Rr}$ gene was used because the introduction of PGPase gene exhibited a positive effect on the polymer production as described below. In the control experiment using pTV118N*pctC1STQKAB*, which does not contain the RuBisCO gene, the cells produced the 33.9 mg/L polymer (Table 2). The cells harboring the RuBisCO$_{Rr}$ gene produced the greater amount of polymer (57.7 mg/L), indicating that the RuBisCO-mediated pathway contributed to the polymer production. The introduction of RuBisCO gene from *S. elongatus* PCC7942 (RuBisCO$_{7942}$) resulted in the similar level of polymer production to the RuBisCO$_{Rr}$ gene. In contrast, the introduction of the RuBisCO gene from *Synechocystis* sp. PCC6803 (RuBisCO$_{6803}$) rather decreased polymer production. The reason for the negative effect is currently unknown. The introduction of the RuBisCO$_{Rr}$ and RuBisCO$_{7942}$ genes increased the amount of GL in the polymer (Table 2), and thus, the RuBisCO pathway served as the GL monomer-supplying route. However, the GL fractions in the polymer was low considering the low CO_2/O_2 specificity of these RubisCOs. The result was partly due to that the CO_2/O_2 relative concentration in the cell was elevated compared to that in atmosphere because of the O_2 consumption and CO_2 generation by *E. coli*. In addition, these RuBisCOs resulted in the similar GL fraction of the polymer. The difference in the catalytic properties of RuBisCO (16) had little effect on the GL fraction, suggesting the presence of other limiting factors in the incorporation of GL units into the polymer.

In order to further increase the expression level of RuBisCO$_{Rr}$, the copy number of the RuBisCO$_{Rr}$ gene was increased by using combination of pBB5RuBisCO$_{Rr}$PRK$_{7942}$PGP$_{Rr}$ and pTV118N*pct*RuBisCO$_{Rr}$*C1STQKAB*. However, the elevated RuBisCO$_{Rr}$ expression (Fig. S2) led to no increase in polymer production (Table 2, No. 2 and Table 3, No. 5), suggesting the presence of other limiting factors in the metabolic pathway. Next, the effect of PGPase on the polymer production was investigated (Table 3). PGP$_{Rr}$ and particularly PGP$_{6803}$ increased polymer production, and PGP$_{Ec}$ and PGP$_{7942}$ exhibited a negative effect on the cell growth and polymer production. The result was not consistent with the result of the extracellular GL production by non-polymer-producing cells (Table 1). The reason for the inconsistency is currently unknown. Under the polymer-producing conditions, the cells produced no extracellular GL, suggesting that carbon fluxes were changed by introducing the polymer biosynthetic genes. Further study is needed to elucidate this issue. The introduction of PGPase genes did not increase the polymer production when the plasmid did not contain RuBisCO gene (Table S1). Therefore, the positive effect of PGPase on the polymer production was probably due to the accelerated 2PG dephosphorylation. The incorporation of GL units into the polymer chain was confirmed by ^{1}H NMR (Fig. S4). The molecular weight of the polymer synthesized via the RuBisCO pathway was measured. The cells of No. 5 in Table 3 were grown on 100 mL media in a shake flask. The weight-averaged molecular weights of the obtained polymer P(3 mol% GL-*co*-3HB) ($M_w = 6.7 \times 10^5$, Table S2) was slightly higher than that of P(5 mol% GL-*co*-3HB) synthesized using exogenous GL ($M_w = 3.3 \times 10^5$) (8), indicating that the RuBisCO pathway had no negative effect on the molecular weight of polymer. The low GL fraction compared to the test-tube culture was presumably due to the low oxygen-supplying rate under the culture conditions.

It was recently reported that four PGPase homologs (Slr0458, Slr0586, Sll1349 and Slr1762) of *Synechocystis* sp. PCC6803, the homologies of which to PGPase from *S. elongatus* PCC7942 are 22.4%,

TABLE 2. GL-based PHA production in recombinant *E. coli* JW2946 harboring different RuBisCO and relevant genes with polymer biosynthetic genes.[a]

No.	Plasmid	CDW (g/L)	Polymer production (mg/L)			GL composition (mol%)
			Total	GL	3HB	
1	pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ + pTV*pct*C1AB	1.47 ± 0.03	33.9 ± 1.2	4.1 ± 0.2	29.8 ± 1.0	12.1 ± 0.4
2	pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ + pTV*pct*RuBisCO$_{Rr}$C1AB	1.45 ± 0.05	57.7 ± 1.7	8.0 ± 0.2	49.8 ± 1.6	13.8 ± 0.2
3	pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ + pTV*pct*RuBisCO$_{6803}$C1AB	0.86 ± 0.03	13.4 ± 1.1	1.8 ± 0.2	11.7 ± 0.8	13.1 ± 0.6
4	pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ + pTV*pct*RuBisCO$_{7942}$C1AB	1.61 ± 0.04	57.4 ± 0.8	8.7 ± 0.6	48.7 ± 1.1	15.1 ± 1.1

[a] Data are mean ± standard deviation of three independent trials.

TABLE 3. GL-based PHA production in recombinant *E. coli* JW2946 harboring different PGPase genes with relevant genes[a].

No.	Plasmid	CDW (g/L)	Polymer production (mg/L)	Monomer composition (mol%)		Extracellular GL (mg/L)
				GL	3HB	
1	pBB5RuBisCO$_{Rr}$PRK$_{7942}$	0.76 ± 0.07	42.6 ± 1.5	Trace	100	ND[b]
2	pBB5RuBisCO$_{Rr}$PRK$_{7942}$PGP$_{Ec}$	0.47 ± 0.02	44.1 ± 5.0	10.6 ± 0.5	89.4 ± 0.5	ND
3	pBB5RuBisCO$_{Rr}$PRK$_{7942}$PGP$_{6803}$	1.33 ± 0.01	67.7 ± 1.6	7.8 ± 0.1	92.2 ± 0.1	ND
4	pBB5RuBisCO$_{Rr}$PRK$_{7942}$PGP$_{7942}$	0.35 ± 0.04	24.4 ± 3.2	Trace	100	ND
5	pBB5RuBisCO$_{Rr}$PRK$_{7942}$PGP$_{Rr}$	0.88 ± 0.01	51.2 ± 0.5	11.4 ± 0.4	88.9 ± 0.4	ND

[a] All cells harbor pTV118N*pct*RuBisCO$_{Rr}$C1STQKAB.
[b] ND, not detected. Data are mean ± standard deviation of three independent trials.

12.5%, 34.9% and 28.1%, respectively, were functionally expressed in *E. coli* and exhibited PGPase activity (18). The result agreed with our result that the expression of PGP_{6803} (Sll1349), which was chosen for the highest homology to PGP_{7942} (CbbZ), increased the polymer production in *E. coli* (Table 2). Based on the phylogenetic analysis by Rai et al, however, the Sll1349 clade is rather distantly related to the CbbZ clade than other homologs (18). Currently the substrate specificity of these PGPase homologs and their physiological roles are not elucidated. The other homologs (Slr0458, Slr0586 and Slr1762) also might be useful for GL-based PHA production.

E. coli JW2946 harboring the control plasmid produced small amount of GL, indicating the presence of intrinsic GL-synthesizing pathway. However, there is no 2PG-generating pathway in *E. coli* based on the KEGG pathway database (http://www.genome.jp/kegg/). Pellicer et al. (15) proposed that *E. coli* PGPase is involved in DNA repair and detected 2PG in *E. coli* cells treated with bleomycin, which induces DNA damage. In the present study, the intracellular GL was detectable without bleomycin, probably because of the deletion of the *glcD* gene. Further study is needed to clarify this problem.

This study aimed at utilizing the RuBisCO-mediated pathway for GL-based PHA production. To this end, the part of the CBB cycle was transferred into *E. coli* and co-expressed with the engineered PHA biosynthetic pathway. The results presented here demonstrated the proof-of-concept that GL generated by RuBisCO-mediated pathway can be utilized for P(GL-*co*-3HB) synthesis in *E. coli* and potentially in autotrophic platforms. The phosphoribulokinase and RuBisCO from cyanobacteria were functionally expressed in *E. coli* in the previous study (19), but the GL-generating pathway has not been utilized. In addition, it should be noted that the RuBisCO-mediated PHA production pathway could serve as an *in vivo* monitoring system of RuBisCO activity. Improving the catalytic properties of RuBisCO by evolutionary engineering and exploring wild enzymes with better properties have been recognized as important research targets (19,20). The *in vivo* monitoring system may provide a rapid and high-throughput assay method of RuBisCO activity compared to the *in vitro* analysis.

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