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

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Short title: Glycolate-based PHA production using RuBisCO pathway

ABSTRACT

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* and but also in photosynthetic organisms.

Keywords; polyhydroxybutyrate; polyglycolic acid; carbon fixation; biodegradable plastic; lactate-polymerizing enzyme

INTRODUCTION

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(3PG) via carboxylation. 3PG is subsequently converted into glyceraldehyde-3-phosphate (GAP), which is withdrawn for the central metabolic pathway.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

55 be recycled into the CBB cycle via photorespiration (4). However, recent studies proposed
56 that the photorespiratory cycle will interact with many other pathways (5).

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

68 In the GL-based PHA synthetic system, a key enzyme is the engineered PHA synthase
69 that is a class II PHA synthase from *Pseudomonas* sp. 61-3 with a pairwise mutation
70 S325T/Q481K (PhaC1$_{Ps}$STQK) (9, 10). PhaC1$_{Ps}$STQK possesses extremely broad
71 substrate specificity toward short-chain-length and medium-chain-length 3-hydroxyacyl-
72 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 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 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 *Escherichia 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′-TGGCGGCCGCTCTAGACTAGACGCTAGCGGCGAC-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′-TATAGGGCGAATTGGAGCTCCTACTGTCGCATCAGTTGC-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′-TATAGGGCCAATTGGAGCTCCTAGGATTTTAATGGATACCA -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′-CCCGGATCCCCAGCCAGGACAGAAATGCCTCG-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 pBB5*pct*PRK$_{7942}$PGP$_{7942}$, pBB5*pct*PRK$_{7942}$PGP$_{Rr}$, pBB5*pct*PRK$_{7942}$PGP$_{6803}$ and pBB5*pct*PRK$_{7942}$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*pctC1STQKAB* 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.

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 using the same preculture and culture conditions.

Intermediate analysis of RuBP and GL using LC-MS 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 13000 *g* for 10 min. The supernatant was concentrated using a centrifugal evaporator (CC-105, Tomy, Japan) 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 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. (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., CA USA) and appropriate amount of the crude proteins were applied to SDS-PAGE analysis (Bio-Rad). The proteins were transferred onto a PVDF membrane. RuBisCO from *R. rubrum* was immunolabeled using a rabbit anti-RuBisCO antibody developed using the peptide CGDADQIYPGWRKALGVEDT (SIGMA Aldrich Japan), and detected by an AmershamTM ECL™ Select Western Blotting Detection Reagents kit (GE Healthcare Life Sciences, 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., Japan).

Gas chromatography (GC) The monomer composition of synthesized polymer was determined by 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.

NMR 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. NMR spectra were acquired with a JEOL ECS400 spectrometer using TMS (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

217 accumulated in cells of the recombinant *E. coli* harboring the PRK gene from
218 *Synechococcus elongatus* PCC7942 (Table 1, No. 2). These results indicate that the
219 metabolic pathway from xylose to RuBP was functional in *E. coli*. *E. coli* JW2946
220 harboring the control plasmid produced 75 mg/L GL in the medium that should be due to
221 the intrinsic GL synthesis in *E. coli* (15). The introduction of PRK_{7942} alone also slightly
222 increased GL production. Currently the reason of the result was unknown. *E. coli* might
223 possess intrinsic RuBP oxygenase activity generating 2PG.
224
225 **Effect of RuBisCO and PGPase expression on GL production** As the next step,
226 RuBisCO gene was introduced into *E. coli*. The RuBisCO gene from *Rhodosprium*
227 *rubrum* ($RuBisCO_{Rr}$) was initially investigated, because the S_{rel} value
228 $[(k_{catCO_2}/k_{catO_2})(K_{mO_2}/K_{mCO_2})]$ of which is reportedly 15, while ones found in cyanobacteria
229 are 35-40 (16). Given the designed the metabolic pathway (Fig. 1), RuBisCO enzyme
230 with relatively low S_{rel} value seemed to be suitable for P(GL-*co*-3HB) production in *E.*
231 *coli*. The introduction of the $RuBisCO_{Rr}$ gene slightly increased extracellular GL
232 production (Table 1), suggesting that $RuBisCO_{Rr}$ was functionally expressed. However,
233 the immunodetection of $RuBisCO_{Rr}$ revealed that the expression level of the enzyme was
234 very low (Fig. 2A, Lane 2). To increase the RuBisCO expression level, the $RuBisCO_{Rr}$

235 gene was expressed using a high-copy-number plasmid pUC19 (Table 1, No. 4). The cells
236 harboring pBB5RuBisCO$_{Rr}$PRK$_{7942}$ and pUCRuBisCO$_{Rr}$ exhibited significantly
237 increased expression of RuBisCO$_{Rr}$ (Fig. 2A, Lane 3). In addition,
238 intracellular/extracellular concentrations of GL were considerably increased (Table 1).
239 Therefore, RuBisCO activity was a dominant factor determining the efficiency of the
240 constructed pathway. This is consistent with the previous knowledge that RuBisCO is the
241 rate-limiting step in the CBB cycle.

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

251

252 **P(GL-*co*-3HB) production via RuBisCO-mediated pathway** The RuBisCO-

253 mediated pathway was applied to the polymer production. At this stage, the effect of
254 RuBisCOs on the polymer production was also investigated. To this end, the double
255 plasmids of pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ and pTV118N*pct*RuBisCO$_X$*C1STQKAB* were used.
256 The RuBisCO genes were inserted into the high-copy-number pTV118N-derived plasmid
257 to increase its expression level. The *pct* gene was inserted into pBB5*pct*PRK$_{7942}$PGP$_{Rr}$ to
258 reduce the expression level of the PRK$_{7942}$ gene. The PGP$_{Rr}$ gene was used because the
259 introduction of PGPase gene exhibited a positive effect on the polymer production as
260 described below. In the control experiment using pTV118N*pctC1STQKAB*, which does
261 not contain the RuBisCO gene, the cells produced the 33.9 mg/L polymer (Table 2). The
262 cells harboring the RuBisCO$_{Rr}$ gene produced the greater amount of polymer (57.7 mg/L),
263 indicating that the RuBisCO-mediated pathway contributed to the polymer production.
264 The introduction of RuBisCO gene from *S. elongatus* PCC 7942 (RuBisCO$_{7942}$) resulted
265 in the similar level of polymer production to the RuBisCO$_{Rr}$ gene. In contrast, the
266 introduction of the RuBisCO gene from *Synechocystis* sp. PCC6803 (RuBisCO$_{6803}$) rather
267 decreased polymer production. The reason for the negative effect is currently unknown.
268 The introduction of the RuBisCO$_{Rr}$ and RuBisCO$_{7942}$ genes increased the amount of GL
269 in the polymer (Table 2), and thus, the RuBisCO pathway served as the GL monomer-
270 supplying route. However, the GL fractions in the polymer was low considering the low

271 CO_2/O_2 specificity of these RubisCOs. The result was partly due to that the CO_2/O_2
272 relative concentration in the cell was elevated compared to that in atmosphere because of
273 the O_2 consumption and CO_2 generation by *E. coli*. In addition, these RuBisCOs resulted
274 in the similar GL fraction of the polymer. The difference in the catalytic properties of
275 RuBisCO (16) had little effect on the GL fraction, suggesting the presence of other
276 limiting factors in the incorporation of GL units into the polymer.

277 In order to further increase the expression level of $RuBisCO_{Rr}$, the copy number of
278 the $RuBisCO_{Rr}$ gene was increased by using combination of
279 pBB5$RuBisCO_{Rr}PRK_{7942}PGP_{Rr}$ and pTV118N*pct*$RuBisCO_{Rr}$*C1STQKAB*. However, the
280 elevated $RuBisCO_{Rr}$ expression (Fig. S2) led to no increase in polymer production (Table
281 2, No. 2 and Table 3, No. 5), suggesting the presence of other limiting factor in the
282 metabolic pathway. Next, the effect of PGPase on the polymer production was
283 investigated (Table 3). PGP_{Rr} and particularly PGP_{6803} increased polymer production, and
284 PGP_{Ec} and PGP_{7942} exhibited a negative effect on the cell growth and polymer production.
285 The result was not consistent with the result of the extracellular GL production by non-
286 polymer-producing cells (Table 1). The reason for the inconsistency is currently unknown.
287 Under the polymer-producing conditions, the cells produced no extracellular GL,
288 suggesting that carbon fluxes were changed by introducing the polymer biosynthetic

289 genes. Further study is needed to elucidate this issue. The introduction of PGPase genes
290 did not increase the polymer production when the plasmid did not contain RuBisCO gene
291 (Table S1). Therefore, the positive effect of PGPase on the polymer production was
292 probably due to the accelerated 2PG dephosphorylation. The incorporation of GL units
293 into the polymer chain was confirmed by ^{1}H NMR (Fig. S4). The molecular weight of the
294 polymer synthesized via the RuBisCO pathway was measured. The cells of No. 5 in Table
295 3 were grown on 100 mL media in a shake flask. The weight-averaged molecular weights
296 of the obtained polymer P(3 mol% GL-*co*-3HB) (M_w = 6.7×10^5, Table S2) was slightly
297 higher than that of P(5 mol% GL-*co*-3HB) synthesized using exogenous GL (M_w =
298 3.3×10^5) (8), indicating that the RuBisCO pathway had no negative effect on the
299 molecular weight of polymer. The low GL fraction compared to the test-tube culture was
300 presumably due to the low oxygen-supplying rate under the culture conditions.
301 It was recently reported that four PGPase homologs (Slr0458, Slr0586, Sll1349 and
302 Slr1762) of *Synechocystis* sp. PCC6803, the homologies of which to PGPase from *S.*
303 *elongatus* PCC7942 are 22.4%, 12.5%, 34.9% and 28.1%, respectively, were functionally
304 expressed in *E. coli* and exhibited PGPase activity (18). The result agreed with our result
305 that the expression of Sll1349 (PGP_{6803}), which was chosen for the highest homology to
306 PGP_{7942} (CbbZ), increased the polymer production in *E. coli* (Table 2). Based on the

307 phylogenetic analysis by Rai *et al*, however, the Sll1349 clade is rather distantly related
308 to the CbbZ clade (18). Currently the substrate specificity of these PGPase homologs and
309 their physiological roles are not elucidated. The other homologs (Slr0458, Slr0586 and
310 Slr1762) also might be useful for GL-based PHA production.

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

319 This study aimed at utilizing the RuBisCO-mediated pathway for GL-based PHA
320 production. To this end, the part of the CBB cycle was transferred into *E. coli* and co-
321 expressed with the engineered PHA biosynthetic pathway. The results presented here
322 demonstrated the proof-of-concept that GL generated by RuBisCO-mediated pathway can
323 be utilized for P(GL-*co*-3HB) synthesis in *E. coli* and potentially in autotrophic platforms.
324 The phosphoribulokinase and RuBisCO from cyanobacteria were functionally expressed

325 in *E. coli* in the previous study (19), but the GL-generating pathway has not been utilized.
326 In addition, it should be noted that the RuBisCO-mediated PHA production pathway
327 could serve as an *in vivo* monitoring system of RuBisCO activity. Improving the catalytic
328 properties of RuBisCO by evolutionary engineering and exploring wild enzymes with
329 better properties have been recognized as important research targets (19, 20). The *in vivo*
330 monitoring system may provide a rapid and high-throughput assay method of RuBisCO
331 activity compared to the *in vitro* analysis.
332
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Figure legends

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).

408 FIG. 2. Immunoblot analysis of $RuBisCO_{Rr}$ in recombinant *E. coli* JW2946 under non-
409 polymer-producing conditions and the relative chemiluminescence intensities of
410 RuBisCO (A). SDS-PAGE image of the same sample (B). Eight microgram of crude
411 protein was loaded in each lane. Lane 1, $pBB5PRK_{7942}$; Lane 2,
412 $pBB5RuBisCO_{Rr}PRK_{7942}$; Lane 3, $pBB5RuBisCO_{Rr}PRK_{7942}$ + $pUCRuBisCO_{Rr}$; M, Size
413 maker. Whole images are shown in Fig. S1.
414

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

No.	Plasmids	CDW (g/L)	Concentration of intracellular intermediates (μmol/g-CDW) [b]		Concentration of extracellular GL (mg/L) [(mmol/g-CDW)] [c]
			RuBP	GL	
1	pBBR1-MCS5	1.9 ± 0.3	ND	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] RuBP: ribulose-1,5-bisphosphate, GL: glycolate. [c] GL concentration in culture supernatant. ND, not detected. Ribulose-5-phosphate (Ru5P) concentration was below the detection limit all conditions tested. Data are mean ± standard deviation of three indepenent trials. CDW, cell dry weight.

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.

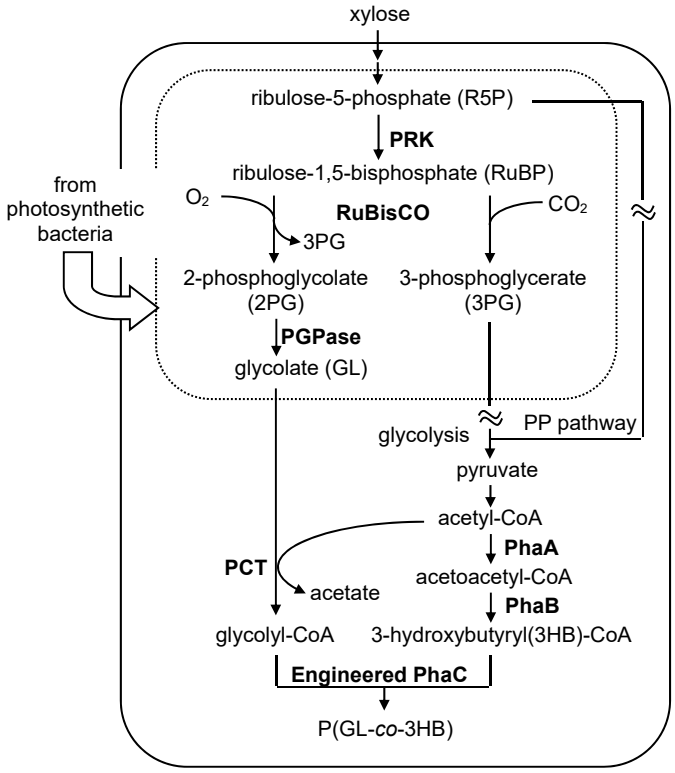
xylose
ribulose-5-phosphate (R5P)
PRK
ribulose-1,5-bisphosphate (RuBP)
from photosynthetic bacteria
O_2
RuBisCO
CO_2
3PG
2-phosphoglycolate (2PG)
3-phosphoglycerate (3PG)
PGPase
glycolate (GL)
glycolysis
PP pathway
pyruvate
acetyl-CoA
PhaA
PCT
acetoacetyl-CoA
acetate
PhaB
glycolyl-CoA
3-hydroxybutyryl(3HB)-CoA
Engineered PhaC
P(GL-co-3HB)
Engineered Escherichia coli

FIG. 1.

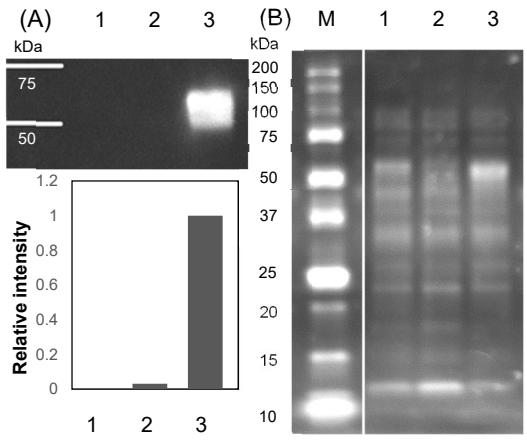
(A)
1
2
3
kDa
75
50
1.2
1
0.8
0.6
0.4
0.2
0
Relative intensity
1
2
3
(B)
M
1
2
3
kDa
200
150
100
75
50
37
25
20
15
10

FIG. 2.