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Dual synthetic pathway for 3-hydroxypropionic acid production in engineered *Escherichia coli*

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3-Hydroxypropionic acid (3-HP) is an important platform C3 chemical; production of 3-HP in recombinant *Escherichia coli* by synthetic pathways has been the focus of a lot of research. When glycerol is used as a substrate to produce 3-HP in *E. coli*, only the ALDH pathway (employing aldehyde dehydrogenase (ALDH) for conversion of 3-hydroxypropionaldehyde (3-HPA) into 3-HP) has been utilized as a synthetic pathway. However, several bacteria (including *Klebsiella pneumoniae*) are known to have the ability to produce 3-HP by the Pdu pathway (employing the PduP, PduL, and PduW enzymes). Here, we report the production of 3-HP in *E. coli* by using the Pdu pathway from *K. pneumoniae* as a synthetic pathway. Moreover, a strain harboring a dual synthetic pathways (ALDH and Pdu) exhibited a 70% increase in 3-HP titer compared to one harboring the ALDH pathway alone (56.1 ± 0.736 mM and 33.1 ± 0.920 mM, respectively). To our knowledge, this is the first report of 3-HP production by *E. coli* harboring the Pdu pathway, with the dual synthetic pathway showing the highest yield ever reported by batch culture [54.1% (mol/mol)].

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[**Key words:** 3-Hydroxypropionic acid; Dual synthetic pathway; *Escherichia coli*; Metabolic engineering; Synthetic biology]

Limited amounts of fossil fuel and the expansion of energy demand have led to research on producing useful materials from biomass. Synthetic pathways, that is, exogenous pathways consisting of genes from many types of organisms, are getting important for this purpose (1). Many studies have demonstrated the use of *Escherichia coli*, a model bacterium, to produce commodity chemicals such as 2-propanol (143 g/L) (2), 1-butanol (552 mg/L) (3), 3-methyl-1-butanol (1.28 g/L) (4), styrene (260 mg/L) (5), isoprene (314 mg/L) (6), polylactic acid (7), and alka(e)nes (8).

3-Hydroxypropionic acid (3-HP) is one of the most valuable platform chemicals; the compound is a precursor of acrylic acid, a raw material used for paint and detergent. Acrylic acid is currently refined from fossil fuel-derived substances such as naphtha and liquefied petroleum gas. In 2004, the United States Department of Energy selected 12 bio-based building block (platform) chemicals that were able to be produced from sugars and to be converted to many types of high valuable chemicals or materials; 3-HP was one of these 12 chemical compounds (9). Glycerol is also one of these 12 chemical compounds, but glycerol is generated as a byproduct in the manufacturing of biodiesel, which causes oversupply of glycerol (10,11). Thus, technologies for production of 3-HP from glycerol are highly attractive.

Several bacterial species, including *Lactobacillus reuteri*, *Klebsiella pneumoniae*, and *Chloroflexus aurantiacus*, are natural producers of 3-HP (12–15). Among these bacteria, *K. pneumoniae* has been well studied for 3-HP production, because this bacterium can produce 3-HP from glycerol (16,17). Not only the natural 3-HP producers, but also engineered *E. coli* with a synthetic pathway for 3-HP production has been employed. (18–24). In this *E. coli* strain, 3-hydroxypropionaldehyde (3-HPA) is generated from glycerol by vitamin B_{12}-dependent glycerol dehydratase (DhaB consisting of three subunits) and its reactivator (GDR consisting of two subunits) derived from *K. pneumoniae*. The resulting 3-HPA then is converted into 3-HP by aldehyde dehydrogenase (ALDH) (Fig. 1). Hereafter, we refer to this synthetic pathway for 3-HP production (consisting of DhaB, GDR, and ALDH) as the ALDH pathway.

In several other bacteria such as *K. pneumoniae* and *L. reuteri*, a pathway composed of the products of the *pdu* genes (*pduP*, *pduL*, and *pduW*) has been shown to convert 3-HPA into 3-HP. These three genes are located as parts of *pdu* operon (25). *pduP*, *pduL*, and *pduW* encode CoA-dependent propionaldehyde dehydrogenase, phosphate propanoyltransferase, and propionate kinase, respectively. In the 3-HP production pathway from 3-HPA using these enzymes, 3-hydroxypropionyl-CoA (3-HP-CoA) and 3-hydroxypropionyl phosphate (3-HP-P) are produced as intermediates (26,27), and a molecule of NADH and ATP are also generated. Hereafter, we refer to this pathway for 3-HP production (consisting of DhaB, GDR, PduP, PduL, and PduW) as the Pdu pathway. Although there are few investigations of the Pdu pathway for 3-HP production, deletion of the *pduP* gene in *K. pneumoniae* has been reported to result in the accumulation of 3-HPA along with a 2-fold decrease in 3-HP titer (28). This result suggests that the Pdu pathway has an important role in 3-HP production in *K. pneumoniae*. Despite the large contribution of the Pdu pathway to 3-HP production in *K. pneumoniae*, introduction of the Pdu pathway into *E. coli* as a synthetic pathway has not been reported in the literature. Here, we

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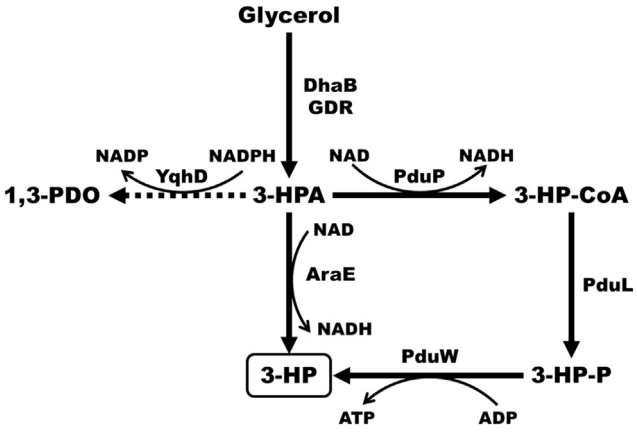

FIG. 1. Metabolic pathways for 3-HP production. The solid lines indicate synthetic pathways. The dashed line indicates an endogenous pathway in *E. coli*. AraE, a type of aldehyde dehydrogenase (ALDH), was provided as α-ketoglutaric semialdehyde dehydrogenase, and was encoded by the *Azospirillum brasilense araE* locus (23). 1,3-PDO means 1,3-propanediol.

report on 3-HP production by the engineered *E. coli* with the *K. pneumoniae* Pdu synthetic pathway. We also demonstrated improvement of the 3-HP productivity by constructing a novel dual synthetic pathway consisting of both the ALDH and Pdu pathways (Fig. 1).

MATERIALS AND METHODS

Chemicals and reagents Unless otherwise specified, all chemicals were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Cloning was performed using commercially available restriction enzymes and phosphatase (New England Biolabs, Ipswich, MA, USA), ligase (Rapid DNA Dephos & Ligation Kit, Roche, Manheim, Germany), and DNA polymerase (KOD-Plus-Neo, Toyobo Co., Ltd., Osaka, Japan). Oligonucleotides were synthesized by Life Technologies Japan, Ltd. (Tokyo, Japan). 3-HP for use as a standard for quantification analysis was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Plasmid and strain construction Table 1 shows strains and plasmids used in this study. *E. coli* strain B (ATCC11303) with *lacI*^{q} introduced from *E. coli* DH5αZ1 (29) by P1 transduction was used as the host strain and designated as TA212. For plasmid construction, we used pZ plasmids created by Lutz and Bujard (29). The same ribosome binding site (RBS) (GAAGGAGATATACAT), derived from pET31b(+) (Invitrogen, Carlsbad, CA, USA), was used for all genes encoding the 3-HP production enzymes, with the exception of *dhaB1* and *pduP* (on pTA1069), which were cloned downstream of the $P_{LlacO-1}$ promoter's RBS.

pTA839 was constructed as follows. *dhaB1*, *dhaB2*, and *dhaB3* (Genbank: AB859215.1) and *gdrA* and *gdrB* (Genbank: U30903.1) were chemically synthesized with codon optimization for *E. coli* (Epoch Biolabs, Missouri City, TX, USA). *dhaB2* and *dhaB3* were synthesized as a single fragment (referred to as *dhaB2+3*) with the RBS flanked between the two genes. To clone *dhaB1*, *dhaB2+3*, *gdrA* and *gdrB*, Acc65I-SalI (*dhaB1*), SalI-ClaI (*dhaB2+3*), ClaI-HindIII (*gdrA*) and HindIII-BamHI (*gdrB*) recognition sites in pZE22-MCS1 were used. *dhaB1* was amplified by PCR using primers T811 (5′ GCCAT CGGTA CCATG AAACG TAGCA AACGC TTCGC) and T812 (5′ GCCAT CGTCG ACTTA TTCGA TGGTG TCCGG CTGC). The PCR product was digested with Acc65I and SalI. *dhaB2+3* was amplified by PCR using primers T1123 (5′ GGGGC CATCG TCGAC GAAGG AGATA TACAA TGCAA CAAAC GACCC AAATC CAACC) and T1124 (5′ CCCAT CGATT CAGGA GCCCT TGCGC AACTT GTG). The PCR product was digested with SalI and ClaI. *gdrA* was amplified by PCR using primers T815 (5′ GCCAT CATCG ATGAA GGAGA TATAC ATATG CCACT GATTG CTGGT ATTGA CAT) and T816 (5′ GCCAT CAAGC TTTTA GTTTG CCTGA CCAGC CAGC). The PCR product was digested with ClaI and HindIII. *gdrB* was amplified by PCR using primers T817 (5′ GCCAT CAAGC TTGAA GGAGA TATAC ATATG TCTCT GTCTC CACCA GGTGT TCG) and T1261 (5′ CCCGG ATCCT TAGTT ACGTT CGCTC AGTGG CAGTA CC). The PCR product was digested with HindIII and BamHI.

pTA867 was constructed as follows. *araE* (GenBank: AB241137) was chemically synthesized with codon optimization for *E. coli* (DNA2.0, Menlo Park, CA, USA). *araE* was amplified by PCR using primers T1549 (5′ GCCAT CGGAT CCGAA GGAGA TATAC ATATG GCGAA CGTGA CCTAC ACCG) and T1550 (5′ GCCAT CACGC GTTTA TACTG CCATG ACCGT CACGC T). The PCR product was digested with BamHI and MluI for cloning into BamHI-MluI site of pTA839.

pTA934 was constructed as follows. *pduP*, *pduL*, and *pduW* (GenBank: CP000647.1) were chemically synthesized with codon optimization for *E. coli* (DNA2.0, Menlo Park). To clone *pduP*, *pduL*, and *pduW*, Acc65I-SalI (*pduP*), SalI-ClaI (*pduL*) and ClaI-HindIII (*pduW*) recognition sites in pZE22-MCS1 were used. *pduP* was amplified by PCR using primers T1582 (5′ GCCAT CGGTA CCATG AACAC GGCTG AGCTG GAAAC) and T1583 (5′ GCCAT CGTCG ACTTA ACGGA TGCTG AAGCC ATTCG T). The PCR product was digested with Acc65I and SalI. *pduL* was amplified by PCR using primers T1584 (5′ GCCAT CGTCG ACGAA GGAGA TATAC ATATG GATAA ACAAC AGCTG GCGAG C) and T1585 (5′ GCCAT CATCG ATTTA CGGCT TGGTG ACCAG CGT). The PCR product was digested with SalI and ClaI. *pduW* was amplified by PCR using primers T1586 (5′ GCCAT CATCG ATGAA GGAGA TATAC ATATG ACTTA CAAAA TCATG GCGAT CAAC) and T1587 (5′ GCCAT CAAGC TTTTA GGCGC TCACC GCCAG C). The PCR product was digested with ClaI and HindIII.

pTA984 was constructed as follows. *pduP*, *pduL*, and *pduW* were amplified altogether by PCR using primers T1735 (5′ GCCAT CGGAT CCGAA GGAGA TATAC ATATG AACAC GGCTG AGCTG GAAAC) and T1736 (5′ GCCAT CACGC GTTTA GGCGC TCACC GCCAG C) and pTA934 was used as template. The PCR product was digested with BamHI and MluI and inserted into BamHI-MluI site of pTA839.

pTA1069 was constructed from pTA934. The replication origin (ColE1) was switched to p15A from pZA21-MCS1 by AvrII and SacI digestions. Antibiotic marker (*kan*^{r}) was switched to *amp*^{r} from pZE12-luc by SacI and AatII digestions.

Culture medium and production conditions For 3-HP production experiments, cells were grown in modified M9 medium. Modified M9 medium contains (per liter) 100 mmol glycerol, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 1 mmol $MgSO_4$, 0.1 mmol $CaCl_2$, 10 g casamino acids, 0.05 mg thiamine hydrochloride, and 1 mL metal solution (H_3BO_3 2.86 g/L, $MnCl_2 \cdot 4H_2O$ 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ 0.222 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g/L, $CuSO_4 \cdot 5H_2O$ 0.79 g/L, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g/L). The appropriate antibiotics (kanamycin 50 μg/mL, ampicillin100 μg/mL) were added.

Preculturing in 3 mL M9 medium was performed at 37°C overnight in a linear shaker (BR-23FP; Taitec, Saitama, Japan; 250 rpm). The overnight culture then was used to inoculate a 60 mL culture of M9 medium (in a 250 mL baffled flask) to an initial OD_{600} of 0.05. This fermentation culture was grown at 37°C using an orbital shaker (Innova 44R; New Brunswick Scientific, NJ, USA). After 3 h at 250 rpm, IPTG was added to 1 mM and the culture was shifted to 100 rpm. 240 μL of 1 mM vitamin B_{12} solution were added at the time of 3, 5, 8, 12 and 24 h. The pH of medium was adjusted to 6–7 using 10 N KOH at each sampling time.

Analytical methods The cell density was determined by measurement of absorption at 600 nm (OD_{600}) using Infinite M200 PRO (Tecan, Männedorf, Switzerland). Glycerol and 1,3-propanediol (1,3-PDO) were quantified using HPLC

TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Strain		
TA212	ATCC11303 with *spec*^{r}, *lacI*^{q}, *tetR*	This study
TA1806	TA212/pTA867	This study
TA2034	TA212/pTA984	This study
TA2142	TA212/pTA867, pTA1069	This study
TA2168	TA212/pTA839, pTA1069	This study
Plasmid		
pZE22-MCS1	$P_{LlacO-1}$::MCS; *kan*^{r}; ColE1	29
pZE12-luc	$P_{LlacO-1}$::*luc*; *amp*^{r}; ColE1	29
pZA21-MCS1	$P_{LtetO-1}$::MCS; *kan*^{r}; p15A	29
pTA839	$P_{LlacO-1}$::*dhaB1*, *dhaB2*, *dhaB3*, *gdrA*, *gdrB*; *kan*^{r}; ColE1	This study
pTA867	$P_{LlacO-1}$::*dhaB1*, *dhaB2*, *dhaB3*, *gdrA*, *gdrB*, *araE*; *kan*^{r}; ColE1	This study
pTA934	$P_{LlacO-1}$::*pduP*, *pduL*, *pduW*; *kan*^{r}; ColE1	This study
pTA984	$P_{LlacO-1}$::*dhaB1*, *dhaB2*, *dhaB3*, *gdrA*, *gdrB*, *pduP*, *pduL*, *pduW*; *kan*^{r}; ColE1	This study
pTA1069	$P_{LlacO-1}$::*pduP*, *pduL*, *pduW*; *amp*^{r}; p15A	This study

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