Electronic Supplementary Information for

Tunable hybrid carbon metabolism coordination for carbon-efficient biosynthesis of 1,3-butanediol in *Escherichia coli*

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Supplementary Note

Yield calculation

The yield calculation is based on the methods described previously by Deepak Dugar *et al.* [1]. Theoretical maximum yield (Y^E) is the maximum amount of a product produced from the carbon source, which is merely calculated from the ratios of degree of reduction of substrate to that of product. The theoretical pathway yield (Y^p) is the maximum amount of a product produced by a specified pathway from its stoichiometry, which can be resolved from the following equations:

$$\text{Glucose} \rightarrow a\ \text{NADPH} + b\ \text{Product} + c\ \text{ATP} + d\ \text{NADH} + e\ CO_2 \quad (1)$$

$$\text{Glucose} \rightarrow 12\ \text{NADPH} + 6\ CO_2 \quad (2)$$

$$\text{Glucose} \rightarrow 28.92\ \text{ATP} + 6\ CO_2 \quad (3)$$

The theoretical pathway yield (Y^p) can be calculated with

$$Y^p = Y * \frac{1}{1 - a/12 - c/28.92} \quad (4)$$

Y is product yield based on equation (1) (Y=b). When a>0, which means NADPH is excessive, a is assigned 0 in the equation (4); when c>0, which means ATP is abundant, c is assigned 0 in the equation (4). To calculate Y^p without cofactor imbalance, in which case NADH and NADPH are interconvertible, a is assigned (a+c) if (a+c)<0, otherwise a is assigned 0 in the equation (4).

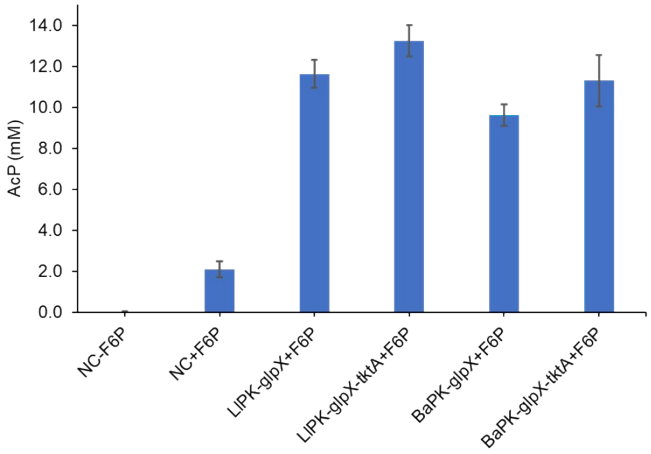

Fig. S1. Enzyme assay of F6P-to-AcP conversion using crude enzyme extracts of wildtype *E. coli* BW25113 (F′) harboring empty plasmid pZE12-luc (NC, negative control), expressing BaPK and *glpX* (BaPK-glpX), expressing BaPK, *glpX* and *tktA* (BaPK-glpX-tktA), expressing LlPK and *glpX* (LlPK-glpX), expressing LlPK, *glpX* and *tktA* (LlPK-glpX-tktA). 10 mM F6P was added as the substrate. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

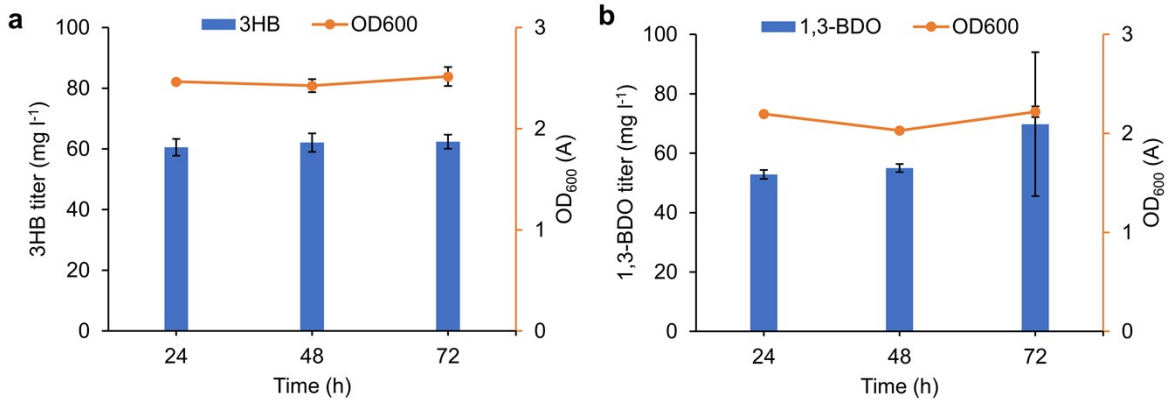

Fig. S2. Shake flask experiments with M9 medium with 5 g l^{-1} yeast extract without glucose in 72 h. (**a**) 3HB production with *E. coli* BW25113 (F′) harboring p3HB. (**b**) 1,3-BDO production with *E. coli* BW25113 (F′) harboring p3HB and pZE-MaCAR. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

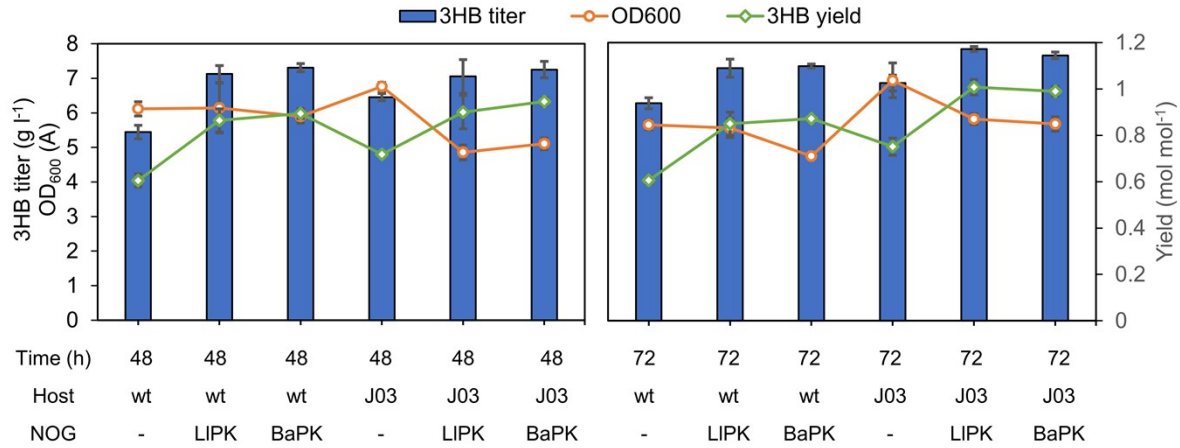

Fig. S3. Comparison of 3HB production with or without P_L*lac*O1-controlled LlPK and BaPK based NOG pathway. Shake flask experiments were performed with wildtype *E. coli* BW25113 (F′) (wt) and its derived mutant strain J03 for 48 h and 72 h. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

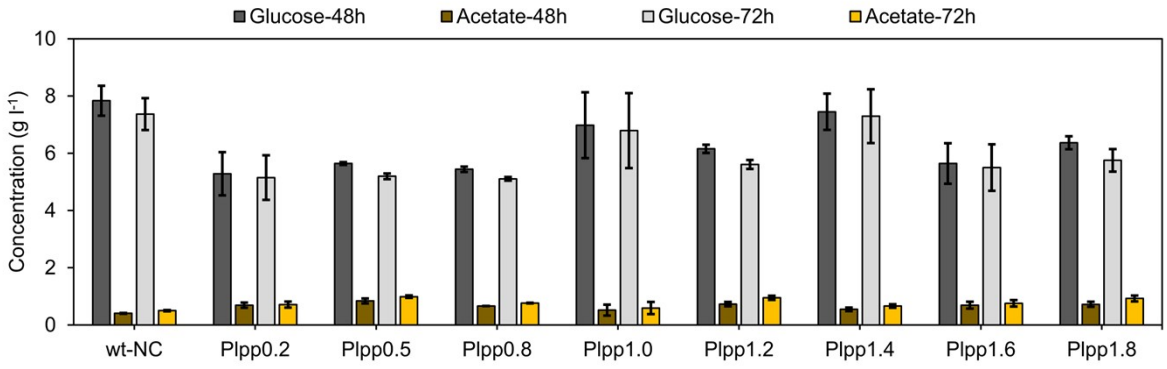

Fig. S4. Glucose remaining and acetate accumulation during 3HB production via P*lppx*-tuned LlPK-based NOG pathway in strain J03. Samples from 48 h and 72 h cultivation were analyzed by HPLC analysis. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

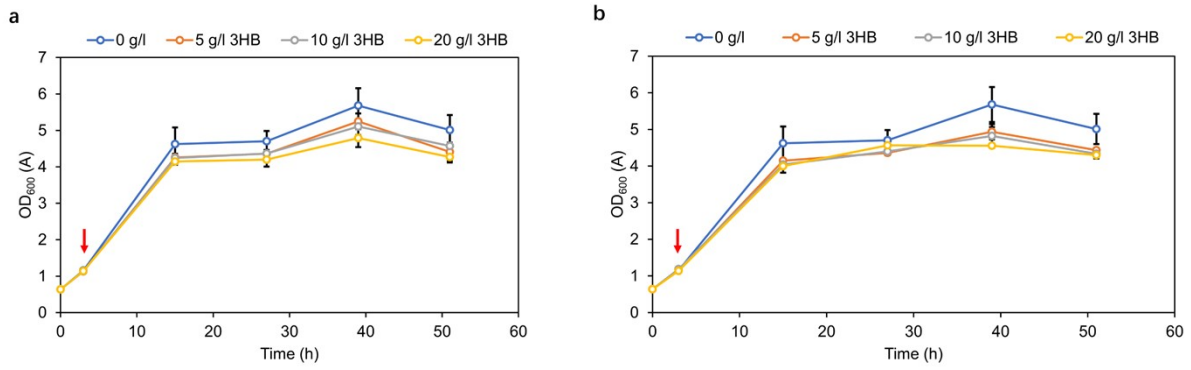

Fig. S5. Test of the 3HB and 1,3-BDO toxicity to *E. coli* BW25113 (F′). 3HB (**a**) or 1,3-BDO (**b**) with different concentrations (0-20 g l^{-1}) was fed to BW25113 (F′) at 3 h post

inoculation as indicated by the red arrows. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

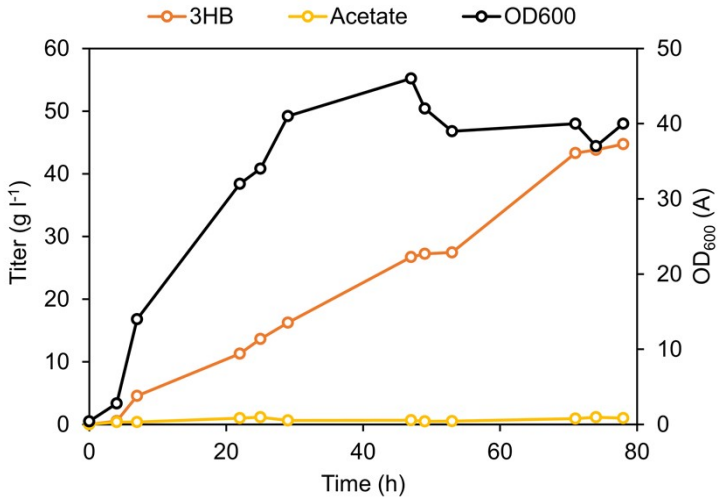

Fig. S6. A duplicate run of fed-batch cultivation of J03 containing p3HB and pZLPK1.4 in 1-l bioreactor.

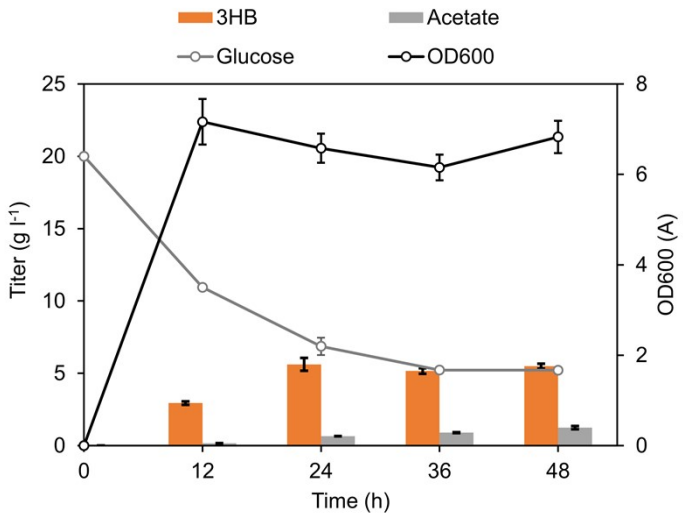

Fig. S7. 3HB production via strain J06 harboring chromosomally integrated 3HB pathway. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

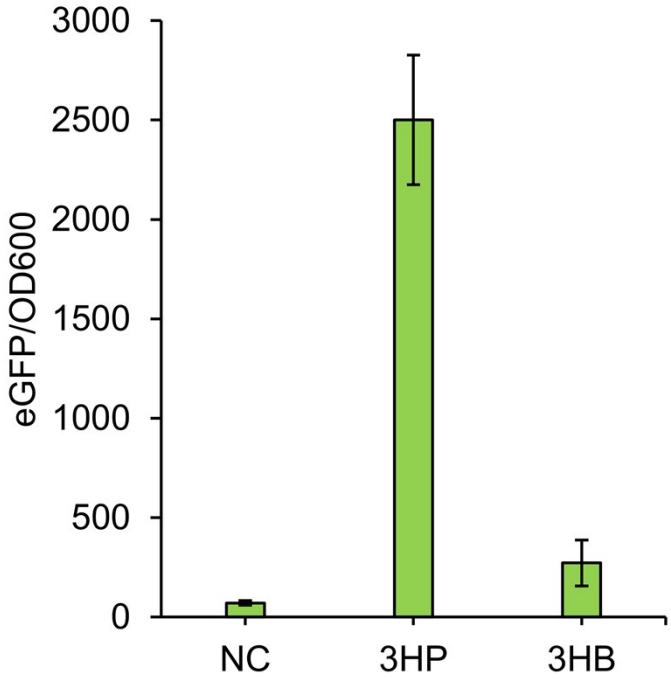

Fig. S8. Induction of wildtype HpdR/P$_{hpdH}$ sensor regulator system by 3HP and 3HB. The eGFP induction assay was performed with strain *E. coli* BW25113 (F′) harboring pZE-HpdR-PhpdH-eGFP. 3HP and 3HB were added at a final concentration of 1 g l^{-1}. NC indicates the negative control without any inducer. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

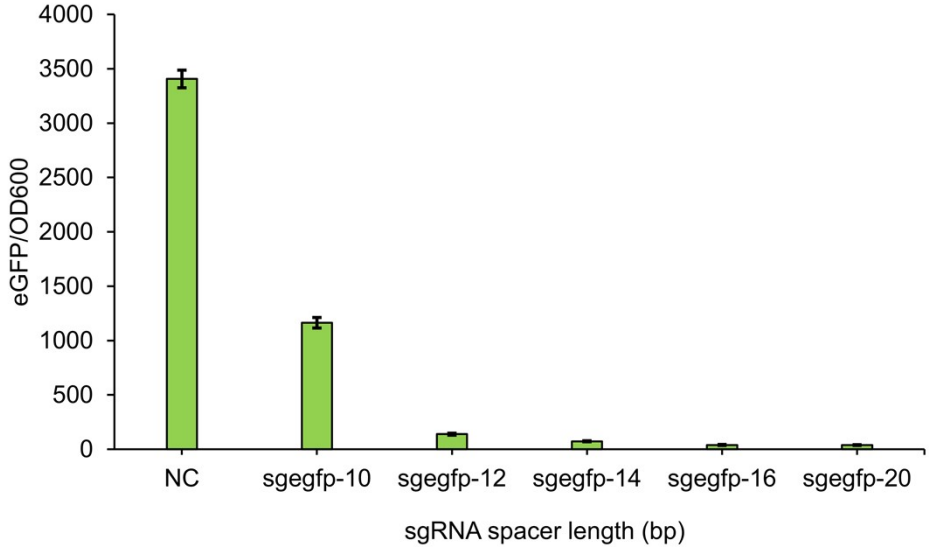

Fig. S9. Effects of spacer length on CRISPRi. The eGFP repression assay was performed with strain J07 transformed with pZE-eGFP and pCS-sgRNA harboring sgegfp with spacer length of 10, 12, 14, 16, and 20 bp. NC indicates the negative control with pZE-eGFP and pCS27. Data indicated the mean ± standard deviation ($n = 3$ independent biological replicates).

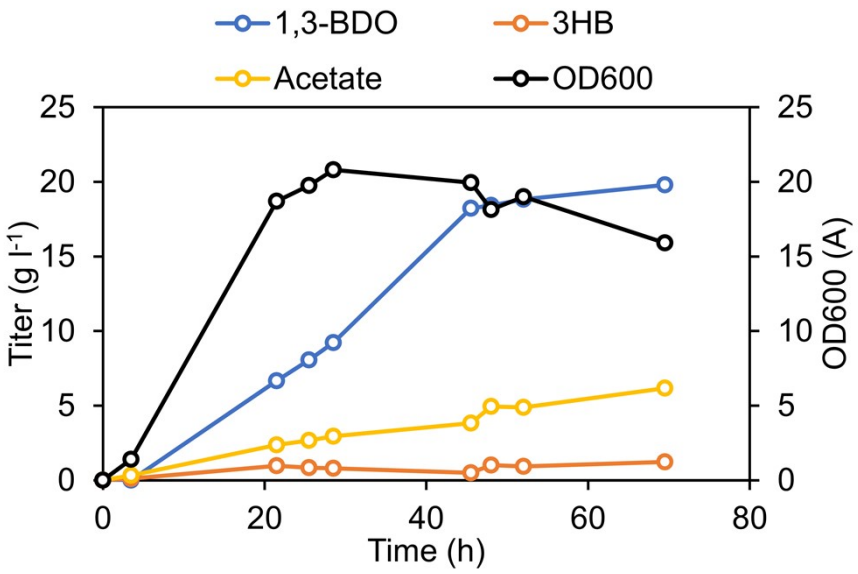

Fig. S10. A duplicate run of fed-batch cultivation of J07 containing pZE-MaCAR, pCS-V1-*sgaccA-14* and pSLPK0.5 in 1-l bioreactor.

Table S1. Reactions and performance of various carbon metabolism involved in this study.

Carbon Metabolism [a]	Metabolites per glucose	N/A stoichiometry [b]	C-yield [c]
EMP	2Acetyl-CoA+2CO_2+4NADH+2ATP	2	66.7%
PP	1.67Acetyl-CoA+2.67CO_2+3.33NADH+2NADPH+1.67ATP	3.19	55.7%
NOG	3Acetyl-CoA-1ATP	0	100%
EMP+PP	1.835Acetyl-CoA+2.335CO_2+3.665NADH+1NADPH+1.835ATP	2.54	61.2%
EMP+NOG	2.5Acetyl-CoA+1CO_2+ 2NADH+0.5ATP	0.8	83.3%
PP+NOG	2.335Acetyl-CoA+1.335CO_2+1.67NADH+1NADPH+0.335ATP	1.14	77.8%
EMP+PP+NOG	2.223Acetyl-CoA+1.556CO_2+2.443NADH+0.67NADPH+0.89ATP	1.4	74.1%

[a] Glucose flux are presumed to equally enter the combined carbon metabolisms referred.
[b] NAD(P)H/acetyl-CoA stoichiometry.
[c] C-yield, or carbon yield, is the ratio of carbon in product acetyl-CoA to carbon in glucose.

111 **Table S2. Metabolic pathways for acetyl-CoA derived products involved in this study.**

Product pathways	Metabolic reactions	N/A stoichiometry
3HB	2 Acetyl-CoA + 1 NADH → 3HB + 1 NAD^+	0.5
1,3-BDO	2 Acetyl-CoA + 1 NADH + 2 NADPH + 1 ATP → 1,3-BDO + 1 NAD^+ + 2 $NADP^+$ + 1 AMP + 1 PPi	1.5

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Table S3. Stoichiometric coefficients in equation (1) with multiple carbon metabolisms [a].

Product pathways	NADPH (a)	Product (b)	ATP (c)	NADH (d)	CO_2 (e)
3HB	2y	-0.5x-0.67y+1.5	3x+2.67y-1	4.5x+4y-1.5	2x+2.67y
1,3-BDO	x+3.33y-3	-0.5x-0.67y+1.5	4x+4y-4 [b]	4.5x+4y-1.5	2x+2.67y

[a] 1 mol glucose is metabolized via three carbon metabolisms including EMP pathway (x mol), PP pathway (y mol) and NOG pathway ((1-x-y) mol). [b] Two ATP inputs were needed considering the additional need of an additional ATP for regeneration of AMP to ADP.

Table S4. Pathway yields for acetyl-CoA derived products with coordinated carbon metabolism.

Products	NADPH (a)	NADPH (a)	Product (b)	ATP (c)	NADH (d)	CO_2 (e)	Pathway yields (Y^p) [b]
3HB	1 EMP [a]	0.00	1.000	2.00	3.00	2.00	1.00
	1 PP	2.00	0.830	1.67	2.50	2.67	0.83
	0.33 EMP+0.67 NOG	0.00	1.33	0.00	0.00	0.67	1.33
	0.38 PP+0.62 NOG	0.76	1.25	0.00	0.00	1.00	1.25
1,3-BDO	1 EMP	-2.00	1.00	0.00	3.00	2.00	1.00
	1 PP	0.33	0.83	0.00	2.50	2.67	0.83
	0.75 PP+0.25 NOG	-0.50	1.00	-1.00	1.50	2.00	0.96
	0.82 EMP+0.18NOG	-2.18	1.09	-0.72	2.19	1.64	1.05
	0.46 EMP+0.27PP+0.27 NOG	-1.64	1.09	-1.08	1.65	1.64	1.05

[a] Mol of glucose entering each carbon metabolism.
[b] The pathway yields (Y^p) were obtained from equation (4) in Supplementary Note considering NADH and NADPH are interconvertible.

Table S5. Biochemical reactions for maximal glucose-to-product conversion with coordinated carbon metabolism.

Product pathways	Biochemical reactions
3HB	Glucose → 1.33 3HB + 0.67 CO_2
1,3-BDO	Glucose → 1.05 1,3-BDO + 1.80 CO_2

Table S6. Specific activities of purified His_6-tagged phosphoketolases from different microorganisms.

One unit of enzyme activity (U) is defined as conversion of 1 μmol of substrate per minute. For R5P as the substrate, additional enzyme Rpe and RpiA was added at ten-fold concentration of PK.

PK	Organisms	Substrates (10 mM)	Specific Activity (μmol/min/mg protein)
BaPK	*Bifidobacterium adolescentis*	F6P	8.42
		R5P	9.17
LlPK	*Lactococcus lactis*	F6P	7.26
		R5P	14.06
LcPK	*Lactobacillus casei*	F6P	6.54
		R5P	11.22
PaPK	*Pseudomonas aeruginosa* PAO1	F6P	5.54
		R5P	5.43
SyPK	*Synechocystis* sp. PC 6803	F6P	4.69
		R5P	5.73

Table S7. Kinetic parameters of CARs towards 3-hydroxybutyrate.

Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)
MmCAR	17.22±3.13	10.86±0.88	0.61
MaCAR	10.87±3.30	19.00±2.86	1.75

Table S8. Nucleotide sequence of HpdR/P_{hpdH} wildtype or variant promoters.

Plasmids	Nucleotide Sequences
wt	(HpdR…)**cat**gcttgtcctttatggcagttcgttccggcctcttaacgggcatgcccacaggtgcggtgaa caccctgaaggtaacgtgatccctgagctgcgggcgagcccgtgaagaggtcggtacaggcttgcccctgt gctaaaacgcacagcggctgcgcgaaatctcgtgtttcatccacgaaattactcactaagatggatcgggac aagaataaaaaacgaattcattaaagaggagaaaggtacc**ATG**(…eGFP)
V1	(HpdR…)**cat**gcttgtcctttatggcagttcgttcGTCGACtacaggcttgcccctgtgctaaaacgc acagcggctgcgcgaaatctcgtgtttcatccacgaaattactcactaagatggatcgggacaagaataaaa aacgaattcattaaagaggagaaaggtacc**ATG**(…eGFP)
V2	(HpdR…)**cat***ggtacctttctcctctttaatcaattccgttacaagtattacacaaagttttttatgttgaga atatttttttgat*GTCGACtacaggcttgcccctgtgctaaaacgcacagcggctgcgcgaaatctcgtgt ttcatccacgaaattactcactaagatggatcgggacaagaataaaaaacgaattcattaaagaggagaaag gtacc**ATG**(…eGFP)
V3	(HpdR…)**cat**gcttgtcctttatggcagttcgttcGTCGACtacaggcttgcccctgtgctaaaacgc acagcggctgcgcgaaatctcgtgttgacaccacgaaattactcacgatactggatcgggacaagaataaaa aacgaattcattaaagaggagaaaggtacc**ATG**(…eGFP)

Note: Bold triple nucleotides are start codons; nucleotides in red lower cases are the putative palindromic sequence (-209 to -118 bp); nucleotides in red upper cases denote *Sal*I restriction site; nucleotides in grey shade denote -35 and -10 boxes in wildtype P_{hpdH} promoter; nucleotides in green shade denote substituted -35 and -10 boxes from $P_L lacO1$ promoter; nucleotide in yellow shade is the transcription initiation site on P_{hpdH} promoter.

147 **Table S9. Strains and plasmids used in this study.**

Strains	**Properties**	**Source**
E. coli XL1-Blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac F'* [*traD36 proAB lacI*^{q}*Z*Δ*M*15 *Tn*10 (*Tet*^{r})]	Stratagene
E. coli BL21 Star(DE3)	F$^-$ *ompT hsdSB (rB-mB-) gal dcm rne131* (DE3)	Invitrogen
E. coli BW25113 (F′)	*rrnBT*14 Δ*lacZWJ*16 *hsdR*514 *ΔaraBADAH*33 *ΔrhaBADLD*78 *F'* [*traD36 proAB lacI*^{q}*ZΔM15 Tn10(Tet*^{r}*)*]	[2]
J01	*E. coli* BW25113 (F′) Δ*edd*	This study
J02	*E. coli* BW25113 (F′) Δ*edd* Δ*pfkA*	This study
J03	*E. coli* BW25113 (F′) Δ*edd* Δ*pfkB*	This study
J04	*E. coli* BW25113 (F′) Δ*edd* Δ*pfkA* Δ*zwf*	This study
J05	*E. coli* BW25113 (F′) Δ*edd* Δ*pfkB* Δ*zwf*	This study
J06	J03 with the 3HB pathway integrated into the genome at *asl* locus	This study
J07	J06 with P_L*lacO1*-dCas9 integrated into the genome at *dkgB* locus	This study
Plasmids	**Properties**	**Source**
pETDuet-1	*PT7*, *pBR322* ori, Amp^r	Novagen
pZE12-luc	P_L*lacO1*, *colE* ori, Amp^r	[3]
pCS27	P_L*lacO1*, *P15A* ori, Kan^r	[4]
pSA74	P_L*lacO1*, *pSC101** ori, Cl^r	[5]
pSA-dCas9	pSA74 harboring dCas9 from *Streptococcus pyogenes*	[6]
pCS-sgRNA	pCS27 harboring sgRNA targeting eGFP	[6]
pZE-eGFP	pZE12-luc harboring eGFP	This study
pZE-MmCAR	pZE12-luc harboring *car* from *Mycobacterium marinum* M and *sfp* amplified from *Bacillus subtilis* 168	[7]
pZE-MaCAR	pZE12-luc harboring *car* from *Mycobacterium abscessus* and *sfp* amplified from *Bacillus subtilis* 168	This study
pZE-LlPK-*glpX*	pZE12-luc harboring phosphoketolase (LlPK, KST82569.1) from *Lactococcus lactis* NCTC 6681, *glpX* from *E. coli*	This study

pZE-BaPK-*glpX*	pZE12-luc harboring phosphoketolase (BaPK, BAF39468.1) from *Bifidobacterium adolescentis* ATCC 15703, *glpX* from *E. coli*	This study
pZLPK	pZE12-luc harboring phosphoketolase (LlPK, KST82569.1) from *Lactococcus lactis* NCTC 6681, *glpX* and *tktA* from *E. coli*	This study
pZBPK	pZE12-luc harboring phosphoketolase (BaPK, BAF39468.1) from *Bifidobacterium adolescentis* ATCC 15703, *glpX* and *tktA* from *E. coli*	This study
pZLPKx	pZE12-luc harboring LlPK, *glpX* and *tktA* from *E. coli* under control of constitutive promoter library Plppx	This study
pSLPKx	pSA74 harboring LlPK, *glpX* and *tktA* from *E. coli* under control of constitutive promoter library Plppx	This study
p3HB	pCS27 carrying *thl* and *hbd* from *Clostridium difficile* 630 and *tesB* from *E. coli*	This study
pETDuet-MmCAR	pETDuet-1 harboring *car* from *Mycobacterium marinum* M and *sfp* amplified from *Bacillus subtilis* 168	This study
pETDuet-MaCAR	pETDuet-1 harboring *car* from *Mycobacterium abscessus* and *sfp* amplified from *Bacillus subtilis* 168	This study
pETDuet-BaPK	pETDuet-1 harboring BaPK from *Bifidobacterium adolescentis*	This study
pETDuet-LlPK	pETDuet-1 harboring LlPK from *Lactococcus lactis*	This study
pETDuet-LcPK	pETDuet-1 harboring LcPK from *Lactobacillus casei*	This study
pETDuet-PaPK	pETDuet-1 harboring PaPK from *Pseudomonas aeruginosa*	This study
pETDuet-SyPK	pETDuet-1 harboring SyPK from *Synechocystis sp.* PCC 6803	This study
pETDuet-Rpi	pETDuet-1 harboring *rpi* from *E. coli*	This study
pETDuet-Rpe	pETDuet-1 harboring *rpe* from *E. coli*	This study
pZE-HpdR-eGFP	pZE12-luc containing HpdR-P_{hpdH} from *Pseudomonas putida* and eGFP	This study
pCS-wt	pCS27 containing HpdR-P_{hpdH}-eGFP	This study
pCS-V1	pCS-wt with deletion of a palindromic sequence (-209 to -118 bp) on the P_{hpdH} promoter	This study
pCS-V2	pCS-V1 with HpdR under control of P*lpp1.0* promoter	This study
pCS-V3	pCS-V1 by substituting the -35 and -10 boxes of P_{hpdH} promoter with those from P_L*lacO1* promoter	This study
pCS-*sgegfp-20*	pCS-sgRNA containing sgegfp with 20 bp spacer targeting eGFP at the start codon region with a TGG PAM site	This study
pCS-*sgegfp-16*	pCS-sgRNA containing sgegfp with 16 bp spacer targeting eGFP at the start codon region with a TGG PAM site	This study
pCS-*sgegfp-14*	pCS-sgRNA containing sgegfp with 14 bp spacer targeting eGFP at the start codon region with a TGG	This study

	PAM site	
pCS-*sgegfp-12*	pCS-sgRNA containing sgegfp with 12 bp spacer targeting eGFP at the start codon region with a TGG PAM site	This study
pCS-*sgegfp-10*	pCS-sgRNA containing sgegfp with 10 bp spacer targeting eGFP at the start codon region with a TGG PAM site	This study
pCS-V1-*sgaccA-14*	pCS-V1 harboring sgRNA targeting *accA* with 14 bp spacer	This study
pCS-V1-*sgaccA-12*	pCS-V1 harboring sgRNA targeting *accA* with 12 bp spacer	This study
pCS-V1-*sgaccA-10*	pCS-V1 harboring sgRNA targeting *accA* with 10 bp spacer	This study
pCS-V2-*sgfabD-14*	pCS-V2 harboring sgRNA targeting *fabD* with 14 bp spacer	This study
pCS-V2-*sgaccA-12*	pCS-V2 harboring sgRNA targeting *fabD* with 12 bp spacer	This study
pCS-V2-*sgaccA-10*	pCS-V2 harboring sgRNA targeting *fabD* with 10 bp spacer	This study

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Reference

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