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Activating Phosphoenolpyruvate Carboxylase and Phosphoenolpyruvate Carboxykinase in Combination for Improvement of Succinate Production

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Phosphoenolpyruvate (PEP) carboxylation is an important step in the production of succinate by *Escherichia coli*. Two enzymes, PEP carboxylase (PPC) and PEP carboxykinase (PCK), are responsible for PEP carboxylation. PPC has high substrate affinity and catalytic velocity but wastes the high energy of PEP. PCK has low substrate affinity and catalytic velocity but can conserve the high energy of PEP for ATP formation. In this work, the expression of both the *ppc* and *pck* genes was modulated, with multiple regulatory parts of different strengths, in order to investigate the relationship between PPC or PCK activity and succinate production. There was a positive correlation between PCK activity and succinate production. In contrast, there was a positive correlation between PPC activity and succinate production only when PPC activity was within a certain range; excessive PPC activity decreased the rates of both cell growth and succinate formation. These two enzymes were also activated in combination in order to recruit the advantages of each for the improvement of succinate production. It was demonstrated that PPC and PCK had a synergistic effect in improving succinate production.

Succinate has many applications in the food, agricultural, pharmaceutical, and biodegradable plastics industries (1). It has been identified as 1 of the 12 most valuable bulk chemicals and has a potential market of $15 billion (1). Currently, succinate is produced mainly from petroleum-derived maleic anhydride. However, due to increasing shortages of petroleum resources and severe environmental problems caused by chemical synthesis processes, microbial production of succinate from renewable biomass has attracted considerable interest in recent years (1–5). Although several rumen bacteria, such as *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Mannheimia succiniciproducens*, can produce large amounts of succinate (2, 6–8), these microorganisms usually require a complex medium and exhibit relatively low yields. On the other hand, *Escherichia coli* has been widely engineered for succinate production with high titers and yields (3–5, 9–12). Through inactivation of the *pflB* (encoding pyruvate-formate lyase), *ldhA* (encoding lactate dehydrogenase), and *ptsG* (encoding enzyme IICB^{Glc} of the phosphoenolpyruvate: carbohydrate phosphotransferase system [PTS]) genes and overexpression of the pyruvate carboxylase gene, strain AFP111/pTrc99A-*pyc* was obtained; it produced 99.2 g/liter succinate with a yield of 1.1 g/g glucose by use of a dual-phase fermentation process (3). Through combined metabolic engineering and metabolic evolution, strain KJ073 was obtained; it produced 668 mM succinate with a yield of 1.2 mol/mol glucose by use of mineral salts medium and a single-batch fermentation process (10). The *pflB* and *ldhA* genes were usually deleted to eliminate the production of acetate, ethanol, and lactate, thus increasing carbon flux toward succinate production (Fig. 1).

Phosphoenolpyruvate (PEP) is an essential precursor for succinate synthesis in *E. coli* (5, 13). PEP can be converted to oxaloacetic acid (OAA) by either PEP carboxylase (PPC) or PEP carboxykinase (PCK) (5), and OAA is further converted to succinate through malate dehydrogenase, fumarase, and fumarate reductase (Fig. 1). PPC has been regarded as the primary catalytic enzyme for the conversion of PEP to OAA during glucose fermentation (14–16). Energetically, the PPC-catalyzed reaction is strongly favored, since the energy contained in PEP is lost in this reaction with the release of inorganic phosphate (5). PPC exhibits high affinity for bicarbonate and high catalytic velocity (17). Previous studies have demonstrated that overexpression of the *ppc* gene in *E. coli* increases succinate production efficiently (13, 18). Overexpression of the native *E. coli ppc* gene in the engineered strain JCL1208 increased the succinate titer 3.5 times (from 3.0 to 10.7 g/liter) and the succinate yield 3.75 times (from 0.12 to 0.45 mol/mol glucose) (13). Overexpression of the *Sorghum vulgare ppc* gene in the engineered *E. coli* strain SB202 increased the succinate titer by 52% (from 12.5 to 19 mM) and the succinate yield by 50% (from 0.12 to 0.18 mol/mol glucose) (18).

On the other hand, expression of the *pck* gene is repressed by glucose in *E. coli* and is activated only under conditions of gluconeogenesis (19, 20). PCK can conserve the high energy of PEP, while it has low affinity for bicarbonate and relatively low catalytic velocity (5, 21). It was recently found that increasing PCK activity through transcriptional activation in *E. coli* could conserve the high energy of PEP, thus leading to net production of ATP for growth and maintenance and improving succinate production significantly (5, 12).

Although PCK and PPC have been engineered individually to improve succinate production, they had never been used in combination. It was even found that increased expression of the *A.*

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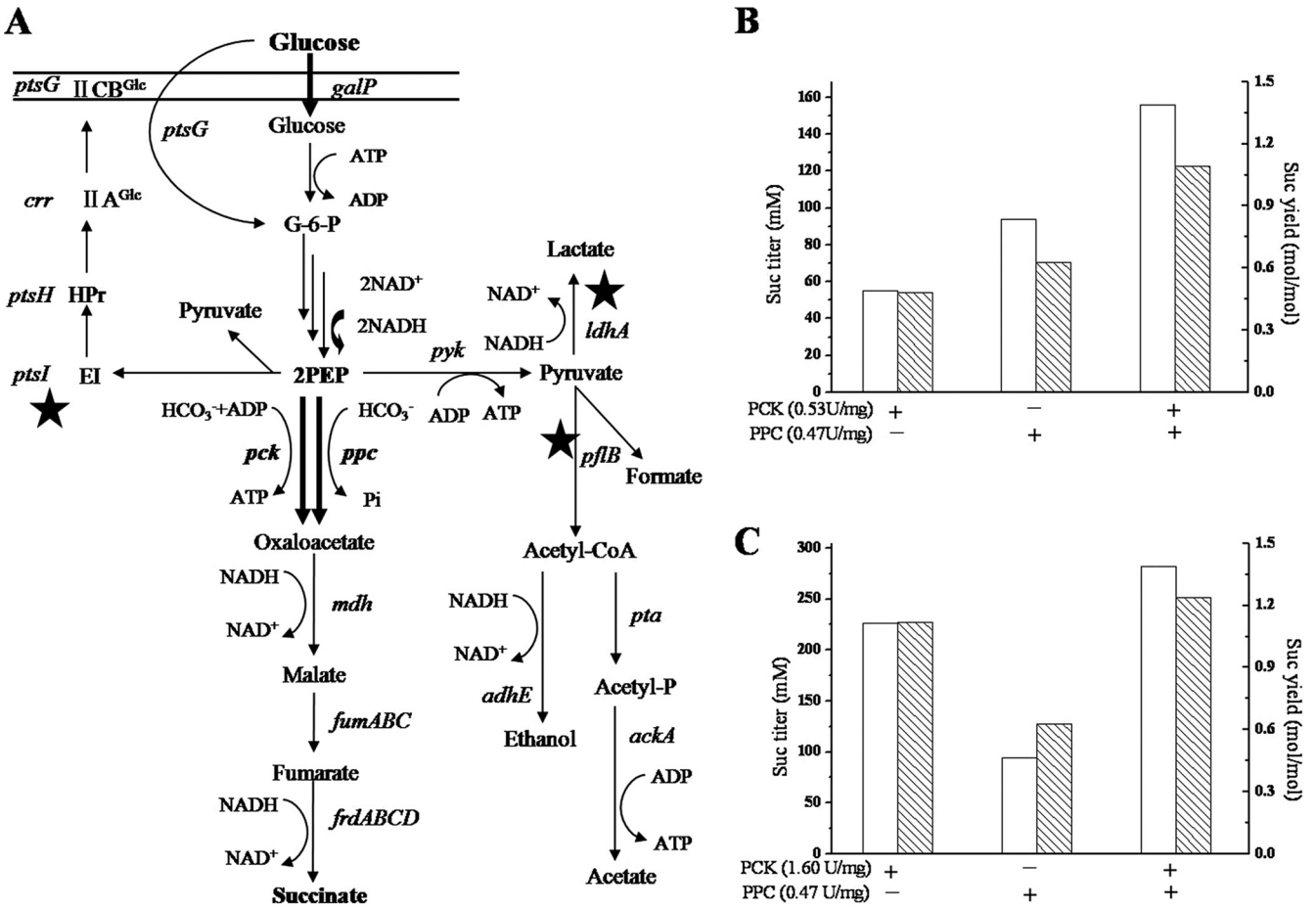

FIG 1 Activation of PPC and PCK in combination to improve succinate production. (A) Mixed-acid fermentation pathways of *E. coli*. Boldface arrows show reactions converting PEP to oxaloacetate and indicate activated gene expression. Stars indicate metabolic reactions that have been blocked by gene deletions. (B) Synergistic effect of PPC and PCK in improving succinate production on a background of moderate PCK activity. (C) Synergistic effect of PPC and PCK in improving succinate production on a background of high PCK activity. Open bars, succinate titers; hatched bars, succinate yield.

succinogenes pck gene increased succinate production only in a PPC mutant *E. coli* strain; it had no effect in wild-type *E. coli* (22). Since each of these carboxylation enzymes has its own advantages for succinate synthesis, they were activated in combination in this work. A synergistic effect of PCK and PPC in improving succinate production was found.

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains constructed in this study are listed in Table 1. During strain construction, strains were cultured aerobically at 30 or 37°C in Luria broth (10 g liter^{-1} Difco tryptone, 5 g liter^{-1} Difco yeast extract, and 10 g liter^{-1} NaCl). Ampicillin (100 mg liter^{-1}), kanamycin (25 mg liter^{-1}), or chloramphenicol (17 mg liter^{-1}) was used where appropriate.

Genetic methods. The *cat* gene was amplified from pACYC184, and the *sacB* gene was amplified from *Bacillus subtilis* 168 using primer pairs 184-cat-up/184-cat-down and Bs-sacB-up/Bs-sacB-down, respectively. These two amplified DNA fragments were digested with SacI and were ligated by T4 ligase, followed by amplification using primer pair 184-cat-up/Bs-sacB-down to produce the *cat-sacB* cassette. This DNA fragment was further ligated with the pEASY-Blunt Simple vector to produce plasmid pXZ-CS.

A two-step recombination method was then used for markerless gene deletion and gene modulation (23, 24). Expression of the *galP* and *pck* genes was modulated with a mutant of the *E. coli pck* promoter (*Ppck**, with a G-to-A mutation at position −64 relative to the ATG start codon) (5). The *pck* promoter was amplified from the genomic DNA of *E. coli* ATCC 8739 by using primer pair P-pck*-up-SpeI/P-pck*-down-KpnI and was subcloned into pTrc99A, resulting in plasmid pXZ602. This plasmid was amplified by primer pair pck*-F/pck*-R and was self-ligated to produce pXZ603. *Ppck** was amplified from pXZ603 by using primer pair P-pck*-up-SpeI/P-pck*-down-KpnI.

Artificial regulatory parts M1-12, M1-46, M1-37, and M1-93, with strengths 0.1, 1.7, 2.5, and 5 times that of the induced *E. coli lacZ* promoter (25), were used for *ppc* and *pck* gene modulation. Red recombinase technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate chromosomal gene deletion and modulation (26, 27). All plasmids are listed in Table 2, and primers are listed in Table 3.

Fermentation. Fresh colonies were picked from New Brunswick Scientific (NBS) mineral salts (5) plates containing 20 g liter^{-1} glucose, inoculated into 250-ml flasks containing 100 ml NBS medium with 50 g liter^{-1} glucose, and grown at 37°C and 120 rpm for 12 h. Cultures were then transferred to a 500-ml fermentation vessel containing 250 ml NBS medium with 50 g liter^{-1} glucose and 100 mM potassium bicarbonate; the initial optical density at 550 nm (OD_{550}) was 0.1. No exogenous gas was supplied. Fermentations were maintained at pH 7.0 by the automatic addition of a base solution containing additional CO_2 (2.4 M potassium carbonate containing 1.2 M potassium hydroxide).

TABLE 1 Strains constructed in this study

Strain	Characteristic(s)[a]	Source or reference
Containing native PCK and PPC		
ATCC 8739	Wild type	Lab collection
Suc-T102	ATCC 8739 Δ*ldhA*	This study
Suc-T104	ATCC 8739 Δ*ldhA* Δ*pflB*	This study
Suc-T106	Suc-T104 Δ*ptsI*	This study
Suc-T108	Suc-T106 *Ppck*-galP*	This study
Activating PCK individually		
Suc-T110	Suc-T108 *Ppck*-pck*	This study
ZT-001	Suc-T108 M1-12-*pck*	This study
ZT-002	Suc-T108 M1-46-*pck*	This study
ZT-003	Suc-T108 M1-37-*pck*	This study
ZT-004	Suc-T108 M1-93-*pck*	This study
Activating PPC individually		
ZT-005	Suc-T108 M1-12-*ppc*	This study
ZT-006	Suc-T108 M1-46-*ppc*	This study
ZT-007	Suc-T108 M1-37-*ppc*	This study
ZT-008	Suc-T108 M1-93-*ppc*	This study
Activating PPC and PCK in combination		
ZT-009	Suc-T108, M1-12-*ppc* M1-37-*pck*	This study
ZT-010	Suc-T108 M1-46-*ppc* M1-37-*pck*	This study
ZT-011	Suc-T108 M1-37-*ppc* M1-37-*pck*	This study
ZT-012	Suc-T108 M1-93-*ppc* M1-37-*pck*	This study
ZT-013	Suc-T108 M1-12-*ppc Ppck*-pck*	This study
ZT-014	Suc-T108 M1-46-*ppc Ppck*-pck*	This study
ZT-015	Suc-T108 M1-37-*ppc Ppck*-pck*	This study
ZT-016	Suc-T108 M1-93-*ppc Ppck*-pck*	This study

[a] *Ppck** is a mutated form of the *pck* promoter (G to A at position −64 relative to the ATG start codon) (5).

TABLE 2 Plasmids used in this study

Plasmid use and designation	Relevant characteristics	Source or reference
Construction of pXZ-CS		
pACYC184	*cat tet*	Lab collection
pEASY-Blunt Simple	*bla kan*	TransGen
pXZ-CS	*cat* gene of pACYC184 and *sacB* gene from *Bacillus subtilis* cloned into the pEASY-Blunt vector	This study
ldhA gene deletion		
pXZ001	*bla kan*; *ldhA* gene (XZ-ldhA-up/XZ-ldhA-down) from *E. coli* ATCC 8739 cloned into the pEASY-Blunt vector	23
pXZ002C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into the *ldhA* fragment of pXZ001 (XZ-ldhA-1/XZ-ldhA-2)	This study
pXZ003	PCR fragment amplified (inside-out product) from pXZ001 (XZ-ldhA-1/XZ-ldhA-2), kinase treated, and then self-ligated	23
pflB gene deletion		
pXZ014	*bla kan*; *pflB* gene (XZ-pflB-up/XZ-pflB-down) from *E. coli* ATCC 8739 cloned into the pEASY-Blunt vector	23
pXZ015C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into the *pflB* fragment of pXZ014 (XZ-pflB-1/XZ-pflB-2)	This study
pXZ016	PCR fragment amplified (inside-out product) from pXZ014 (XZ-pflB-1/XZ-pflB-2), kinase treated, and then self-ligated	23
ptsI gene deletion		
pXZ008	*bla kan*; *ptsI* gene (XZ-ptsI-up/XZ-ptsI-down) from *E. coli* ATCC 8739 cloned into the pEASY-Blunt vector	This study
pXZ009C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into the *ptsI* fragment of pXZ008 (XZ-ptsI-1/XZ-ptsI-2)	This study
pXZ010	PCR fragment amplified (inside-out product) from pXZ008 (XZ-ptsI-1/XZ-ptsI-2), kinase treated, and then self-ligated	This study
galP promoter replacement		
pTrc99A	*bla*; expression vector with *trc* promoter	Lab collection
pXZ602	*bla*; *pck* promoter amplified from *E. coli* ATCC 8739 (P-pck*-up-SpeI/P-pck*-down-KpnI) and cloned into the pTrc99A vector	This study
pXZ603	PCR fragment amplified (inside-out product) from pXZ602 (pck*-F/pck*-R), kinase treated, and then self-ligated	This study
pXZ011	*bla kan*; *galP* gene (XZ-galP-P-up/XZ-galP-P-down) from *E. coli* ATCC 8739 cloned into the pEASY-Blunt vector	This study
pXZ012C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into *galP* of pXZ011 (XZ-galP-P-1/XZ-galP-P-2)	This study
pXZ013	*Ppck** fragment amplified from pXZ603 (P-pck*-up-SpeI/P-pck*-down-KpnI) and cloned into *galP* of pXZ011 (XZ-galP-P-1/XZ-galP-P-2)	This study

Enzyme assay. Cells were collected at the late-exponential stage (72 h) for an enzyme assay. The activities of PPC and PCK were determined as described previously (5). Activity was reported in micromoles per milligram of protein per minute.

Analysis. The dry weight of cells was calculated by measuring the OD_{550}. Organic acids and residual glucose in fermentation broth were measured by high-performance liquid chromatography (5).

RESULTS

Construction of strain Suc-T108 for succinate production. Under anaerobic conditions, wild-type *E. coli* produced mixed acids, including lactate, formate, acetate, and succinate (28). The succinate yield was only 0.17 mol/mol glucose during glucose fermentation for wild-type *E. coli* ATCC 8739 (data not shown). In order to produce succinate as the sole product, the *pflB* and *ldhA* genes were deleted in *E. coli* ATCC 8739, eliminating the formation of by-products (formate, lactate, ethanol, and acetate) and resulting in strain Suc-T104. However, deletion of these two genes led to very slow growth of *E. coli* under anaerobic conditions, since the native succinate synthetic pathway of *E. coli* was not very active, and NADH produced during glycolysis could not be recycled back to NAD^+ efficiently for further glucose metabolism (12).

Wild-type *E. coli* utilized the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) for glucose uptake and phosphorylation (29–32). One PEP molecule is required for the transport and phosphorylation of one glucose molecule (24, 25). Since PEP is an essential precursor for succinate synthesis, the PTS needs to be inactivated in order to increase the PEP supply for the improvement of succinate production (5, 12). In a previous study, activation of PCK in strain ATCC 8739 (Δ*ldhA* Δ*pflB*) resulted in only a little improvement in the cell mass and succinate titer. In contrast, activation of PCK combined with inactivation of

TABLE 3 Primers used in this study

Primer use and designation	Sequence
Construction of pXZ-CS	
184-cat-up	GCTAGGTACCTGTGACGGAAGATCACTTCG
184-cat-down	GCTAGAGCTCGCGGCTATTTAACGACCCT (SacI)
Bs-sacB-up	GCTAGAGCTCAAGTAAATCGCGCGGGTTT (SacI)
Bs-sacB-down	GCTAGGATCCTTATTTGTTAACTGTTAATTGTC
ldhA gene deletion	
XZ-ldhA-up	GATAACGGAGATCGGGAATG
XZ-ldhA-down	CTTTGGCTGTCAGTTCACCA
XZ-ldhA-1	TCTGGAAAAAGGCGAAACCT
XZ-ldhA-2	TTTGTGCTATAAACGGCGAGT
cat-sacB-up	TGTGACGGAAGATCACTTCGCA
cat-sacB-down	TTATTTGTTAACTGTTAATTGTCCT
pflB gene deletion	
XZ-pflB-up	TGTCCGAGCTTAATGAAAAGTT
XZ-pflB-down	CGAGTAATAACGTCCTGCTGCT
XZ-pflB-1	AAACGGGTAACACCCCAGAC
XZ-pflB-2	CGGAGTGTAAACGTCGAACA
ptsI gene deletion	
XZ-ptsI-up	CGCATTATGTTCCCGATGAT
XZ-ptsI-down	CACCAATCAGCGTGACAACT
XZ-ptsI-1	GCCACCATCGTAATCCTGTT
XZ-ptsI-2	ATAGCGCACCACCTCAATTT
galP promoter replacement	
XZ-galP-P-up	ATCTGCTGCACCCGATCTAC
XZ-galP-P-down	GAACCGGCAACAAACAAAAT
XZ-galP-P-1	ATGCCTGACGCTAAAAAACAGGG
XZ-galP-P-2	GATTAAACGCTGTTATCTGCAA
P-pck*-up-SpeI	GCATACTAGTGTTGGTTATCCAGAATCAAA
P-pck*-down-KpnI	GCATGGTACCAGCCAATATGTATTGCCTGAATAG
pck*-F	ACGGTTAACACCCCCAAAAAG
pck*-R	GACAAGGCTCATAGATTTACGTATC
Modulation of *pck* gene	
pck-cat-sacB-up	CGCCATATAAACCAAGATTTAACCTTTTGAGAAC ATTTTCCACACCTAATGTGACGGAAGATCACT TCGCA
pck-cat-sacB-down	ATACCATAAGCCTCGAGTTCTTGCGGGGTCAAAC CATTGTTAACGCGCATTTATTTGTTAACTGTTA ATTGTCCT
pck-up-P	CGCCATATAAACCAAGATTTAACCTTTTGAGAAC ATTTTCCACACCTAATTATCTCTGGCGGTGT TGAC
pck-RBS-down	ATACCATAAGCCTCGAGTTCTTGCGGGGTCAAAC CATTGTTAACGCGCATAGCTGTTTCCTGGTT
Modulation of *ppc* gene	
ppc-cat-sacB-up	GTTTGCTGAAGCGATTTCGCAGCATTTGACGTCA CCGCTTTTACGTGGCTTTATAAAATGTGACGG AAGATCACTTCGCA
ppc-cat-sacB-down	TTGCCGAGCATACTGACATTACTACGCAATGCGG AATATTGTTCGTTCATTTATTTGTTAACTGTTA ATTGTCCT
ppc-up-P	GTTTGCTGAAGCGATTTCGCAGCATTTGACGTCA CCGCTTTTACGTGGCTTTATAAAATTATCTCTG GCGGTGTTGAC
ppc-RBS-down	TTGCCGAGCATACTGACATTACTACGCAATGCGG AATATTGTTCGTTCATAGCTGTTTCCTGGTT

the PTS resulted in significant increases in cell mass and succinate production (12). Thus, in this work, the *pstI* gene (encoding PTS enzyme I) was deleted in strain Suc-T104 for succinate production, resulting in strain Suc-T106.

Although inactivation of the PTS could increase the PEP supply, *E. coli* strains with inactivated PTSs usually exhibited slow cell growth and limited capacities for glucose transport and phosphorylation (24, 30, 32). In order to enhance the glucose utilization of strain Suc-T106, the expression of the *galP* gene (encoding galactose permease) was modulated by replacing its native promoter with the strong promoter *Ppck** (5), resulting in strain Suc-T108. The amounts of by-products, such as lactate, formate, and acetate, were significantly lower in strain Suc-T108 than in the wild-type strain ATCC 8739 (Table 4). However, this strain exhibited remarkably slow growth in mineral salts medium under anaerobic conditions and produced only 4 mM succinate after 4 days, with a yield of 0.17 mol/mol glucose (Table 4).

Activation of PCK for the improvement of succinate production. PEP carboxylation had been regarded as a rate-limiting reaction for succinate production (5). It was thought that the PEP carboxylation efficiency of strain Suc-T108 was low and that NADH produced during glycolysis could not be converted back to NAD^+ efficiently, thus leading to slow cell growth.

In order to increase PCK activity so as to improve succinate production, a previously identified point mutation (5) was introduced into the *pck* promoter region (G to A at position −64 relative to the ATG start codon) of strain Suc-T108, resulting in strain Suc-T110. PEP carboxykinase activity increased 16-fold, from 0.1 to 1.6 U/mg protein. The succinate titer increased 57-fold (from 4 to 226 mM), while the succinate yield increased 6.6-fold (from 0.17 to 1.12 mol/mol glucose) (Table 4).

In addition, in order to investigate the relationship between PCK activity and succinate production, multiple artificial regulatory parts with different expression strengths were used to modulate the expression of the *pck* gene. These regulatory parts were selected from a previously constructed mRNA-stabilizing region (mRS) library (25) and had the same promoter (P2-15) and ribosome binding site (RBS) (*E. coli lacZ*) sequence but differed in the sequence between the promoter and the RBS region. The mRNA-stabilizing region could form a stem-loop structure with transcribed mRNA to control its stability, thus modulating the strength of expression of the target gene. Four representative regulatory parts (M1-12, M1-46, M1-37, and M1-93) in the mRS library, with strengths 0.1, 1.7, 2.5, and 5 times that of the induced *E. coli lacZ* promoter, were selected for the modulation of *pck* gene expression, resulting in strains ZT-001, ZT-002, ZT-003, and ZT-004, respectively. The PCK activities of strains ZT-001, ZT-002, ZT-003, and ZT-004 were 0.24, 0.38, 0.53, and 0.21 U/mg, while the succinate titers were 17, 31, 55, and 12 mM, respectively (Table 4). It was suggested that there was a positive correlation between PCK activity and the succinate titer. In addition, the succinate yields of strains ZT-001, ZT-002, ZT-003, and ZT-004 were 0.26, 0.33, 0.48, and 0.18 mol/mol glucose, respectively (Table 4), suggesting that PCK activity was also positively correlated with the succinate yield.

Activation of PPC for the improvement of succinate production. In order to investigate the relationship between PPC activity and succinate production, the expression of the *ppc* gene in strain Suc-T108 was modulated by four artificial regulatory parts, M1-12, M1-46, M1-37, and M1-93, resulting in strains ZT-005, ZT-006, ZT-007, and ZT-008, respectively. The PPC activities of strains Suc-T108, ZT-005, ZT-006, ZT-007, and ZT-008 were 0.1, 0.34, 0.47, 0.6, and 1.01 U/mg protein, while their succinate titers were 4, 75, 94, 58, and 56 mM, respectively (Table 5). There was a positive correlation between PPC activity and the succinate titer when the PPC activity was equal to or less than 0.47 U/mg protein

TABLE 4 Effect of PCK activation on succinate production[a]

Strain	Cell mass (g/liter)[b]	Glu concn used (mM)	Suc titer (mM)	Suc yield (mol/mol Glu)	PCK activity (U/mg)	Concn (mM) of the following fermentation product[c]:				
						Pyr	Lac	For	Ace	EtOH
Suc-T108	0.17	24 ± 1	4 ± 0	0.17 ± 0.00	0.11 ± 0.01	35	3	0	0	0
ZT-001	0.35	66 ± 1	17 ± 1	0.26 ± 0.02	0.24 ± 0.02	4	26	43	29	20
ZT-002	0.48	95 ± 3	31 ± 2	0.33 ± 0.02	0.38 ± 0.04	3	36	52	48	28
ZT-003	0.52	114 ± 3	55 ± 4	0.48 ± 0.04	0.53 ± 0.08	4	42	35	42	21
ZT-004	0.32	67 ± 3	12 ± 1	0.18 ± 0.01	0.21 ± 0.03	1	7	38	35	15
Suc-T110	1.33	202 ± 3	226 ± 5	1.12 ± 0.02	1.60 ± 0.00	12	4	5	105	20

[a] Fermentation was performed in NBS mineral salts medium containing 5% glucose and 100 mM potassium bicarbonate (37°C, pH 7.0, 150 rpm, 4 days). Suc, succinate.
[b] Calculated from the highest OD_{550} value obtained during fermentation (1 OD_{550} unit = 0.33 g [dry weight of cells] $liter^{-1}$).
[c] Pyr, pyruvate; Lac, lactate; For, formate; Ace, acetate; EtOH, ethanol.

(Fig. 2). However, excessive PPC activity (higher than 0.47 U/mg) decreased the level of succinate production (Fig. 2).

Activation of PCK and PPC in combination for the improvement of succinate production. In order to investigate whether the activation of PCK and PPC in combination had a synergistic effect in improving succinate production, the expression of the *ppc* gene in strain ZT-003, which had moderate PCK activity (0.53 U/mg protein), was modulated by four artificial regulatory parts, M1-12, M1-46, M1-37, and M1-93, resulting in strains ZT-009, ZT-010, ZT-011, and ZT-012, respectively. The succinate titers of strains ZT-003, ZT-009, ZT-010, ZT-011, and ZT-012 were 55, 119, 156, 138, and 130 mM, respectively (Table 5; Fig. 2B). The relationship between PPC activity and succinate production in strains derived from ZT-003 (Fig. 2B) was similar to that in strains derived from Suc-T108 (Fig. 2A). The highest titer and yield were obtained for strain ZT-010, which had moderate PPC activity (0.47 U/mg). The succinate titer of strain ZT-010 (M1-37-*pck* M1-46-*ppc*) was 66% higher than that of strain ZT-006 (M1-46-*ppc*) and 184% higher than that of strain ZT-003 (M1-37-*pck*) (Fig. 1B). In addition, the succinate yield of strain ZT-010 was 73% higher than that of strain ZT-006 and 127% higher than that of strain ZT-003 (Fig. 1B).

The expression of the *ppc* gene in strain Suc-T110, which had high PCK activity (1.6 U/mg protein), was also modulated by four artificial regulatory parts, M1-12, M1-46, M1-37, and M1-93, resulting in strains ZT-013, ZT-014, ZT-015, and ZT-016, respectively. The succinate titers of strains ZT-013, ZT-014, ZT-015, and ZT-016 were 270, 282, 237, and 210 mM, respectively (Table 5; Fig. 2C). The relationship between PPC activity and succinate production in strains derived from Suc-T110 (Fig. 2C) was similar to that in strains derived from Suc-T108 (Fig. 2A). The highest titer and yield was obtained for strain ZT-014, which had moderate PPC activity (0.47 U/mg). The succinate titer of strain ZT-014 (M1-46-*ppc Ppck**-*pck*) was 200% higher than that of strain ZT-006 (M1-46-*ppc*) and 24% higher than that of strain Suc-T110

TABLE 5 Effects of PPC activation on succinate production on different PCK activity backgrounds[a]

Strain	Cell mass (g/liter)[b]	Glu concn used (mM)	Suc titer (mM)	Suc yield (mol/mol Glu)	PPC activity (U/mg)	Concn (mM) of the following fermentation product[c]:				
						Pyr	Lac	For	Ace	EtOH
Low PCK activity										
Suc-T108	0.17	24 ± 1	4 ± 0	0.17 ± 0.01	0.10 ± 0.00	35	3	0	0	0
ZT-005	0.65	115 ± 2	75 ± 1	0.65 ± 0.01	0.34 ± 0.03	1	18	23	53	18
ZT-006	0.71	150 ± 2	94 ± 3	0.63 ± 0.02	0.47 ± 0.03	0	39	53	67	30
ZT-007	0.62	98 ± 1	58 ± 2	0.59 ± 0.02	0.60 ± 0.04	0	4	28	43	7
ZT-008	0.55	129 ± 2	56 ± 1	0.44 ± 0.01	1.01 ± 0.08	0	40	14	45	26
Moderate PCK activity										
ZT-003	0.52	114 ± 3	55 ± 4	0.48 ± 0.04	0.10 ± 0.00	4	42	35	42	21
ZT-009	0.61	126 ± 0	119 ± 1	0.94 ± 0.01	0.34 ± 0.03	0	27	25	68	15
ZT-010	0.72	143 ± 2	156 ± 3	1.09 ± 0.02	0.47 ± 0.03	0	24	17	77	11
ZT-011	0.62	136 ± 2	138 ± 2	1.01 ± 0.01	0.60 ± 0.04	0	24	20	76	12
ZT-012	0.55	129 ± 1	130 ± 3	1.01 ± 0.02	1.01 ± 0.08	0	20	19	72	11
High PCK activity										
Suc-T110	1.33	202 ± 3	226 ± 5	1.12 ± 0.02	0.10 ± 0.00	12	4	5	105	20
ZT-013	1.31	229 ± 3	270 ± 3	1.18 ± 0.01	0.34 ± 0.03	12	0	0	120	20
ZT-014	1.52	227 ± 5	282 ± 6	1.24 ± 0.03	0.47 ± 0.03	0	0	0	101	12
ZT-015	1.28	204 ± 4	237 ± 3	1.16 ± 0.01	0.60 ± 0.04	22	0	0	91	16
ZT-016	1.22	181 ± 1	210 ± 0	1.16 ± 0.00	1.01 ± 0.08	11	12	0	92	23

[a] Fermentation was performed in NBS mineral salts medium containing 5% glucose and 100 mM potassium bicarbonate (37°C, pH 7.0, 150 rpm, 4 days). Suc, succinate.
[b] Calculated from the highest OD_{550} value obtained during fermentation (1 OD_{550} unit = 0.33 g [dry weight of cells] $liter^{-1}$).
[c] Pyr, pyruvate; Lac, lactate; For, formate; Ace, acetate; EtOH, ethanol.

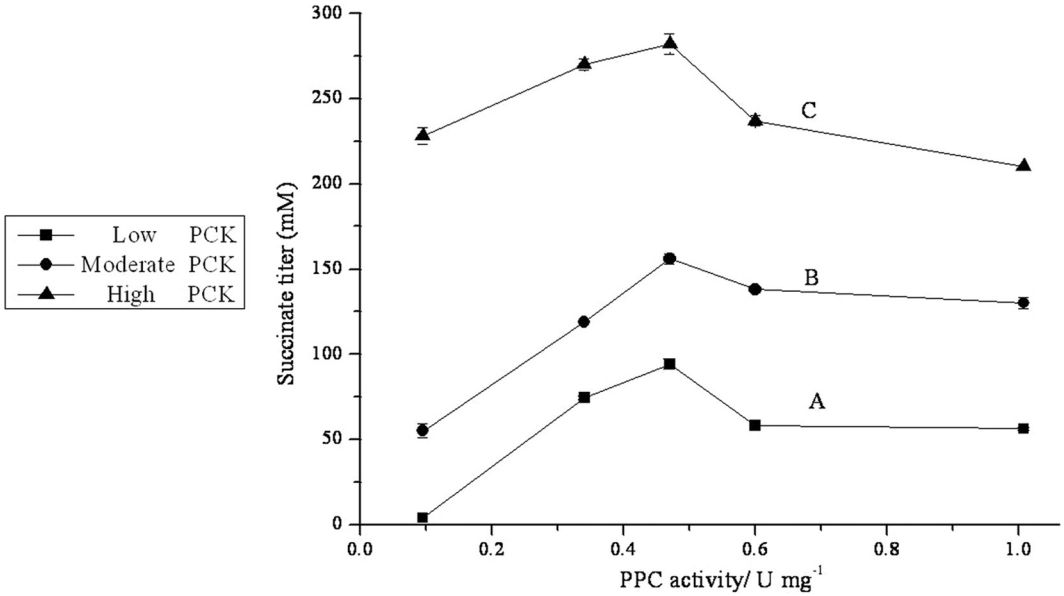

FIG 2 Relationship between PPC activity and the succinate titer on different PCK activity backgrounds. (A) Low PCK activity; (B) moderate PCK activity; (C) high PCK activity.

(*Ppck***-pck*) (Fig. 1C). In addition, the succinate yield of strain ZT-014 was 97% higher than that of strain ZT-006 and 11% higher than that of strain Suc-T110 (Fig. 1C).

DISCUSSION

PPC and PCK are two important carboxylation enzymes within the succinate synthetic pathway. PPC has a K_m for bicarbonate of 0.1 μM and a specific activity of 250 μmol min^{-1} g^{-1}, whereas PCK has a K_m for bicarbonate of 13 μM and a specific activity of 28 μmol min^{-1} g^{-1} (17, 21). Each of these two enzymes has advantages and disadvantages in the carboxylation of PEP to OAA for succinate production. PPC has high substrate affinity and catalytic velocity but wastes the high energy of PEP, so that ATP is not formed during succinate production. PCK has low substrate affinity and catalytic velocity but can conserve the high energy of PEP for ATP formation (5). For the first time, we modulated the expression of either the *ppc* or the *pck* gene by using multiple regulatory parts with different strengths, and we investigated the relationship between PPC or PCK activity and succinate production.

There was a positive correlation between PCK activity and succinate production. Higher PCK activity was coupled with higher succinate titers. It was suggested that higher PCK activity would lead to more OAA and ATP formation. The energy conserved in this reaction would be beneficial for cell growth. Increased cell mass would also cause the production of more succinate. In contrast, there was a positive correlation between PPC activity and succinate production only when PPC activity fell within a certain range; excessive PPC activity (higher than 0.47 U/mg protein) decreased both cell growth and succinate formation (Table 5).

If reducing equivalent supply were enough (for example, by using exogenous hydrogen or electricity), 2 molecules of ATP would be produced from the conversion of 1 molecule of glucose to 2 molecules of succinate when only PCK was used, while there would be no ATP when only PPC was used. When both PCK and PPC were used for PEP carboxylation, net ATP production would be reduced (from 2 molecules of ATP to none). ATP production would depend on the ratio of carbon fluxes going through these two carboxylation reactions. It should be noted that only 1 molecule of ATP was produced when 1 molecule of glucose was converted to 2 molecules of ethanol in *Zymomonas mobilis* through the Entner-Doudoroff pathway (33, 34). The reduced ATP formation accelerated the glucose utilization rate to produce more ATP, resulting in high cell growth and ethanol productivity (34).

It was assumed that when PPC activity was equal to or lower than 0.47 U/mg protein, a low level of carbon flux went through this reaction. The ATP formed through PCK (5) was enough to support cell growth and maintenance. However, when PPC activity exceeded a threshold, more carbon flux went through this reaction and less carbon flux would go through PCK. Under this condition, the ATP supply was below the threshold for supporting normal cell growth and maintenance, causing reduced cell mass, which would further lead to reduced succinate production. Among strains with wild-type PCK and a modulated *ppc* gene, the highest cell mass and succinate titer were obtained for strain ZT-006, which had an optimal PPC activity (0.47 U/mg protein) and a good balance between a high carboxylation rate and a supply of ATP for cell growth. For strains ZT-007 and ZT-008, which had excessive PPC activities, cell masses were 0.62 and 0.55 g/liter, respectively, lower than that of strain ZT-006 (0.71 g/liter) (Table 5). It was suggested that when PPC activity exceeded the threshold, higher PPC activity led to reduced cell mass.

In order to utilize the advantages of both PCK and PPC for the improvement of succinate production, these two enzymes were activated in combination. On a moderate PCK activity background, the succinate titer of the strain with combined activation was 66% or 184% higher than that of the strain individually activated with PPC or PCK, respectively (Fig. 1B; Table 5). On a high PCK activity background, the succinate titer of the strain with combined activation was 200% or 24% higher than that of the strain individually activated with PPC or PCK, respectively

(Fig. 1C; Table 5). On both moderate and high PCK activity backgrounds, when the PPC activity exceeded the threshold (0.47 U/mg protein), higher PPC activity led to reductions in cell mass and succinate production. On a moderate PCK activity background, the cell mass decreased from 0.72 to 0.62 or 0.55 g/liter, while the succinate titer decreased from 156 to 138 or 130 mM. On a high PCK activity background, the cell mass decreased from 1.52 to 1.28 or 1.22 g/liter, while the succinate titer decreased from 282 to 237 or 210 mM (Table 5). Our results demonstrated that activating PPC and PCK in combination could lead to both high catalytic velocity and energy conservation and that PPC and PCK had a synergistic effect in improving succinate production.

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REFERENCES

1. **McKinlay JB, Vieille C, Zeikus JG.** 2007. Prospects for a bio-based succinate industry. Appl. Microbiol. Biotechnol. **76:**727–740.
2. **Lee PC, Lee SY, Hong SH, Chang HN.** 2002. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl. Microbiol. Biotechnol. **58:**663–668.
3. **Vemuri GN, Eiteman MA, Altman E.** 2002. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J. Ind. Microbiol. Biotechnol. **28:**325–332.
4. **Lin H, Bennett GN, San KY.** 2005. Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. Biotechnol. Bioeng. **90:**775–779.
5. **Zhang X, Jantama K, Moore JC, Jarboe LR, Shanmugam KT, Ingram LO.** 2009. Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **106:**20180–20185.
6. **Glassner DA, Datta R.** September 1992. Process for the production and purification of succinic acid. US patent 5143834.
7. **Guettler MV, Jain MK, Soni BK.** April 1996. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms. US patent 5504004.
8. **Scholten E, Renz T, Thomas J.** 2009. Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1. Biotechnol. Lett. **31:**1947–1951.
9. **Lee SJ, Lee DY, Kim TY, Kim BH, Lee J, Lee SY.** 2005. Metabolic engineering of *Escherichia coli* for enhanced production of succinic acid, based on genome comparison and *in silico* gene knockout simulation. Appl. Environ. Microbiol. **71:**7880–7887.
10. **Jantama K, Haupt MJ, Svoronos SA, Zhang X, Moore JC, Shanmugam KT, Ingram LO.** 2008. Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. Biotechnol. Bioeng. **99:**1140–1153.
11. **Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO.** 2008. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol. Bioeng. **101:**881–893.
12. **Zhang X, Jantama K, Shanmugam KT, Ingram LO.** 2009. Reengineering *Escherichia coli* for succinate production in mineral salts medium. Appl. Environ. Microbiol. **75:**7807–7813.
13. **Millard CS, Chao YP, Liao JC, Donnelly MI.** 1996. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl. Environ. Microbiol. **62:**1808–1810.
14. **Fraenkel DG.** 1996. Glycolysis, p 189–198. *In* Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol 1. ASM Press, Washington, DC.
15. **Unden G, Kleefeld A.** July 2004, posting date. Chapter 3.4.5. C_4-dicarboxylate degradation in aerobic and anaerobic growth. In Böck A, Curtiss R, III, Kaper JB, Karp PD, Neidhardt FC, Nyström T, Slauch JM, Squires CL, Ussery D (ed), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. doi:10.1128/ecosal.3.4.5.
16. **Keseler IM, Collado-Vides J, Gama-Castro S, Ingraham J, Paley S, Paulsen IT, Peralta-Gil M, Karp PD.** 2005. EcoCyc: a comprehensive database resource for *Escherichia coli*. Nucleic Acids Res. **33:**D334–D337.
17. **Kai Y, Matsumura H, Inoue T, Terada K, Nagara Y, Yoshinaga T, Kihara A, Tsumura K, Izui K.** 1999. Three-dimensional structure of phosphoenolpyruvate carboxylase: a proposed mechanism for allosteric inhibition. Proc. Natl. Acad. Sci. U. S. A. **96:**823–828.
18. **Lin H, San KY, Bennett GN.** 2005. Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Appl. Microbiol. Biotechnol. **67:**515–523.
19. **Oh MK, Rohlin L, Kao KC, Liao JC.** 2002. Global expression profiling of acetate-grown *Escherichia coli*. J. Biol. Chem. **277:**13175–13183.
20. **Kao KC, Tran LM, Liao JC.** 2005. A global regulatory role of gluconeogenic genes in *Escherichia coli* revealed by transcriptome network analysis. J. Biol. Chem. **280:**36079–36087.
21. **Krebs A, Bridger WA.** 1980. The kinetic properties of phosphoenolpyruvate carboxykinase of *Escherichia coli*. Can. J. Biochem. **58:**309–318.
22. **Kim P, Laivenieks M, Vieille C, Zeikus JG.** 2004. Effect of overexpression of *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase on succinate production in *Escherichia coli*. Appl. Environ. Microbiol. **70:**1238–1241.
23. **Shi A, Zhu X, Lu J, Zhang X, Ma Y.** 2013. Activating transhydrogenase and NAD kinase in combination for improving isobutanol production. Metab. Eng. **16:**1–10.
24. **Tang J, Zhu X, Lu J, Liu P, Xu H, Tan Z, Zhang X.** 2013. Recruiting alternative glucose utilization pathways for improving succinate production. Appl. Microbiol. Biotechnol. **97:**2513–2520.
25. **Lu J, Tang J, Liu Y, Zhu X, Zhang T, Zhang X.** 2012. Combinatorial modulation of *galP* and *glk* gene expression for improved alternative glucose utilization. Appl. Microbiol. Biotechnol. **93:**2455–2462.
26. **Murphy KC.** 1998. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. J. Bacteriol. **180:**2063–2071.
27. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
28. **Clark DP.** 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol. Rev. **5:**223–234.
29. **Postma PW, Lengeler JW, Jacobson GR.** 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
30. **Flores N, Xiao J, Berry A, Bolivar F, Valle F.** 1996. Pathway engineering for the production of aromatic compounds in *Escherichia coli*. Nat. Biotechnol. **14:**620–623.
31. **Hernandez-Montalvo V, Martinez A, Hernandez-Chavez G, Bolivar F, Valle F, Gosset G.** 2003. Expression of *galP* and *glk* in a *Escherichia coli* PTS mutant restores glucose transport and increases glycolytic flux to fermentation products. Biotechnol. Bioeng. **83:**687–694.
32. **Gosset G.** 2005. Improvement of *Escherichia coli* production strains by modification of the phosphoenolpyruvate:sugar phosphotransferase system. Microb. Cell Fact. **4:**14. doi:10.1186/1475-2859-4-14.
33. **Swings J, De Ley J.** 1977. The biology of *Zymomonas*. Bacteriol. Rev. **41:**1–46.
34. **Seo JS, Chong H, Park HS, Yoon KO, Jung C, Kim JJ, Hong JH, Kim H, Kim JH, Kil JI, Park CJ, Oh HM, Lee JS, Jin SJ, Um HW, Lee HJ, Oh SJ, Kim JY, Kang HL, Lee SY, Lee KJ, Kang HS.** 2005. The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. Nat. Biotechnol. **23:**63–68.