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Influence of Gluconeogenic Phosphoenolpyruvate Carboxykinase (PCK) Expression on Succinic Acid Fermentation in *Escherichia coli* Under High Bicarbonate Condition

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Abstract The effects of amplifying the gluconeogenic phosphoenolpyruvate carboxykinase of *Escherichia coli* (pck_{Ec}) on succinic acid production in *E. coli* were examined under anaerobic condition. No significant increase in succinic acid production was observed in *E. coli* overexpressing the pck_{Ec} gene without supplementing $NaHCO_3$ or $MgCO_3$. On the other hand, succinic acid production was enhanced as the $NaHCO_3$ concentration was increased. When 20 g/l of $NaHCO_3$ was added, succinic acid production in recombinant *E. coli* overexpressing PCK was 2.2-fold higher than that observed in the wild-type strain. It was concluded that the gluconeogenic pck_{Ec} overexpression enabled *E. coli* to enhance succinic acid production only under the high bicarbonate supplementation condition.

Key words: *Escherichia coli*, succinic acid, phosphoenolpyruvate carboxykinase, $NaHCO_3$

Succinic acid, a four-carbon metabolite of the tricarboxylic acid cycle and also one of the mixed acids produced during the fermentation, has been considered to be an important intermediary chemical feedstock, as it finds numerous applications in agricultural, food, and pharmaceutical industries [23]. Succinic acid has mostly been synthesized from petrochemical-based maleic acid, but its fermentative production is drawing much attention in response to the current need to develop sustainable processes using renewable resources. This is an important point, as succinic acid can be produced from renewable environmentally sound carbohydrates rather than relying on limited petrochemical hydrocarbons. Succinic acid (C4) is synthesized by CO_2 fixation-based carboxylation of C3 metabolites [12]. This unique CO_2 fixation makes fermentative succinic acid production even more attractive, as it copes well with the Kyoto Protocol.

Metabolic engineering studies have been performed to develop a recombinant *Escherichia coli* strain capable of producing succinic acid with high productivity and yield [9, 17]. The enzymes overexpressed in *E. coli* for enhanced succinic acid production include phosphoenolpyruvate carboxylase (PPC) [3], pyruvate carboxylase (PYC) [20], and malic enzyme (MAE) [7]. Succinic acid production in *E. coli* could also be increased by mutations in the genes for lactate dehydrogenase (*ldh*) and pyruvate formate-lyase (*pfl*) [2], and the gene for the glucose-specific transporter of the phosphotransferase system (*ptsG*) [1]. The *ldh*$^-$ *pfl*$^-$ *ptsG*$^-$ mutant *E. coli* strain (AFP111) overexpressing the *Rhizobium etli pyc* gene was able to produce succinic acid as its primary end-product [22]. Recently, San and co-workers have reported strategies for the metabolic engineering of *E. coli* for the production of succinic acid under aerobic condition [21].

In addition to recombinant *E. coli* strains, ruminal bacteria have also been studied for succinic acid fermentation. Three ruminal bacterial species, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Mannheimia succiniciproducens* [5, 6, 19], naturally produce succinic acid as a major fermentation product. In these ruminal bacteria, phosphoenolpyruvate (PEP) is converted to oxaloacetic acid (OAA) mainly by PEP carboxykinase (PCK); thus, the ruminal bacterial PCK was suggested as a target enzyme for possible enhancing succinic acid fermentation in *E. coli*. Kim *et al.* [10] recently showed that succinic acid production could be enhanced in a *ppc* mutant *E. coli* strain expressing the *A. succinogenes* pck_{Ac} gene. On the other hand, Millard *et al.* [14] reported that the overexpression of the *E. coli* pck_{Ec} gene in *E. coli* had no effect on succinic acid production. In this study,

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we reevaluated succinic acid production by *E. coli* overexpressing its own pck_{Ec} gene in a medium with or without supplementing a carbonate source. It was found that succinic acid production can be enhanced in the presence of $NaHCO_3$.

MATERIALS AND METHODS

Plasmids and Strains

The plasmids and strains used in this study are listed in Table 1. Routine DNA manipulations were performed as described in Sambrook and Russell [18]. The pck_{Ec} gene was amplified by PCR using *E. coli* K12 (Korean Collection of Type Culture, KCTC 2223) genomic DNA as a template. PCR amplification was performed using the following primer pair: the forward primer, 5-GAATTCATGCGCG-TTAACAATGGTTTGACCCC-3 (EcoRI site underlined), and the backward primer, 5-CTGCAGTTACAGTTTCG-GACCAGCCGCTAC-3 (PstI site underlined). The 1.6-kb PCR product corresponding to the pck_{Ec} gene was cloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.), and the pck_{Ec} gene was subcloned into the EcoRI-PstI sites of pTrc99A expression vector to make pEcPCK. The *E. coli* DH5α (Invitrogen Co., Carlsbad, CA, U.S.A.) was used for plasmid construction, and the wild-type K12 strain and a *ppc* mutant strain were used for the production of succinic acid. Cells were transformed by electroporation (Gene Pulser, Bio-Rad, Hercules, CA, U.S.A.) to express the pck_{Ec} gene. The *ppc-5::kan* gene from JCL1242 (CGSC 7728) was introduced into K12 by P1 transduction to construct K12*ppc*- strain, as previously described [15].

Culture Conditions

Luria-Bertani medium (LB medium; tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) was used in all experiments. Ampicillin (Ap, 50 μg/ml) or kanamycin (Km, 20 μg/ml) was added when necessary. A single bacterial colony was inoculated into a 15-ml tube containing 4 ml of LB medium. After 12 h cultivation in a shaking incubator at 37°C, 120 μl of the preculture was transferred to a polytetrafluorethylene (PTFE) tube with a silicon-rubber septum, which contained 12 ml of LB medium supplemented with 9 g/l glucose, 20 g/l of $NaHCO_3$, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The source of carbonate in the medium was varied (0.5, 5, 10, 20, or 50 g/l of $NaHCO_3$, or 0.15 or 15 g/l of $MgCO_3$) as necessary. The tube was placed in an anaerobic jar (GasPak 150 System, Becton Dickinson & Co., Franklin Lakes, NJ, U.S.A.), and the air in the headspace was removed by using an aspirator and three oxygen trap packs (GasPak CO_2 System Envelope, Becton Dickinson & Co.). The anaerobic jar was placed in a static incubator for 24 h at 37°C. All experiments were performed in at least triplicates.

Analysis

To measure the concentrations of fermentation products and enzyme activities, the cells were harvested and disrupted on ice using a sonicator (Vibracell, Sonics & Materials Inc., Danbury, CT, U.S.A.) at 30 W for 1 min at 1-sec intervals. Protein contents in the extracts were determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) using bovine serum albumin as a standard. The PCK activity was determined by measuring ATP formation at 37°C with an ATP bioluminescent assay kit (FL-AA, Sigma Chemical Co., St. Louis, MO, U.S.A.). The mixture (200 μl) for PCK assay was composed of 100 mM Tris-HCl (pH 7.8), 5 mM PEP, 35 mM $NaHCO_3$, 16 mM $MgCl_2$, 10 mM ADP, and the ATP assay premix containing luciferase and luciferin. The reaction was initiated by adding 100 μl of the cell extract and the mixture was allowed to form ATP for 3 min at room temperature. The

Table 1. Plasmids and strains used in this study.

Plasmids and strains	Description	Source
Plasmids		
pGEM-T	PCR cloning vector	Promega[a]
pTrc99A	Expression vector, trc promoter, Ap^R	AP Biotech[b]
pEcPck	pTrc99A with pck_{Ec} gene at the EcoRI-PstI sites	This study
Strains		
DH5α	F- Φ80 d*lacZ* ΔM15 Δ (*lacZYA-argF*) U169 *endA1 recA1 hsdR17* ($r_K^- m_K^+$) *deoR thi-1 phoA supE44* λ^- *gyrA96 relA1*	Invitrogen[c]
K12	Wild-type *E. coli* strain	KCTC[d] 2223
K12 *ppc*-	K12 *ppc*-5::*kan*, Km^R	This study
K12 (pEcPCK)	K12 harboring pEcPCK	This study
K12 *ppc*-(pEcPCK)	K12 *ppc*- harboring pEcPCK	This study

[a]Madison, WI, U.S.A.
[b]Uppsala, Sweden.
[c]Carlsbad, CA, U.S.A.
[d]Korean Collection of Type Culture, Daejeon, Korea.

ATP formation was estimated by a luminometer (20/20n Luminometer System, Turner Biosystem Inc., Sunnyvale, CA, U.S.A.) and by ATP standard curve. The PCK activity was expressed as mmole of ATP produced per mg-protein per min. The PPC activity was estimated by measuring OAA formation coupled with malate dehydrogenase and NADH. The reaction mixture (1 ml) was composed of 50 mM HEPES (pH 7.3), 10 mM $MgCl_2$, 5 mM PEP, 5 mM $NaHCO_3$, 0.2 mM NADH, 4 units of malate dehydrogenase, and 100 µl of the cell extract. The absorbance at 340 nm was measured after incubating the reaction mixture for 15 min at 37°C. The extinction coefficient for NADH was 6.22 cm^{-1} mM^{-1} at 340 nm. The PPC activity was corrected by subtracting PCK activity from the above OAA-forming activity.

Fermentation products were analyzed by a high performance liquid chromatography (Waters, Milford, MA, U.S.A.) equipped with a cation-exchange column (HPX-87H, Bio-Rad, Hercules, CA, U.S.A.) and a refractive index (RI) detector. The mobile phase used was 0.5 mM H_2SO_4 solution, which was isocratically controlled at a rate of 0.6 ml/min. The column temperature was maintained at 60°C. Glucose consumption was confirmed by the dinitrosalicilic acid (DNS) method, and the succinic acid concentration was verified using a succinic acid analysis kit (Roche/Boehringer Mannheim, Basel, Switzerland). Biomass was estimated by measuring the optical density at 600 nm (OD_{600}). The expression of PCK was confirmed by SDS-polyacrylamide gel (10%) electrophoresis followed by Coomassie Brilliant Blue staining.

Results and Discussion

The wild-type K12 strain and its *ppc*-negative mutant strain were transformed with pEcPCK; and we investigated succinic acid production in *E. coli* strains overexpressing the *E. coli* pck_{Ec} gene. The culture medium was the LB medium supplemented with 9 g/l glucose and 20 g/l of $NaHCO_3$, and the anaerobic culture was maintained for 24 h. The succinic acid productions of the strains were represented with time course in Fig. 1, and the metabolites of the strains at 24 h were shown in Table 2. The *ppc* knockout *E. coli* mutant strain expressing the pck_{Ec} gene [K12*ppc*$^-$(pEcPCK)] showed enhanced succinic acid production (14.0 mM) compared with that of wild-type K12 (6.4 mM), suggesting that the *E. coli* PCK can also enhance succinic acid production in the same manner as the *A. succinogenes* PCK [10]. On the other hand, K12*ppc*$^-$(pEcPCK) produced 20.9 mM of lactic acid, which is considerably lower than that produced in K12 (36.7 mM) or K12*ppc*$^-$ (32.7 mM) strains. Surprisingly, the wild-type K12 strain overexpressing the pck_{Ec} gene [K12(pEcPCK)] also showed increased succinic acid production (14.2 mM). It should be noted that K12(pEcPCK) cells express both PCK and PPC for PEP carboxylation, whereas K12 cells express only PPC, and K12*ppc*$^-$(pEcPCK) cells express only PCK.

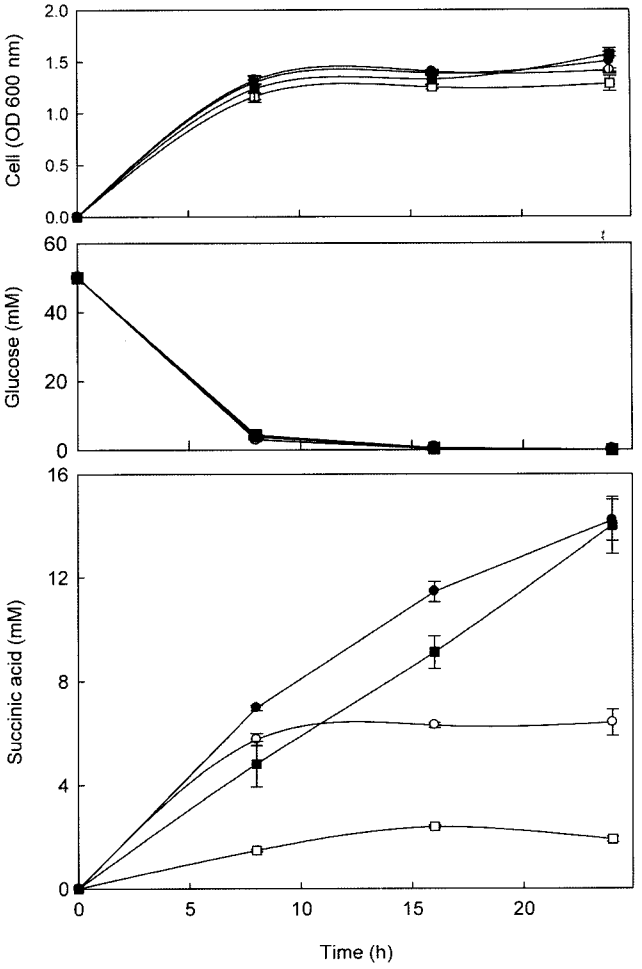

Fig. 1. Anaerobic succinic acid fermentation profile with 20 g/l $NaHCO_3$ and 9 g/l glucose supplementation.
Symbols represent K12 for (○), K12(pEcPCK) for (●), K12*ppc*- for (□), and K12*ppc*-(pEcPCK) for (■).

The reason for enhanced succinic acid production in K12(pEcPCK) cells was unclear, as the *E. coli* PCK has been known to be a gluconeogenic enzyme [4]. Millard *et al.* [14] reported that the overexpression of the pck_{Ec} gene in *E. coli* had no effect on succinic acid production. These contradictory results obtained by Millard *et al.* [14] and this study were assumed to be attributable to the availability of carbon dioxide in the two studies; we supplemented the culture medium with soluble $NaHCO_3$ (solubility at 25°C, 10 g/100 g), whereas Millard *et al.* [14] used $MgCO_3$ (solubility at 25°C, 17.5 mg/l) and CO_2 gas in the headspace as carbon dioxide sources. To test this hypothesis, fermentation of K12(pEcPCK) was carried out for 24 h in LB medium supplemented with varying concentrations of $NaHCO_3$ (0–50 g/l) or $MgCO_3$ (0–15 g/l).

Table 2. Effect of the pck_{Ec} gene expression on the production of fermentation products.

Strain	Fermentation products (mM)[a]				Biomass ($OD600_{nm}$)	PCK activity[b]	PPC activity[c]
	Succinic acid	Lactic acid	Acetic acid	Formic acid			
K12	$6.4_{\pm 0.5}$	$36.7_{\pm 4.0}$	$14.6_{\pm 0.7}$	$33.1_{\pm 2.3}$	$1.40_{\pm 0.01}$	<0.01	$2.58_{\pm 0.60}$
K12 *ppc*-	$1.9_{\pm 0.1}$	$32.7_{\pm 0.7}$	$14.7_{\pm 0.7}$	$37.5_{\pm 2.4}$	$1.32_{\pm 0.04}$	<0.03	ND[d]
K12 (pEcPCK)	$14.2_{\pm 0.8}$	$19.8_{\pm 3.1}$	$15.1_{\pm 0.1}$	$29.6_{\pm 0.8}$	$1.44_{\pm 0.06}$	$0.48_{\pm 0.07}$	$1.40_{\pm 0.02}$
K12*ppc* (pEcPCK)	$14.0_{\pm 1.1}$	$20.9_{\pm 1.0}$	$16.9_{\pm 0.9}$	$37.9_{\pm 3.3}$	$1.56_{\pm 0.01}$	$0.21_{\pm 0.03}$	ND

[a]Values are shown as mean±standard deviation. Fermentation products were harvested and analyzed at 24-h culture.
[b]PCK activity was measured as μmol ATP produced per mg-protein per min.
[c]PPC activity was measured as mmol NADH used per mg-protein per min when coupled with malate dehydrogenase.
[d] Not determined.

The final succinic acid concentrations obtained are shown in Fig. 2. Compared with the control (no carbonate supplementation), the differences in succinic acid concentrations obtained with wild-type K12 and K12(pEcPCK) were not significant when the $NaHCO_3$ concentration was less than 5 g/l or when 15 g/l of $MgCO_3$ was used. The positive effect of pck_{Ec} gene overexpression on succinic acid production was observed at $NaHCO_3$ concentrations greater than 10 g/l. The succinic acid concentration obtained by cultivating K12(pEcPCK) in a medium supplemented with 20 g/l $NaHCO_3$ was 14.2 mM, which was 2.2 times higher than that obtained with K12 cells (6.4 mM). These results suggest that the overexpression of the pck_{Ec} gene can enhance succinic acid production in *E. coli* only at sufficiently high concentrations of HCO_3^-. Considering the insolubility of $MgCO_3$, PCK would not have sufficient HCO_3^- ions for the carboxylation of PEP, resulting in no increase of succinic acid as reported by Millard *et al.* [14].

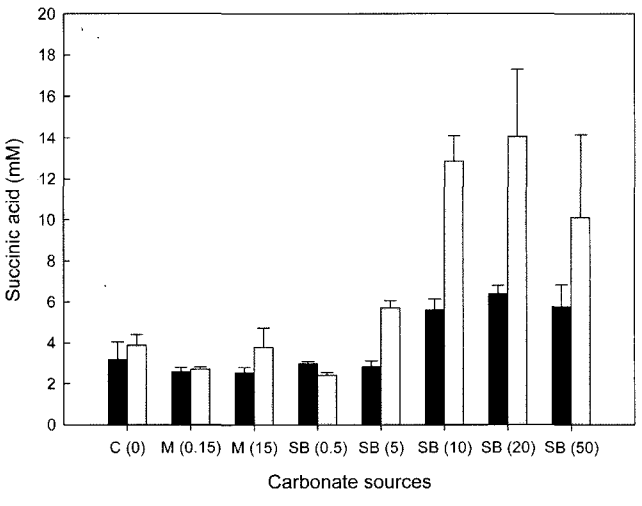

Fig. 2. Succinic acid production (mM) by K12 (black bar) and K12 (pEcPCK) (gray bar) in media supplemented with varying concentrations of $MgCO_3$ or $NaHCO_3$.
The C, M, and SB on the x-axis stand for control, $MgCO_3$, or $NaHCO_3$, respectively. Numbers in parentheses represent the supplemented concentrations (g/l).

The enhanced succinic acid productions in both K12 and K12(pEcPCK) cells were dependent on the bicarbonate concentration, as in ruminal bacteria [13, 19]. High bicarbonate concentration enabled ruminal bacteria and recombinant *E. coli* to produce more succinic acid because succinic acid is synthesized via CO_2 fixation of C3 metabolites. K12(pEcPCK) cells express both PCK and PPC for PEP carboxylation, whereas K12 cells expressed only PPC. It was reported that the K_m value of PCK for HCO_3^- is 13 mM, whereas that of PPC for HCO_3^- is 0.15 mM [8, 11, 16]. Thus, in the presence of both PPC and PCK, PPC would perform PEP carboxylation at a low concentration of HCO_3^-. On the other hand, PCK would be able to catalyze the reaction at a high concentration of HCO_3^-. In addition, the PCK reaction appears to be more suitable for succinic acid production because it generates nucleotide triphosphate, thus conserving the energy, whereas the PPC reaction generates no nucleotide triphosphate.

Succinic acid production in K12(pEcPCK) cells was lower at 50 g/l $NaHCO_3$ (10.1 mM) than at 20 g/l $NaHCO_3$ (14.2 mM). The reason for the inhibition of succinic acid production at high HCO_3^- concentrations is not clear. It may be due to the inhibitory effect of monovalent ion accumulation in *E. coli* cells, preventing proper metabolization. An unknown inhibitory effect of high bicarbonate concentrations on PPC and PCK activities could be another contributing factor.

By taking into consideration the intracellular bicarbonate concentration, enzyme affinity for bicarbonate, and enzyme inhibition by high concentration of bicarbonate, the recombinant *E. coli* system can be further developed for the more efficient production of succinic acid.

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