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

KWON, YEONG DEOK¹,³, SANG YUP LEE², and PIL KIM³*

¹Department of Life Science, ³Department of Biotechnology, The Catholic University of Korea, Bucheon 420-743, Korea
²Department of Chemical and Biomolecular Engineering, KAIST, Daejeon 305-701, Korea

Received: April 17, 2006
Accepted: May 18, 2006

**Abstract**

The effects of amplifying the gluconeogenic phosphoenolpyruvate carboxykinase of *Escherichia coli* (*pck*<sub>Ec</sub>) 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*<sub>Ec</sub> gene without supplementing NaHCO<sub>3</sub> or MgCO<sub>3</sub>. On the other hand, succinic acid production was enhanced as the NaHCO<sub>3</sub> concentration was increased. When 20 g/l of NaHCO<sub>3</sub> 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*<sub>Ec</sub> 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<sub>3</sub>

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<sub>2</sub> fixation-based carboxylation of C3 metabolites [12]. This unique CO<sub>2</sub> 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*<sup>−</sup>*pfl*<sup>−</sup>*ptsG*<sup>−</sup> mutant *E. coli* strain (AFP1) 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. succiniciproducens pck*<sub>Ec</sub> gene. On the other hand, Millard et al. [14] reported that the overexpression of the *E. coli pck*<sub>Ec</sub> gene in *E. coli* had no effect on succinic acid production. In this study,
we reevaluated succinic acid production by E. coli overexpressing its own pckEc 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 NaHCO₃.

**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 pckEc gene was amplified by PCR using E. coli K12 (Korean Collection of Type Culture, KCTC 2223) genomic DNA as a template. The PCR product corresponding to the pckEc gene was cloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.), and the pckEc gene was subcloned into the EcoRI-PstI sites of pT_hosts99A 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 DH5α (Invitrogen Co., Carlsbad, CA, U.S.A.) was used for the production of succinic acid. Cells were transformed by electroporation into pGEM-T easy vector (Promega, Madison, WI, U.S.A.), 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.) 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-IA, Sigma Chemical Co., St. Louis, MO, U.S.A.).

**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. Isopropyl-β-D-thiogalactopyranoside (IPTG) 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 NaHCO₃, 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 NaHCO₃, or 0.15 or 15 g/l of MgCO₃) 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 CO₂ 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.

**Table 1. Plasmids and strains used in this study.**

<table>
<thead>
<tr>
<th>Plasmids and strains</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>PCR cloning vector</td>
<td>Promega&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pT_hosts99A</td>
<td>Expression vector, trc promoter, Ap&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AP Biotech&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>pEcPck</td>
<td>pT_hosts99A with pckEc gene at the EcoRI-PstI sites</td>
<td>This study</td>
</tr>
<tr>
<td>DH5α</td>
<td>F- ΔacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 (rK&lt;sup&gt;−&lt;/sup&gt; mK&lt;sup&gt;−&lt;/sup&gt;) deoR thi-1 phoA supE44 thi-1 gyrA96 relA1</td>
<td>Invitrogen&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>K12</td>
<td>Wild-type E. coli strain</td>
<td>KCTC&lt;sup&gt;d&lt;/sup&gt; 2223</td>
</tr>
<tr>
<td>K12 ppc&lt;sup&gt;−&lt;/sup&gt;</td>
<td>K12 ppc&lt;sup&gt;−&lt;/sup&gt;:kan, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>K12 (pEcPCK)</td>
<td>K12 harboring pEcPCK</td>
<td>This study</td>
</tr>
<tr>
<td>K12 ppc&lt;sup&gt;−&lt;/sup&gt;(pEcPCK)</td>
<td>K12 ppc&lt;sup&gt;−&lt;/sup&gt; harboring pEcPCK</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Madison, WI, U.S.A.
<sup>b</sup>Uppsala, Sweden.
<sup>c</sup>Carlsbad, CA, U.S.A.
<sup>d</sup>Korean Collection of Type Culture, Daejeon, Korea.
ATP formation was estimated by a luminometer (20/20n Luminometer System, Turner Biosystem Inc., Sunnyvale, CA, USA) and by ATP standard curve. The PCK activity was expressed as mmol 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$_2$SO$_4$ 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 [K12ppc$^-$ (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, K12ppc$^+$ (pEcPCK) produced 20.9 mM of lactic acid, which is considerably lower than that produced in K12 (36.7 mM) or K12ppc$^-$ (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 K12ppc$^-$ (pEcPCK) cells express only PCK.

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).
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 NaHCO₃ concentration was less than 5 g/l or when 15 g/l of MgCO₃ was used. The positive effect of pckE gene overexpression on succinic acid production was observed at NaHCO₃ concentrations greater than 10 g/l. The succinic acid concentration obtained by cultivating K12(pEcPCK) in a medium supplemented with 20 g/l NaHCO₃ 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 pckE gene can enhance succinic acid production in E. coli only at sufficiently high concentrations of HCO₃⁻. Considering the insolubility of MgCO₃, PCK would not have sufficient HCO₃⁻ ions for the carboxylation of PEP, resulting in no increase of succinic acid as reported by Millard et al. [14].

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 CO₂ 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 Kₘ value of PCK for HCO₃⁻ is 13 mM, whereas that of PPC for HCO₃⁻ is 0.15 mM [8, 11, 16]. Thus, in the presence of both PCK and PPC, PPC would perform PEP carboxylation at a low concentration of HCO₃⁻. On the other hand, PCK would be able to catalyze the reaction at a high concentration of HCO₃⁻. In addition, the PCK reaction appears to be more suitable for succinic acid production because it generates nucleotide triphosphate, thus conserving energy, whereas the PPC reaction generates no nucleotide triphosphate.

Succinic acid production in K12(pEcPCK) cells was lower at 50 g/l NaHCO₃ (10.1 mM) than at 20 g/l NaHCO₃ (14.2 mM). The reason for the inhibition of succinic acid production at high HCO₃⁻ concentrations is not clear. It may be due to the inhibitory effect of monovalent ion accumulation in E. coli cells, preventing proper metabolism. 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.

**Acknowledgments**

This work was funded by the Genome-based Integrated Bioprocess Development Project from the Korean Ministry of Science and Technology. P. Kim was partly supported by the Catholic University of Korea research fund 2006.

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**Table 2.** Effect of the pckE gene expression on the production of fermentation products.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fermentation products (mM)</th>
<th>Biomass (OD₆₀₀nm)</th>
<th>PCK activity</th>
<th>PPC activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinic acid</td>
<td>Lactic acid</td>
<td>Acetic acid</td>
<td>Formic acid</td>
</tr>
<tr>
<td>K12</td>
<td>6.4±0.5</td>
<td>36.7±0.8</td>
<td>14.6±0.7</td>
<td>33.1±2.3</td>
</tr>
<tr>
<td>K12 ppc</td>
<td>1.9±0.1</td>
<td>32.7±0.7</td>
<td>14.7±0.7</td>
<td>37.5±2.4</td>
</tr>
<tr>
<td>K12 (pEcPCK)</td>
<td>14.2±0.8</td>
<td>19.8±3.1</td>
<td>15.1±0.2</td>
<td>29.6±0.8</td>
</tr>
<tr>
<td>K12ppc (pEcPCK)</td>
<td>14.0±1.1</td>
<td>20.9±4.0</td>
<td>16.9±1.9</td>
<td>37.9±3.3</td>
</tr>
</tbody>
</table>

*Values are shown as mean±standard deviation. Fermentation products were harvested and analyzed at 24-h culture.

PPC activity was measured as µmol ATP produced per mg-protein per min.

PCK activity was measured as mmol NADH used per mg-protein per min when coupled with malate dehydrogenase.

ND Not determined.