To determine whether intracellular ATP levels can be affected, *Escherichia coli* overexpressing phosphoenolpyruvate carboxykinase (**pck**) or phosphoenolpyruvate carboxylase (**ppc**) were grown in glucose minimal medium. The Pck-overexpressing cells showed approximately twice the intracellular ATP concentration, with 22% slower growth than the Ppc-overexpressing strain. This unexpected result of higher ATP coupled with slower growth is discussed based on transcriptome analysis.

**Key words:** PEP carboxykinase; PEP carboxylase; intracellular ATP

Many attempts have been made to produce four-carbon (C4) dicarboxylic acid metabolites, found in TCA cycle, through metabolic engineering using renewable resources.1-3 Because C4 metabolites are synthesized in the same pathways in which C3 metabolites are carboxylated, overexpression of anaplerotic enzymes is of interest with a view to C4 metabolite production. Phosphoenolpyruvate carboxylase (**ppc**) is an anaplerotic enzyme that mediates the carboxylation of phosphoenolpyruvate (**PEP**) into oxaloacetate (**OAA**) under glycolysis conditions in *Escherichia coli*. Under gluconeogenesis conditions, **ppc** is repressed and phosphoenolpyruvate carboxykinase (**pck**) is expressed.4-6 Although carboxylation of **PEP** into **OAA** mediated by **Pck** occurs only with a supplemental CO2 source or in a complex medium, conditions that result in higher succinate production, it has been proposed that the **pck** reaction to lead to increased cellular growth and biomolecule production, since it produces more energy than the **ppc** reaction.3,5,6 This is because the **ppc** reaction releases a phosphate, in contrast to the **pck** reaction, which produces a high-energy ATP (Fig. 1). The intracellular ATP concentration is one of the most important factors in cellular physiology. It regulates many events. For example, ribosomal RNA synthesis is enhanced by high NTP concentrations,7 and a larger ATP pool is present in rapidly growing *E. coli* in chemostat cultures.8 Cellular metabolic pathways, such as glycolysis, the phosphotransferase system, and the glyoxylate shunt, are also affected by the intracellular ATP concentration.9-12 In this paper, we report the intracellular ATP concentrations in *E. coli* overexpressing Pck and Ppc grown in minimal glucose medium and complex glucose medium. The genes upregulated and downregulated in the above strains were identified to determine the physiology of the cells. Succinate production caused by Pck/Ppc overexpression is discussed based on the cellular physiology results.

*E. coli* W3110 (KCTC 2223) was used in subsequent experiments. Ppc expression vector pEcPPC and Pck expression vector pEcPCK were described previously.5,13 Two different media were used in the fermentation of Pck- and Ppc-overexpressing *E. coli*, both containing glucose as the main carbon source and supplemental bicarbonate. The minimal glucose medium, based on M9 medium, consisted of (per liter) 0.8 g of NH4Cl, 0.5 g of NaCl, 7.5 g of Na2HPO4·2H2O, and 3 g of KH2PO4, with trace elements (0.2 g of MgSO4·7H2O, 0.1 g of CaCl2, 1 mg of thiamine, 10 mg of FeCl3·6H2O, 1.8 mg of CoCl3·6H2O, 2.2 mg of MnSO4·H2O, and 1.8 mg of Na2EDTA·6H2O), 9 g of sterilized glucose, 10 g of NaHCO3, 50 mg of ampicillin, and 0.1 nm isopropyl-β-D-thiogalactopyranoside (IPTG), added sequentially. The complex glucose medium contained 5 g of NaCl, 10 g of yeast extract, 9 g of glucose, 10 g of NaHCO3, 50 mg of ampicillin, and 0.1 mmol of IPTG per liter. The initial pH of the complex glucose medium was adjusted to 7.3 with 1 N HCl. For initial comparison of fermentation behavior, cells were inoculated (1% v/v) into 12 ml of medium in a 15-ml polytetrafluoroethylene (PTFE) tube with a silicone septum, and cultured in an anaerobic jar (GasPak 150 System, Becton Dickinson, Franklin Lakes, NJ) at 37°C for 24 h. Cells in exponential phase, obtained with a 250-ml fermentor containing 100 ml of medium (Mini-Chemostat Fermentor; Biotron, Bucheon, Korea),
Korea), were subjected to physiological analysis. Active growing cells in LB medium (1 ml) were transferred into a 250-ml fermentor containing 100 ml of minimal glucose medium, and the fermentor was maintained at 37 °C at 350 rpm, with CO2 gas flushing at 20 ml/min. The sample in the fermentor was collected after 6h of cultivation and frozen rapidly for further analysis. For complex glucose medium cultures, the feeding and outlet pump flow rates were set at 0.166 ml/min (dilution rate = 0.1 h⁻¹) after 8h of batch-mode cultivation to obtain steady-state cells. After three turnover times (30h), 5-ml aliquots of the chemostat culture were frozen rapidly in liquid nitrogen for later analysis.

The biomass, enzyme activities, and fermentation products were determined as previously described. Intracellular ATP concentrations were determined using an ATP determination kit (FL-AA; Sigma Chemical, St. Louis, MO) and a luminometer (20/20n Luminometer System, Turner Biosystems, Sunnyvale, CA) with an ATP standard. Total mRNA from Pck- and Ppc-overexpressing E. coli W3110 was compared to that of the control using DNA microarrays (Frontier Project team, KIRIBB, Daejon, Korea). Transcriptomes of the cells were prepared using an RNA extraction kit (RNeasy Mini Kit; Qiagen, Hilden, Germany) following the manufacturer’s instructions. Hybridization and washing were performed at Digital Genomics (Seoul, Korea). The DNA microarray results were deposited in the Gene Expression Omnibus.

Here we report that overexpression of Pck and of Ppc in E. coli growing in LB-glucose medium containing supplemental CO2 resulted in cells with enhanced succinate levels. To study the physiology of these cells, chemostat cultures of E. coli strains overexpressing Pck or Ppc were grown in complex glucose medium and minimal glucose medium at various dilution rates. At a dilution rate of 0.3 h⁻¹, Pck-overexpressing E. coli (W3110/Pck) grown in complex glucose medium was washed out of the chemostat, but control (W3110) and Ppc-overexpressing E. coli (W3110/Ppc) achieved steady-state growth after 30h. Only a lower dilution rate (D = 0.1 h⁻¹) led to steady-state growth of W3110/Pck, and of W3110/Ppc and control cells, in complex glucose medium. In minimal glucose medium, W3110/Pck was washed out more rapidly than in complex glucose medium, even at D = 0.1 h⁻¹, although the control and W3110/Ppc strains achieved steady-state growth. The finding that steady-state growth of E. coli overexpressing Pck at D = 0.1 h⁻¹ could be achieved only in complex glucose medium, whereas steady-state growth could be reached for all of the chemostat cultures of E. coli overexpressing Ppc, which mediates the same reaction as Pck except for ATP generation, was surprising. This result encouraged us to investigate batch-mode fermentation of E. coli strains overexpressing Pck and Ppc under anaerobic conditions (Table 1). Under these conditions, both Pck- and Ppc-overexpressing E. coli grown in complex glucose medium consumed 9 g/l of glucose in 24 h, and produced higher levels of succinate than the control. The final biomass was in the range of 0.46–0.48 g/l for both strains and control. In minimal glucose medium, however, W3110/Pck consumed less glucose (6.0 g/l) than W3110/Ppc (8.4 g/l) or the control (8.2 g/l). The succinate yield of W3110/Pck was even lower (12.0%) than that of the control (13.0%), in contrast to the results in complex glucose medium.

Since W3110/Pck did not show steady-state growth in minimal glucose medium, the strain was cultured in batch mode in this medium and harvested at the middle log phase at 6h after inoculation for physiological analysis (Table 2A). The most biomass, 0.23 g/l, was observed in the control cells, and the growth of the Ppc-overexpressing strain was 14% lower (0.20 g/l) than that of the control, which is understandable given the metabolic load of the overexpressing cells. The growth of Pck-overexpressing cells (0.16 g/l) was even lower than that of the control or the Ppc-expressing cells. The glucose consumption trend of these strains over 6h was similar to the growth trend, with the greatest glucose consumption in the control (3.7 g/l), somewhat

### Table 1. Batch-Mode Succinate Fermentation of E. coli-Overexpressing Pck and Ppc

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biomass (g/l)</th>
<th>Glucose consumption (g)</th>
<th>aSuc yield (%)</th>
<th>bBiomass yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal glucose medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>0.25 ± 0.002</td>
<td>8.2 ± 0.10</td>
<td>13.0 ± 0.01</td>
<td>3.0</td>
</tr>
<tr>
<td>W3110/Ppc</td>
<td>0.24 ± 0.01</td>
<td>8.4 ± 0.15</td>
<td>25.0 ± 0.03</td>
<td>2.8</td>
</tr>
<tr>
<td>W3110/Pck</td>
<td>0.24 ± 0.01</td>
<td>6.0 ± 0.69</td>
<td>12.0 ± 0.01</td>
<td>4.0</td>
</tr>
<tr>
<td>Complex glucose medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>0.46 ± 0.01</td>
<td>9.0 ± 0.01</td>
<td>12.8 ± 0.14</td>
<td>5.1</td>
</tr>
<tr>
<td>W3110/Ppc</td>
<td>0.46 ± 0.01</td>
<td>9.0 ± 0.01</td>
<td>22.0 ± 0.04</td>
<td>5.1</td>
</tr>
<tr>
<td>W3110/Pck</td>
<td>0.48 ± 0.01</td>
<td>9.0 ± 0.01</td>
<td>36.2 ± 1.62</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Cells were static-cultured anaerobically in test tubes at 37 °C and harvested after 24h. The initial medium contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 ml IPTG, and 50 μg/ml of ampicillin. Data are represented as means ± SD for three independent experiments.

aSuc yield, mole ratio of [mmol succinate production]/[mmol glucose consumption]
bBiomass yield, ratio of [g DCW]/[g glucose consumption]
lower consumption in Ppc-overexpressing cells (3.6 g/l), and the lowest consumption in Pck-overexpressing cells (2.5 g/l). The biomass yields and succinate production of all of the strains were similar, at about 5.6–6.4% and about 0.19–0.26%, respectively. The intracellular ATP levels in the control (0.58 mmol/g) and Ppc-overexpressing (0.69 mmol/g) lines were similar, but the Pck-overexpressing (0.58 mmol/g) lines were different. Analysis of the transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211798. Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested 6 h after inoculation. The initial medium was based on minimal medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211801.

To compare the transcription regulation in these cells at different ATP levels and growth rates, the transcriptomes of Pck-overexpressing W3110 and wild-type E. coli grown in minimal glucose medium were compared using DNA microarrays (Gene Expression Omnibus access code, GSM211798). More than 3-fold upregulation of genes related to energy metabolism (mgtA, adhE) and building-block biosynthesis were found. Genes related to utilization of carbon sources other than glucose were downregulated more than 3-fold. This result was expected because of the presence of glucose in the medium. Surprisingly, genes categorized as related to the SOS response, such as sulA, recN, dinI, recA, recX, were upregulated. No upregulation of glycolysis enzyme expression was found, even though glucose was the only carbon source in the medium.

Fermentation of E. coli strains overexpressing Pck or Ppc in complex glucose medium with suplemental CO₂ has been used in the metabolic engineering of succinate production, and Pck overexpression has been proposed to lead to better growth than Ppc overexpression, since Pck yields more ATP during carbon fixation.5,6,13) We confirmed that intracellular ATP levels increased with overexpression of Pck, which mediates the ATP generation step, but we also observed that Pck overexpression in minimal glucose medium did not cause high succinate production. The low succinate production might have been due to low glucose consumption and thereby to low growth.

It is unclear why Pck-overexpressing cells showed enhanced succinate production and normal growth in complex glucose medium but no increased succinate production in minimal glucose medium. In a chemostat culture in complex glucose medium, cells overexpressing Pck (D = 0.1 h⁻¹) showed the same growth as Ppc-overexpressing cells (0.45 g/l), but approximately twice the intracellular ATP content (0.38 vs. 0.65 mmol/g respectively) (Table 2B). Analysis of the transcriptome of Pck-overexpressing cells grown in complex glucose medium in a chemostat (GEO code, GSM211801) revealed upregulation of many genes involved in motility, glycolysis, and biosynthesis, as compared to their expression in control cells. Minimal glucose medium-grown cells in early log phase did not show activation of glycolysis genes (GEO code, GSM211798), unlike the transcriptome of complex glucose medium-grown cells. Even though the cells contained high levels of ATP, glycolysis flux might have been insufficient for the biosynthesis of building blocks and succinate production in minimal glucose medium, since glycolysis was the only gateway for carbon flow. Koebmann et al. reported that low ATP levels triggered an increase in glycolytic flux in E. coli,10) and Noda et al. found that ATPase-defective E. coli increased in glucose consumption.10) Based on the recent reports, we hypothesize that the high ATP levels caused by Pck overexpression slowed the rate of glucose consumption and, consequently, the cellular growth and succinate production in minimal glucose medium. In contrast, an unknown regulation mechanism active in complex medium might upregulate glycolysis-related genes when high ATP levels are also present, providing enough metabolite resources for growth and an increase in succinate production. A hypothetical factor present in complex medium might have triggered an increase in glycolysis even though large amounts of ATP were available, and the high ATP levels provided extra potential for growth and succinate production.

Pck overexpression might be useful in the metabolic engineering of biomolecule synthesis if the pathway contains a carboxylation step, due to the greater ATP availability it causes, if glucose consumption can be enhanced. We are currently attempting to metabolically engineer cells that contain high ATP levels derived from Pck overexpression but that do not show decreased glucose consumption.

### Table 2. Intracellular ATP and Growth of E. coli-Overexpressing Pck and Ppc in Minimal Glucose Medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biomass yield (g/L)</th>
<th>Glucose consumption yield (g)</th>
<th>Suc yield (%)</th>
<th>Intracellular ATP (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>0.23</td>
<td>3.7</td>
<td>0.19</td>
<td>6.2</td>
</tr>
<tr>
<td>W3110/Ppc</td>
<td>0.20</td>
<td>3.6</td>
<td>0.19</td>
<td>5.6</td>
</tr>
<tr>
<td>W3110/Pck</td>
<td>0.16</td>
<td>2.5</td>
<td>0.26</td>
<td>6.4</td>
</tr>
</tbody>
</table>

### Notes:
- Suc yield, mole percentage of [mmol succinate production]/[mmol glucose consumption]
- Biomass yield, percentage of [g DCW]/[g glucose consumption]
- Intracellular ATP, means ± SD of three measurements

5Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested 6 h after inoculation. The initial medium was based on minimal medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211798.

6Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested after 30 h of chemostat culture (D = 0.1 h⁻¹). The initial medium was based on LB medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data for the chemostat culture are available at GEO under access code GSM211801.

7Biomass yield, percentage of [g DCW]/[g glucose consumption]

8Intracellular ATP, means ± SD of three measurements

9Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested after 30 h of chemostat culture (D = 0.1 h⁻¹). The initial medium was based on LB medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211798.

10Biomass yield, percentage of [g DCW]/[g glucose consumption]

11Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested after 30 h of chemostat culture (D = 0.1 h⁻¹). The initial medium was based on LB medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211801.

12Cells were cultured anaerobically in a 100-ml mini-fermentor at 37 °C and harvested after 30 h of chemostat culture (D = 0.1 h⁻¹). The initial medium was based on LB medium and also contained 9 g/l of glucose, 10 g/l of bicarbonate, 0.1 mM IPTG, and 50 g/ml of ampicillin. The transcriptome raw data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) under access code GSM211801.

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Acknowledgments

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