Pyruvate production using engineered Escherichia coli

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Abstract
Pyruvate plays an essential role in the central carbon metabolism of multiple organisms and is used as a raw material in the chemical, biochemical and pharmaceutical industries. To meet demand, large amounts of pyruvate are produced through fermentation processes. Here we describe a simple and efficient method for producing pyruvate in Escherichia coli. To stop carbon flux from pyruvate to fatty acids, the accBC genes, which encode the enzyme that catalyzes the first step of fatty acid biosynthesis and is essential for vegetative growth, were manipulated within the genome; its native promoter was replaced with the tetracycline (or doxycycline)-regulated promoter and the corresponding transcriptional regulator genes. The resulting strain grew normally in the presence of doxycycline, but showed poor growth upon withdrawal of doxycycline. Using this strain, we developed a high pyruvate producing strain (strain LAFCPCPt-accBC-aceE), in which the tetracycline-regulated promoter was also introduced upstream of aceE, and the ackA-pta, adhE, cra, ldhA, pflB and poxB genes were deleted. After determining the optimal culture conditions for this strain, the final pyruvate concentration reached 26.1 g L\(^{-1}\) after 72 h with a theoretical yield of 55.6 %. These levels are high enough to indicate that the developed strain has the potential for application to industrial production of pyruvate.

Keywords: Pyruvate, Escherichia coli, Promoter regulation, Tetracycline, Doxycycline, Fermenter

Introduction
Pyruvate is a key metabolite in such catabolic and anabolic pathways as glycolysis, gluconeogenesis, and amino and fatty acid metabolism. In addition, commercial demand for pyruvate has been expanding every year. In biochemical industries, for example, pyruvate is used as a building block for the synthesis of antioxidants (Wang et al. 2007), food additives (EFSA 2009), and dietary and weight control supplements (Kalman et al. 1999; Saper et al. 2004). Pyruvate is also used as a raw material in the pharmaceutical industry. For example, various pharmaceutical precursors, including N-acetyl-d-neuraminic acid (Zhang et al. 2010), l-3,4-dihydroxyphenylalanine (Park et al. 1998) and R-phenylacetylcarnbol (Rosche et al. 2001) are produced from pyruvate.

Commercial methods of pyruvate production can be roughly classified as chemical, enzymatic or fermentation processes (Li et al. 2001a). In the chemical process, pyruvate is synthesized mainly through dehydration and decarboxylation of tartrate. But while this method is easy to implement, it is not cost effective (Li et al. 2001a). Compared with the chemical process, the enzymatic process effectively reduces the production cost and improves yield (Li et al. 2001a). The fermentation process is the most widely used in industry, because it is sustainable, cost-effective and achieves high yields and productivity (Li et al. 2001a; Xu et al. 2008). For the fermentation process, a multi-vitamin auxotroph strain of Torulopsis glabrata is primarily used (Li et al. 2001a). With T. glabrata strains, a pyruvate concentration of more than 40 g L\(^{-1}\) was reached (Li et al. 2001a), though maintenance of the concentration balance among biotin, nicotinic acid, pyridoxine and thiamine was required (Li et al. 2001b). Consequently, the availability of this method is limited by the necessity for special expertise and expensive equipment. On the other hand,
a lipoic acid auxotroph strain of *Escherichia coli* has also developed as a producer (Table 1). For example, *E. coli* CGSG 7916 showed high pyruvate accumulation in controlled fermentations (Tomar et al. 2003). This strain was constructed through *aceE* mutation. The *aceEF* and *lpd* genes express the pyruvate dehydrogenase complex, which catalyzes conversion of pyruvate into acetyl-CoA (Fig. 1; Calacob et al. 1985). Thus, pyruvate accumulation was achieved by decreasing the activity of the pyruvate dehydrogenase complex. In addition, *E. coli* YYC202, an *aceEF* deletion strain, showed excellent pyruvate productivity (Zelić et al. 2003). However, this strain required acetate for growth due to its weak capacity for acetyl-CoA production.

*E. coli* TBLA-1 was constructed by transduction of a F1-ATPase-defective gene into *E. coli* W1485 lip2, which is a derivative strain from *E. coli* K-12 (Yokota et al. 1994). This mutation enhanced both glucose consumption and pyruvate production.

*E. coli* TC44 was constructed through decreasing the ATP yield, cell growth and CO₂ production as well as deletion of the acetate, ethanol and lactate production pathways (Causey et al. 2004). During *E. coli* TC44 fermentations, changing the oxygen saturation from 5 to 50 % enhanced the pyruvate productivity. In *E. coli* TC44, mutation of the *poxB* gene, which encodes pyruvate oxidase, was most beneficial for growth and pyruvate production, as the mutation enhanced the NAD⁺ concentration in the cells and activated several enzymes involved in the glycolysis pathway. Thus construction of *E. coli* strains for pyruvate production involves introducing mutations that reduce utilization of pyruvate for cell growth and deletion of nonessential pathways through pyruvate metabolism.

Here we report an effective method for using promoter regulation for pyruvate production. To control essential gene expressions, tetracycline-regulated promoter (*P₄₄*) was inserted upstream of the *accBC* genes, and gene expressions were regulated by the absence of doxycycline. After the pyruvate producing strain was constructed, optimal culture conditions for pyruvate production were determined.

### Table 1 Comparison of the pyruvate productivities of *E. coli* strains

| Strain | Genotype | Carbon source | Concentrations (g L⁻¹) | Productivity (g L⁻¹ h⁻¹) | Yield* (%) | References |
|--------|----------|---------------|------------------------|--------------------------|------------|------------|
| *E. coli* LAFCPCPt-accBC-aceE | MG(1655) ΔdhA ΔϕlB Δpta-ackA ΔpoxB Δcra P₄₄-accBC P₄₄-aceE | Glucose | 4.2b | 0.0584 | 10.6 | This study |
| *E. coli* LAFCPCPt-accBC-aceE | MG(1655) ΔdhA ΔϕlB Δpta-ackA ΔpoxB Δcra P₄₄-accBC P₄₄-aceE | Glucose | 26.1c | 0.363 | 55.6 | This study |
| *E. coli* CGSG (6162) | F⁺ aceF10 fadR200 tyrT58(AS) adhE80 mel1 | Glucose, acetate | 37.0 | 1.03 | ND | Tomar et al. (2003) |
| *E. coli* CGSG (7916) | CGSC(6162) ppc::Kan | Glucose, acetate | 35.0 | 0.972 | ND | Tomar et al. (2003) |
| *E. coli* TC44 | W(3110) (Succ⁺), ΔfoaC-prlB::FRT ΔfrdBC ΔldhA Δatp(FH) ΔadhE::FRT ΔacS::FRT ΔacK::FRT | Glucose | 52.0 | 1.21 | 77.9 | Causey et al. (2004) |
| *E. coli* W(1485)lip2 | W(1485) F+ λ⁻ lipA2 | Glucose | 25.5 | 0.797 | 52.2 | Yokota et al. (1994) |
| *E. coli* TBLA-1 | W(1485)lip2 bglF401 | Glucose | 31.5 | 0.984 | 64.4 | Yokota et al. (1994) |
| *E. coli* YYC202 | Hfr zbi::Tn10 poxB1 Δ(aceEF) rpsL pps-4 pfl-1 | Glucose, acetate | 62.0 | 1.75 | ND | Zelić et al. (2003) |
| *E. coli* ALS929 | YYC202 ldhA::Kan | Glucose, acetate | 70.0 | 2.06 | ND | Zhu et al. (2008) |
| *E. coli* ALS(1059) | YYC202 ldhA::Kan | Glucose, acetate | 90 | 2.05 | ND | Zhu et al. (2008) |

ND not described

* Yield was the theoretical maximum value

b Pyruvate was produced from N5G medium under standard culture conditions for 72 h

c Pyruvate was produced from N5G medium under optimized culture conditions for 72 h
Fig. 1 Glucose metabolism in *Escherichia coli*
Materials and methods
General genetic techniques
*E. coli* strain MG1655 (wild-type, The Coli Genetic Stock Center; CGS6300) and its derivative strain LAF was described earlier (Nakashima and Tamura 2012). Gene deletion and knock-in were carried out as described earlier (Emmerison et al. 2006; Nakashima et al. 2014a, b; Nakashima and Miyazaki 2014). The sequences of all primers are shown in Table 2.

Unless stated otherwise, *E. coli* cells were cultured in Luria–Bertani medium at 37 °C. To evaluate cell growth, culture pre-grown overnight was diluted 1:500, and doxycycline was added if necessary. Cell density was measured at 600 nm in 200 µL aliquots of culture in a 96-well plate (Nalge Nunc International, Rochester, NY, USA; product no. 269,620) using a Safire microplate reader (Tecan, Männedorf, Switzerland) and a LS-PLATEmanager 2004 data analysis program (Wako, Osaka, Japan).

Construction of a plasmid for deletion of poxB
The 5′- and 3′-flanking regions of poxB were PCR-amplified from genomic DNA from strain MG1655 using specific primer sets: sSN1185/sSN1186 and sSN1187/sSN1188, respectively. The 5′ end of sSN1185 was phosphorylated using T4 DNA kinase before use. The termini of the amplified fragments were treated with *NcoI* and *NsiI*, respectively, and the two fragments were cloned into the *PstI*–*NcoI* site of pHN1234, yielding pHN1243. Plasmid pHN1243 was used for disruption of poxB.

Construction of plasmids for knock-in of tetracycline promoters
A DNA fragment containing the 5′-flanking region of accBC was PCR-amplified using primers sSN1892-accN and sSN1893-accN. The amplified fragment was then cut with *PstI* and *XbaI* and cloned into the *PstI*–*XbaI* site of pHN1234 (Nakashima and Tamura 2012), yielding pHN2125. A DNA fragment containing *tetR* and transcriptional regulator gene (*tetR*) was PCR-amplified using primers sSN1894-tetR and sSN1895-tetR from pHN1234 (Nakashima and Tamura 2013). That amplified fragment was then cut with *BamHI* and *NcoI* and cloned into the *BamHI*–*NcoI* site of pHN2125, yielding pHN2127. A DNA fragment containing the 3′-flanking region of accBC was PCR-amplified using primers sSN1896-accORF and sSN1897-accORF. The amplified fragment was then cut with *SpeI* and *BamHI* and cloned into the Spel–BamHI site of pHN2125, yielding pHN2128. Plasmid pHN2128 was used to knock in *Ptet* and *tetR* into the upstream of accBC.

To introduce *Ptet* upstream of *aceE* within the genome, pHN2198 was constructed. A DNA fragment containing the 5′-flanking region of *aceE* was PCR-amplified using primers sSN1997aceEDox1 and sSN1998aceEDox2. The amplified fragment was then cut with *PstI* and *XbaI* and cloned into the *PstI*–*XbaI* site of pHN1234 (Nakashima and Tamura 2012), yielding pHN2187. A DNA fragment containing *Ptet* was PCR-amplified using primers sSN1998aceEDox3 and sSN1999aceEDox4, with pHN2127 serving as a template. The amplified fragment was then cut with *SpeI* and *PstI* and ligated into the Spel–BamHI site of pBluScriptII KS(+) using T4 DNA ligase, yielding pHN2189. A DNA fragment containing the 3′-flanking region of *aceE* was PCR-amplified using primers sSN2000aceEDox5 and sSN2001aceEDox6, after which the amplified fragment was cut with *PstI* and *EcoRV* and cloned into the *PstI*–*EcoRV* site of pHN2189, yielding pHN2191. A DNA fragment cut from pHN2191 using *SpeI* and *NcoI* was cloned into the *XbaI*–*NcoI* site of pHN2187, yielding pHN2198.

Standard culture conditions for pyruvate production
Strain LAF6PCP-ttaccBC-aceE was pre-grown overnight in Luria–Bertani medium and then diluted 1:100 with fresh N5G medium (pH 7.2) containing 60 g L⁻¹ glucose, 10 g L⁻¹ (NH₄)₂SO₄, 2 g L⁻¹ NaCl, 1 g L⁻¹ KH₂PO₄, 0.24 g L⁻¹ MgSO₄·7H₂O and 0.011 g L⁻¹ CaCl₂·2H₂O. Pyruvate production was performed in a small-scale

### Table 2 PCR primers for construction of plasmids

| Primers       | Sequences                                                                 |
|---------------|---------------------------------------------------------------------------|
| sSN1185       | 5′-ATCCATGTCGTCTACCTAGTCTGGAATTCTG-3′                                   |
| sSN1186       | 5′-GTGGAATCCAGATGCTTCATCACTGGAATTCTG-3′                                  |
| sSN1187       | 5′-AATCTAGACACGGCGTAGCTCCTTAGACGCGCGCGGAACTG-3′                         |
| sSN1188       | 5′-GATTAGATCCGCGCTTACCACTGCGGTAG-3′                                     |
| sSN1892-accN  | 5′-AAATCCTGTCGCCGTTACCACTGCGGTAG-3′                                     |
| sSN1893-accN  | 5′-AAATCCTAGACACGGCGTAGCTCCTTAGACGCGCGCGGAACTG-3′                      |
| sSN1894-tetR  | 5′-TCTAGCTAGACACGGCGTAGCTCCTTAGACGCGCGCGGAACTG-3′                      |
| sSN1895-tetR  | 5′-AAATCCTAGACACGGCGTAGCTCCTTAGACGCGCGCGGAACTG-3′                      |
| sSN1896-accORF| 5′-AAATCCTAGACACGGCGTAGCTCCTTAGACGCGCGCGGAACTG-3′                      |
| sSN1897-accORF| 5′-CCGACATGTCAGATGCTTCATCACTGCGGTAG-3′                                  |
| sSN1997aceEDox1| 5′-TCACTAGCTAGACACGGCGTAGCTCCTTAGACGCGCGGAACTG-3′                      |
| sSN1998aceEDox2| 5′-AGACTAGCTAGACACGGCGTAGCTCCTTAGACGCGCGGAACTG-3′                      |
| sSN1998aceEDox3| 5′-AAATCCTAGACACGGCGTAGCTCCTTAGACGCGCGGAACTG-3′                      |
| sSN1999aceEDox4| 5′-ATGGATCCGCGGTAGCTCCTTAGACGCGCGGAACTG-3′                             |
| sSN2000aceEDox5| 5′-CCGACATGTCAGATGCTTCATCACTGCGGTAG-3′                                  |
| sSN2001aceEDox6| 5′-GGGATCCGCGGTAGCTCCTTAGACGCGCGGAACTG-3′                             |

The restriction enzyme site is underlined.
multistage fermentor Bio Jr. 8 (Able & Biott, Tokyo, Japan) with a working volume of 80 mL. The standard culture conditions were as follows: culture temperature, 37 °C; culture pH, 7.2; stirrer speed, 1200 rpm; airflow rate, 50 mL min⁻¹.

Optimization of the culture conditions
The effect of culture temperature was examined at 29–37 °C. The culture pH was tested at pH 5.4–7.2 through automatic addition of 4 M NaOH solution. The stirrer speed and airflow rate were examined at 1200–2000 rpm and 50–150 mL min⁻¹, respectively. The OD₆₀₀ was measured by monitoring the difference between the cell and cell-free turbidity values using an Eppendorf Bio-Spectrometer (Eppendorf, Hamburg, Germany).

Quantification of pyruvate and glucose
After clarifying the culture by centrifugation and filtration, pyruvate and glucose were quantified using a high performance liquid chromatograph equipped with a Jasco UV-2070 Plus Intelligent UV/VIS Detector at 210 nm (Jasco, Tokyo, Japan), a Jasco RI-2031 Plus Intelligent Refractive Index Detector (Jasco) and an Aminex HPX-87H cationic exchange column connected to an Aminex 85H Micro-Guard Column (Bio-Rad Labs, Hercules, CA, USA). The chromatographic conditions were as follows: mobile phase, 4 mM H₂SO₄; flow rate, 0.5 mL min⁻¹; column oven temperature, 65 °C.

Results
Control of accBC expressions by the tetracycline-regulated promoter
In E. coli cells, the accABCD genes are annotated as essential genes for growth. The accABCD gene products comprise the acetyl-CoA carboxylase complex, which catalyzes the biotin-dependent carboxylation of acetyl-CoA to produce malonyl-CoA via two half-reactions (Broussard et al. 2013). In the first half-reaction, biotin carboxylase, which is encoded by accC, catalyzes the ATP-dependent carboxylation of biotin. Immediately after the carboxylation, the carboxylated biotin is attached to the biotin carboxyl carrier protein, which is encoded by accB, resulting in the synthesis of carboxybiotin. In the second half-reaction, carboxyltransferase, which is encoded by accAD, transfers the carboxyl group from carboxy-biotin to acetyl-CoA, yielding malonyl-CoA. In an earlier study, we observed that silencing aceE expression increased the accumulation of pyruvate (Nakashima et al. 2014b). In addition, we also reported that silencing both accA and aceE led to even greater pyruvate accumulation (Nakashima et al. 2014b). Those results indicate that pyruvate accumulation is achieved by significantly decreasing the activities of both the pyruvate dehydrogenase complex and the acetyl-CoA carboxylase complex. In the present study, therefore, we used Ptet, which is controlled by the presence or absence of doxycycline, to regulate the expressions of accBC and aceE.

To confirm the effect of Ptet on gene expression, we assessed our ability to control accBC expressions. When doxycycline was present in the culture, the growth curves obtained with strain Pt-accBC were similar to those obtained with the wild-type strain (Fig. 2). On the other hand, growth of strain Pt-accBC was retarded in the absence of doxycycline (Fig. 2b). These results demonstrate that Ptet is useful for controlling essential gene expression.

Development of a pyruvate producing strain
In E. coli cells, acetate is produced via two primary routes (Fig. 1): (1) conversion of acetyl-CoA to acetate by phosphotransacetylase (pta) and acetate kinase (ackA) (Kakuda et al. 1994) and (2) oxidation of pyruvate to acetate by pyruvate oxidase (poxB) (Abdel-Hamid et al. 2001). To block acetate production from pyruvate in E. coli cells, the ackA–pta and poxB genes were deleted.

The Cra protein, global transcriptional regulator, controls transcriptional expression of genes involved in sugar catabolism (Saier and Rameier 1996). Moreover, we previously reported that disruption of cra was also beneficial for accumulating pyruvate in E. coli cells, since this disruption led to a more rapid rate of pyruvate production (Nakashima et al. 2014b). Therefore, cra was also deleted. The resultant strain, LAFCPCPt-accBC-aceE, was then used for experimentation.

Pyruvate production using standard and optimized conditions
When 60 g L⁻¹ glucose was provided as the carbon source under the standard culture conditions (see “Materials and methods” section), strain LAFCPCPt-accBC-aceE pyruvate levels reached 4.2 g L⁻¹ in 72 h with consumption of 40.5 g L⁻¹ glucose (Fig. 3).

To optimize the culture conditions for pyruvate production by strain LAFCPCPt-accBC-aceE, the effects of temperature, initial culture pH, stirrer speed and airflow rate were evaluated. The maximum pyruvate concentration was obtained at 35 °C (Fig. 4a). When the effect of initial culture pH was assessed, the highest product concentration was obtained at pH 5.7 (Fig. 4b). When the stirrer speed was investigated, the highest final product concentration was obtained at 1800 rpm (Fig. 4c). Finally, examination of the airflow rate conditions showed that 150 mL min⁻¹ produced the highest product concentration (Fig. 4d). In sum, the optimal culture conditions for pyruvate production were as follows;
culture temperature, 35 °C; culture pH, 5.7; stirrer speed, 1800 rpm; airflow rate, 150 mL min⁻¹. Under these optimized conditions, the pyruvate concentration reached 26.1 g L⁻¹ after 72 h with a theoretical yield of 55.6 %. In our knowledge, this yield was similar compared with the yields of another engineered E. coli strains (Table 1). However, our pyruvate producing strain was not required supplemental carbon additives and special expertise for pyruvate production, which was easy to perform. These results indicate that strain LAFPCP-t-accBC-aceE also has an industrial potential for pyruvate production.

With pyruvate production using strain LAFPCP-t-accBC-aceE, we think the culture pH is the most important factor. pH is known to have a significant effect on gene expression in E. coli cells, and expression of aceE is promoted at acidic pH (Maurer et al. 2005). On the other hand, after deletion of the ackA–pta pathway, cell growth is accelerated at acidic pH and the activity of the poxB gene product is enhanced (Dittrich et al. 2005). Similarly, pyruvate productivity in a poxB-deleted strain is higher at acidic pH than neutral pH (Dittrich et al. 2005). Taken together, these observations suggest that both the production and degradation of pyruvate is stimulated at acidic pH, and thus pH 5.7 was optimal in our study. Note that pyruvate degradation is limited in our study due to gene manipulation.

We also observed that both stirrer speed and airflow rate influenced pyruvate production. Transcription of several genes involved in the gluconeogenesis and anaplerosis pathways (pckA, ppsA, ppc and sfcA), the TCA cycle (gltA), the glyoxylate cycle (aceA), and acetate metabolic pathways (acs, ackA, pta and poxB) are affected by the dissolved oxygen concentration (Phue and Shiloach...
In our study, more aerobic conditions showed improved growth rate (Fig. 5), presumably to support overall carbon flux (Matsuoka and Shimizu 2013).

We found that the optimal culture temperature was 35 °C (Fig. 3a), though the reason is unclear. This temperature has no effect on expression of the genes involved in glycolysis or the pentose phosphate pathway (Gadgil et al. 2005). Perhaps this temperature contributes to enhancing the growth rate. For example, when *E. coli* strain ML30G was cultured in glucose minimal medium at various temperatures, the maximum growth rate was observed at 35 °C (Shehata and Marr 1975).

**Fig. 4** Effects of culture conditions on pyruvate production. *E. coli* strain LAFCPCPt-accBC-aceE was cultivated for 24 h in N5G medium. Shown are the effects of temperature (a), culture pH (b), stirrer speed (c), and airflow rate (d) on pyruvate production. Error bars indicate SE (*n* = 3).

**Fig. 5** Growth of *E. coli* strain LAFCPCPt-accBC-aceE under standard and optimized culture conditions. The strains were cultured in N5G medium under standard (open symbols) and optimized (filled symbols) culture conditions. Error bars indicate SE (*n* = 3).

**Abbreviations**
Prt: tetracycline-regulated promoter; tetR: transcriptional regulator gene.
Authors’ contributions

HA designed this study, performed experiments, participated in the interpretation of the results and drafted the manuscript. NN participated in the design and coordination of this study and helped to revise the manuscript. TH conceived and designed this study, coordinated the experiments, interpreted the results and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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