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Contributions of the anaplerotic reaction enzymes pyruvate carboxylase and phosphoenolpyruvate carboxylase to L-lysine production in *Corynebacterium glutamicum*.

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Short title: PC and PEPC Contributions to Lysine Production

ABSTRACT

Anaplerotic reactions catalyzed by pyruvate carboxylase (PC) and phosphoenolpyruvate carboxylase (PEPC) have important roles in the production of L-lysine to replenish oxaloacetic acid (OAA) in *Corynebacterium glutamicum*. However, the relative contributions of these enzymes to L-lysine production in *C. glutamicum* are not fully understood. In this study, using a parent strain (P) carrying a feedback inhibition-resistant aspartokinase with the T311I mutation, we constructed a PC gene-deleted mutant strain (P Δ PC) and a PEPC gene-deleted mutant strain (P Δ PEPC). Although the growth of both mutant strains was comparable to the growth of strain P, the maximum L-lysine production in strains P Δ PC and P Δ PEPC decreased by 14% and 49%, respectively, indicating that PEPC strongly contributed to OAA supply. L-Lysine production in strain P Δ PC slightly decreased during the logarithmic phase, while production during the early stationary phase was comparable to production in strain P. By contrast, strain P Δ PEPC produced L-lysine in an amount comparable to the production of strain P during the logarithmic phase; L-lysine production after the early stationary phase was completely stopped in strain P Δ PEPC. These results indicate that OAA is supplied by both PC and PEPC during the logarithmic phase, while only PEPC can continuously supply OAA after the logarithmic phase.

Key words: Anaplerotic reaction; oxaloacetic acid; lysine; *Corynebacterium glutamicum*; pyruvate carboxylase; phosphoenolpyruvate carboxylase

INTRODUCTION

L-Lysine is an essential amino acid that has been utilized as a feed additive to increase the bioefficacy of protein sources. With the increasing global demand for livestock production, approximately 2.6 million tons of L-lysine is produced annually by fermentation using industrial workhorses e.g. *Corynebacterium glutamicum* and *Escherichia coli* (1). *C. glutamicum* is a Gram-positive bacterium that belongs to mycolic-acid containing actinomycetes (2). This bacterium was originally isolated as an L-glutamate-secreting bacterium; it has been used for the production of various metabolites including L-lysine (1). During the approximately 50-year history of strain development for L-lysine production using *C. glutamicum*, three main strategies for L-lysine production have been identified. First, feedback inhibition of aspartokinase (AK) which catalyzes the phosphorylation of aspartate, is released to facilitate L-lysine production (3,4). Second, an enhanced supply of oxaloacetic acid (OAA; a precursor of L-lysine) significantly increases L-lysine production (3,4). Finally, enhanced NADPH supply increases L-lysine yield because one mol of L-lysine biosynthesis requires four moles of NADPH (5). Metabolic pathways related to phosphoenolpyruvate (PEP) and OAA, which lead to L-lysine biosynthesis in *C. glutamicum* are shown in Fig. 1.

C. glutamicum has two anaplerotic enzymes: pyruvate carboxylase (PC; converts pyruvate to OAA) and phosphoenolpyruvate carboxylase (PEPC; converts PEP to OAA), thus replenishing of OAA (6). On the other hand, *C. glutamicum* can catalyze C3-directed decarboxylation by PEP carboxykinase (PEPCK) (7), oxaloacetate decarboxylase (ODx) (8), and malic enzyme (ME) (9) (Fig.1). Because OAA supply is a bottleneck in the production of both L-

glutamate (through the tricarboxylic acid (TCA) cycle) and L-aspartate family amino acids, (i.e., L-asparagine, L-lysine, L-threonine, L-isoleucine, and L-methionine), anaplerotic enzymes have been extensively studied for use in strain development (10). Furthermore, OAA is a core chemical in the production of several industrially important chemicals: succinic acid (11), β -alanine (12), ectoine (13), glutarate (14), and 4-hydroxyisoleucine (15). Despite the importance of enhanced OAA supply for metabolic engineering, the effects of PC and PEPC on OAA supply in *C. glutamicum* have not yet been fully elucidated.

In *C. glutamicum*, PC catalyzes the irreversible carboxylation of pyruvate to OAA coupled to the hydrolysis of ATP (6), while PEPC fixes bicarbonate on PEP to form OAA and inorganic phosphate (6). Because PC exhibits considerable lability, measurement of PC activity has been reported only using either permeabilized cells (16,17) or purified enzymes derived from *C. glutamicum* PC gene (*pyc*)-overexpressing strains (18). The study that used purified enzymes showed that PC activity was inhibited by ADP and aspartate with K_i values of 1.5 mM and 9.3 mM, respectively (18). By contrast, PEPC activity is activated by acetyl-CoA and fructose-1,6-bisphosphate: it is inhibited by aspartate (19,20). Measurement of *C. glutamicum* PEPC activity showed that the half-maximal inhibitory concentration of aspartate was 0.5 mM; 5 mM aspartate-induced complete inhibition of PEPC activity could be relieved by 0.2 mM acetyl-CoA or 1 mM fructose-1,6-bisphosphate (21).

Previous studies showed that the inactivation of either PC or PEPC did not cause a growth defect in glucose minimal medium, while the inactivation of both PC and PEPC resulted in the loss of growth ability in glucose minimal medium (16,22). Thus, one of the two anaplerotic enzymes is sufficient to supply a quantity of OAA that supports growth on glucose

minimal medium, while no other enzyme can compensate for the inactivation of both anaplerotic enzymes in *C. glutamicum*. Furthermore, PC and PEPC reactions can be rate-limiting steps in metabolite production. A recent laboratory evolution experiment showed that the lethality of the double inactivation of PC and PEPC in glucose minimal medium can be suppressed by an up-regulation of the glyoxylate shunt, potentially in combination with the 2-methylcitrate cycle (23). The operation of glyoxylate shunt coupling with the 2-methylcitrate cycle is thought to channel the increase in PEP and pyruvate amount as well as succinate resulting in replenishing OAA (23).

For example, the production of L-glutamate using *C. glutamicum* can be induced by either biotin limitation or adding a surfactant (e.g., Tween 40 and Tween 60). Because PC is a biotin-dependent enzyme, PC activity is drastically downregulated during biotin limitation-induced L-glutamate production (24–26). Under such conditions, PEPC becomes the rate-limiting anaplerotic enzyme for L-glutamate production (25,26). When L-glutamate production is induced by surfactant addition, both enzymes are active during L-glutamate production (25). Under these conditions, the overexpression of *pyc* (rather than *ppc* encoding PEPC) has been reported to enhance Tween 60-induced L-glutamate production (27). Increases in PC-catalyzed anaplerotic flux in response to Tween 40 addition were also detected by metabolic flux analysis during L-glutamate production by *C. glutamicum* (28). However, another study that used knockout mutant strains concluded that PEPC, rather than PC, provides the major anaplerotic reaction during Tween 40-induced L-glutamate production (29). Besides, a recent comprehensive analysis of selected single or double deletion mutants in the anaplerosis of wild-type *C. glutamicum* under glucose minimal medium also concluded the majority role of PC in the

anaplerotic reaction (23). Therefore, the contributions of PC and PEPC to OAA supply during L-glutamate production remain unclear.

During L-lysine production by *C. glutamicum* with feedback inhibition-resistant AK (AKR), the overexpression of *pyc* was found to increase L-lysine production by approximately 50%, while *pyc* inactivation resulted in approximately 60% reduction of L-lysine production (27). Another study reported that the inactivation of *ppc* had no effect on L-lysine yield (30). These findings suggest that PC functions as the major anaplerotic enzyme. However, there remains potential for optimization with respect to the genetic background of the experimental strains and the culture conditions for evaluating L-lysine production.

MATERIALS AND METHODS

Media The complete medium, Medium 7, was previously described (31). The sucrose-containing medium, Medium S10 (32), was used to confirm the completion of double-crossover replacement during strain construction. For L-lysine production in a 2 L jar fermentor, Medium S2+ and Medium L1+ were used as seed medium and fermentation medium, respectively. Medium S2+ contained (per liter) 40 g glucose, 4 g urea, 1 g KH_2PO_4 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 200 μg thiamine·HCl, 60 μg biotin, 0.02 mL anti-foaming agent, Adekanol LG-109 (Asahi Denka Kogyo, Tokyo, Japan), and 24.9 mL soybean-meal hydrolysate (total nitrogen, 30.4 g/L). The pH was adjusted to 7.0 using 5 N KOH; the medium was then autoclaved at 115°C for 10 min. Medium L1+ contained (per liter) 100 g glucose, 40 g $(\text{NH}_4)_2\text{SO}_4$, 1 g KH_2PO_4 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8 mg

MnSO₄·5H₂O, 200 µg thiamine·HCl, 300 µg biotin, and 24.9 mL soy-bean-meal hydrolysate. The pH was adjusted to 7.0 using 28% (w/w) ammonia solution; the medium was then autoclaved at 121°C for 10 min. Bacterial growth was monitored by determining the optical density at 660 nm (OD₆₆₀) of the appropriate dilution. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium (Miller's) supplemented with 20 µg/mL kanamycin, when necessary.

Bacterial strains, plasmids, and primers Bacterial strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers used in this study are listed in Table 2. *E. coli* DH5α was used for plasmid construction. The plasmid pBS4S (33) harboring both a kanamycin-resistance gene and *sacB* was used for the construction of *pyc* and *ppc* deletion mutant strains, PΔPC and PΔPEPC, respectively. Using the parent strain P (34), which carries an AKR with T311I mutation, PΔPC and PΔPEPC were constructed by two-step homologous recombination. The upstream and downstream regions of *pyc* or *ppc* were amplified using oligonucleotide pairs *pyc_df1* and *pyc_dr1*, *pyc_df2* and *pyc_dr2*, *ppc_1* and *ppc_2*, and *ppc_3* and *ppc_4*, respectively. The resulting PCR products and SacI digested pBS4S plasmid were subsequently fused using the In-fusion Cloning kit (Takara Bio Inc. Shiga, Japan) to generate pBS4SΔ*pyc* and pBS4SΔ*ppc* plasmids, respectively. Subsequently, the plasmids were electroporated into strain P and the transformation mixture was plated on Medium 7 agar plates containing 20 µg/mL kanamycin. After selection for the first and second recombination events, kanamycin-sensitive and sucrose-resistant clones were analyzed by colony-PCR with the primer pair *pyc_df1* and *pyc_dr2* or *ppc_1* and *ppc_4* to select strains PΔPC and PΔPEPC, respectively.

L-Lysine production L-Lysine production was conducted as previously described (34). Strains P, PΔPC, and PΔPEPC were cultured for L-lysine production in Medium L1+

containing 100 g/L glucose as the fermentation medium using a 2 L jar fermentor (BMJ-02PI; ABLE Corporation, Tokyo, Japan). Cells grown on Medium 7 agar plates were inoculated into 2 L Sakaguchi flasks, each containing 150 mL of Medium S2+; the flasks were incubated at 94 rpm for 10 h. The precultured cells were harvested, washed twice, and resuspended in 0.9% NaCl solution. The washed cells were inoculated in 1.2 L Medium L1+ at an initial OD₆₆₀ of 1.0, using a 2 L jar fermentor. The cultures were aerated at 1 volume air·culture volume⁻¹·min⁻¹ (vvm) with stirring at 750 rpm. The pH was maintained at 7.0 using 28% (w/w) ammonia solution. All cultures were incubated at 30°C.

Fermentation analysis The concentrations of residual glucose and organic acids were determined as previously described (31). The concentrations of amino acids (e.g., L-lysine, L-aspartate, and L-alanine) in the culture supernatant were determined using a JLC-500S amino acid analyzer (JEOL Ltd., Tokyo, Japan). The L-lysine yields were expressed as free form L-lysine, rather than L-lysine monohydrochloride.

Enzyme activity assay Enzyme activities were measured in extracts from cells during L-lysine production in a jar fermentor in the early stationary phase (10.5 h for strains P, PΔPC, and PΔPEPC). Cells were harvested by centrifugation, washed twice in 0.2% KCl solution, and stored at -80 °C until use. Briefly, cells were disrupted with 0.5 g glass beads by the multi-bead shocker (Yasui kikai, Osaka, Japan) at 2,000 rpm for 20 min at 4°C. Cell debris and glass beads were removed by centrifugation; the supernatant was subjected to gel filtration using a Sephadex-G25 PD10 desalting column (GE Healthcare, Chicago, IL, USA). PEPC, PEPCK, pyruvate kinase (PYK), citrate synthase (CS), malate dehydrogenase (MDH), and L-lactate dehydrogenase (LDH) were measured in accordance with previously described methods (34–36).

For pyruvate dehydrogenase complex (PDHc) activity assays, 50 mM Tris-HCl (pH 7.5) containing 100 mM $(\text{NH}_4)_2\text{SO}_4$ was used as the extraction buffer during treatment with the multi-bead shocker; the reaction mixture consisted of 100 mM TES-NaOH (pH 7.0), 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 mM thiamine pyrophosphate, 0.2 mM coenzyme A, 3 mM cysteine, and 1 mM 3-acetylpyridine adenine dinucleotide (APAD), and the crude enzyme, along with 1 mM sodium pyruvate as the substrate. APAD was used in place of NAD to avoid the effect of concomitant oxidase. The reactions were initiated by adding pyruvate, and the increase in APADH was monitored spectrophotometrically at 365 nm using a V-730 spectrophotometer (JASCO Corporation, Tokyo, Japan) at 25°C. The protein concentrations in crude extracts were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as the standard. The enzyme activity was expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

Quantitative reverse transcription PCR (qRT-PCR) To quantify the expression levels of *pyc* and *ppc*, total cellular RNA from *C. glutamicum* cells was isolated as follows. Cultures grown on Medium L1+ containing 100 g/L glucose, using a 2 L jar fermentor, were collected in the early stational phase (at time point 10.5 h). Two volumes of RNA Protect bacterial reagent (Qiagen, Valencia, CA, USA) were added directly to one volume of culture to stabilize cellular RNAs. The cells were harvested by centrifugation at $6,000 \times g$ for 10 min at 4°C; total cellular RNAs were isolated using a RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Total RNA was treated with RNase-Free DNase Set (Qiagen) in accordance with the manufacturer's instructions. qRT-PCR analysis was performed using the following primer sets: RT-16S_df2 and RT-16S_dr2 (16S rRNA), RT-pyc_df and RT-pyc_dr

(ppc), and RT-ppc_df2 and RT-ppc_dr2 (ppc) (Table 2). cDNA synthesis was performed with each primer and 2 µg of total RNA, using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA). The synthesized cDNAs were then quantified using the Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analyzed using RNA isolated from three independent samples.

Western blotting analysis

Western blotting analysis was conducted as previously described (24). Cultures grown on Medium L1+ containing 100 g/L glucose, using a 2 L jar fermentor, were collected at early stationary phase (i.e., time point 10.5 h). Cells were pelleted by centrifugation at $6,000 \times g$ for 10 min at 4°C; then they were washed twice with 0.2% KCl, resuspended in 1 mL of 150 mM Tris-HCl (pH 7.8) and disrupted by 80 cycles of shaking at 2,500 rpm for 30 s at 4°C with 0.5 g glass beads in a multi-bead shocker (Yasui Kikai). After centrifugation at $20,400 \times g$ for 10 min at 4°C, supernatants were collected and the protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin as the standard. Twenty micrograms of protein extracts per well were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%, w/v acrylamide) and transferred to Sequi-Blot™ PVDF Membranes (Bio-Rad) by electroblotting. PC was detected with Streptavidin-HRP Conjugate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and the avidin-bound proteins were visualized by the Lumino image analyzer LAS-4000 (Fujifilm Corporation, Tokyo, Japan). The amount of PC was measured as the band intensity according to Image J software (<https://imagej.nih.gov/ij/>).

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RESULTS

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Enzyme activities related to OAA supply in *pyc*- and *ppc*-deleted mutant strains.

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Effects of *pyc*- and *ppc*- deletion mutations on L-lysine production.

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examine the effects of PC and PEPC inactivation on L-lysine production, strains P, PΔPC, and

PΔPEPC were cultured in Medium L1+ containing 100 g/L glucose, using 2 L jar fermentors, as described in the Materials and Methods. The time courses of the fermentation profiles are shown in Fig. 2. Because all three strains consumed almost all glucose after they had been cultured for 18 h, we regarded the amount of L-lysine production at 18 h as the maximum. Growth, glucose consumption, and concentrations of aspartate and 2-oxoglutarate in strains PΔPC and PΔPEPC were comparable to those values in strain P. However, approximately 14% and 49% reductions in the amounts of maximum L-lysine production were observed in strains PΔPC and PΔPEPC, respectively. The maximum amounts of L-lysine produced by strains P, PΔPC, and PΔPEPC were 8.82 ± 0.23 , 7.59 ± 0.20 , and 4.34 ± 0.17 g/L, respectively. These results indicate that the overall contribution of PC to L-lysine production is much lower than the overall contribution of PEPC, in contrast, PC has been reported to play a greater role in L-lysine production as an anaplerotic enzyme, compared with PEPC (23). During L-lysine production, larger amounts of pyruvate (Fig. 2F), and its derivatives via one-step reaction (alanine [Fig. 2E] and lactate [Fig. 2G]), were detected in the culture supernatants of strain PΔPEPC, compared with the culture supernatants of other strains. These results suggest that carbon flux from PEP to pyruvate increased because of PEPC inactivation.

Strain PΔPC showed approximately 32% reduction of L-lysine production during the logarithmic phase (~9 h) compared with strain P (3.07 ± 0.10 g/L in P vs. 2.09 ± 0.09 g/L in PΔPC), while strain PΔPC produced amount of L-lysine comparable to the amount produced by strain P during the early stationary to stationary phase (between 9 h and 18 h) (6.12 ± 0.47 g/L in P vs 5.71 ± 0.20 g/L in PΔPC) (Fig. 2C). Conversely, PEPC inactivation caused a severe reduction of L-lysine production. In strain PΔPEPC, the amount of L-lysine production gradually

declined after 9 h; the L-lysine production completely stopped before glucose had been completely consumed (Fig. 2BC). The amounts of L-lysine production in strain PΔPEPC before 9 h and after 9 h (i.e., between 9 h and 18 h) were 2.67 ± 0.14 g/L (13% reduction compared with strain P) and 1.98 ± 0.23 g/L (68% reduction compared with strain P), respectively. Although the amount of L-lysine production in strain PΔPEPC significantly decreased at the stationary phase, the amount of L-lysine production during the logarithmic phase was less affected; these findings indicate that PC activity was critical for OAA supply during the logarithmic phase. At 15 h, strain PΔPEPC produced 5.67-fold and 7.76-fold greater amounts of L-alanine and pyruvate, respectively, compared to strain P (Fig. 2EF). These results suggest that the reduction of OAA supply after PEPC inactivation led to increased pyruvate formation, followed by enhanced L-alanine production. Overall, these experiments showed that while both PC and PEPC supplied OAA during the logarithmic phase, the anaplerotic reaction during the early stationary phase is mainly performed by PEPC, rather than PC.

Expression of PC during the early stationary phase in strain PΔPEPC Because the contribution of PC to the anaplerotic reaction significantly decreased during the early stationary phase, we hypothesized that the expression of *pyc* may be suppressed during the early stationary phase. Therefore, we performed qRT-PCR and Western blotting analyses. Total RNA was prepared from the early stationary phase and subjected to qRT-PCR analysis using the specific primer sets for *pyc* and *ppc* (Table 2). Surprisingly, the *ppc* deletion resulted in a 2.44-fold increase in *pyc* transcripts, compared with strain P (Fig. 3A). Western blotting analysis confirmed the expression of PC protein during the early stationary phase in both strains P and

PΔPEPC, such that the amount of PC in strain PΔPEPC was almost comparable to the amount in strain P (mean 1.1-fold increase [n=3], Fig. 3B). As expected, PC expression was not observed in strain PΔPC as a negative control for this assay (Fig. 3B). Thus, somewhat increased *pyc* transcription possibly due to the compensating for *ppc* deletion did not result in increased PC translation. These results imply posttranscriptional downregulation of PC during the early stationary phase. Consistent with enzyme activity measurements (Table 3), *ppc* transcripts were comparable between strains P and PΔPC (Fig. 3A).

DISCUSSION

In previous studies that involved construction and evaluation of *pyc*-overexpressed or inactivated strains (27), and *ppc*-inactivated strains (30), PC demonstrated a greater role in the anaplerotic reaction, compared with PEPC, during L-lysine production as well as when cells are grown in glucose minimal medium (23) in *C. glutamicum*. The contribution of PC to Tween 60-induced L-glutamate production was also demonstrated by overexpression or inactivation of *pyc* in *C. glutamicum* (27), while *ppc*-overexpression and inactivation did not affect L-glutamate yields (27). However, another study reported that PEPC (rather than PC) functions as the major anaplerotic enzyme in Tween 40-induced L-glutamate production (29). This discrepancy in L-glutamate production might be related to differences in culture conditions such as production medium (i.e., minimal medium (27) vs. complex medium (29)), and/or variations in medium pH and insufficient aeration during L-glutamate production associated with flask cultures (27–29); these differences might have prevented consistent evaluation of the effects of the introduced

genetic modifications into anaplerotic enzyme genes.

Here, we re-examined the contributions of PC- and PEPC-catalyzed anaplerotic reactions to L-lysine production in *C. glutamicum*. We constructed *pyc*- or *ppc*-deleted mutant strains using a simple L-lysine-producing *C. glutamicum* strain, which carried only AKR (T311I), as the parent strain to evaluate the contributions of PC and PEPC to L-lysine production. We used this approach because of the genetic ambiguity of previously used L-lysine producers, which may carry unnecessary mutations associated with mutagen treatment during strain development (37). Our method used genetically defined mutant strains that carried the minimum required mutations for evaluation. Furthermore, our culture conditions for L-lysine production involved the use of a complex medium containing soybean-meal hydrolysate, which is generally employed for industrial L-lysine production. In addition, the evaluation of L-lysine production was conducted using jar-fermentor cultures with controlled pH and generally consistent dissolved oxygen concentrations (data not shown).

Our results showed that, contrary to previous findings (27,30), PEPC (rather than PC) is the major anaplerotic enzyme under L-lysine production conditions (Fig. 2C). In our culture conditions, all strains immediately began exponential growth (Fig. 2A), rapidly consumed glucose (Fig. 2B), and reached maximum growth level over 100 at OD₆₆₀ only after they had been cultured for 15 h (Fig. 2A); at the 15 h time point, the 100 g/L starting concentration of glucose in the culture medium had been completely depleted (Fig. 2B). No apparent differences in growth or glucose consumption profiles were observed among the three strains throughout the culture period (Fig. 2AB), which confirmed the previous observation that PEPC and PC can compensate for each other's loss with respect to growth (16,22); these findings indicate that our

fermentation data are reliable.

The results also showed the relative contributions of PC and PEPC to L-lysine production over time. Both PC and PEPC have roles in anaplerotic reactions during the logarithmic phase (Fig. 2C). On the other hand, during the early stationary to stationary phase, only PEPC is continuously active and contributes to L-lysine production (Fig. 2C). PEPC enzyme activity was comparable between strains P Δ PC and P (Table 3); the maximum L-lysine production in strain P Δ PC was 0.86-fold that of the maximum production in strain P (Fig. 2C). These results suggest that the maximum contribution of PEPC to the anaplerotic reaction is 86%. Because this estimate was derived from the results using single deletion mutants, the contribution of PEPC in the wild-type strain may differ from the contribution in strain P Δ PC. It should be noted that the relative contributions of PC and PEPC to L-lysine production can be changed when different medium compositions including different biotin concentrations, different strains, and/or different fermentation protocols e.g. batch, fed-batch, and continuous culture. Indeed, a recent study reported that the major contribution of PC in glucose minimal medium (23). This implies that the difference between the minimal medium and the complex medium containing soybean-meal hydrolysate affects the relative contributions of PC and PEPC. Also, the contributions may be changed when additional mutational effects e.g. enhanced NADPH supply other than the feedback desensitization of AK are introduced for improving L-lysine production.

Strain P Δ PEPC almost ceased L-lysine production after the late exponential phase (Fig. 2C), although *pyc* is transcribed at higher levels in strain P Δ PEPC than in the parent strain P (Fig. 3A); in addition, the amount of the expressed PC protein was comparable between strain P Δ PEPC and P (Fig. 3B). These results suggest that PC activity is subject to be downregulated

post-transcriptionally and/or post-translationally. The pyruvate accumulation (Fig. 2F) with no significant change in PDHc activity (Table 3) would also support the idea that PC is dysfunctional at the timing in a PEPC-deleted strain. The possibility of post-transcriptional regulation may suggest the existence of regulation mediated by sRNAs that inhibit the translation of *pyc* mRNA. In *Bacillus subtilis*, the non-coding RNA known as RoxS sRNA is involved in regulating the expression of genes related to central metabolism (38). It has been reported that RoxS inhibits translation by forming base pairs near the SD sequence of *pycA* mRNA encoding pyruvate carboxylase (38). At present, sRNAs like RoxS have not been identified in *C. glutamicum*, but there may be sRNAs with similar functions. On the other hand, the possibility of post-translational regulation may involve allosteric inhibition by aspartate. However, our assessment of aspartate concentration in the culture supernatant (Fig. 2D) suggests that the intracellular aspartate concentration did not specifically increase in strain PΔPEPC. It was also reported that PEPC rather than PC provides the major anaplerotic reaction under oxygen deprivation conditions (39). In our study, considering that the transition in the contribution of the anaplerotic reactions of PC and PEPC occurred after the late exponential phase, it is possible that the decrease in dissolved oxygen concentration after the late exponential phase may influence the differential utilization of PC and PEPC.

Another possible mechanism involves the control of enzyme activity by acetylation of the ε-amino group of lysine residues in the target enzyme protein. Protein acetylation was originally studied in eukaryotes (until the late 1990s). More recently, many examples have been reported in bacterial proteins involved in central and secondary metabolism (40). In *C. glutamicum*, lysine acyl modifications by acetylation/succinylation occur in a posttranslational

manner in many enzymes in the glycolytic pathway, TCA cycle, and anaplerotic pathway (including PEPC, PC, and PEPCk) (41). Moreover, profiles of lysine acyl modification are significantly altered during Tween 40-induced L-glutamate production (decreased acetylation and increased succinylation), suggesting the involvement of these modifications in L-glutamate overproduction (41). In addition, the acetylation of PEPC at lysine 653 led to reduced enzyme activity, resulting in decreased L-glutamate production (29). Acetyl-CoA and acetyl phosphate are utilized as acetyl group donors during N ϵ -lysine acylation. Therefore, the accumulation of these metabolites is presumed to facilitate protein acetylation in bacteria (40). A label-free semi-quantitative proteomic analysis identified acetylation sites of PC (41); thus, inhibition of PC activity through the acetylation mechanism may occur after the late exponential phase in P Δ PEPC. Further studies are needed to more fully elucidate the regulation of PC activity and test our proposed underlying mechanism.

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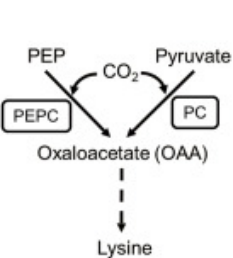
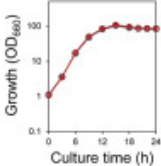
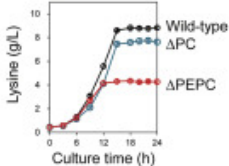
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Fig. 1. Central metabolic pathway leading to lysine formation in *C. glutamicum*, with a focus on OAA supply. PEP, phosphoenolpyruvate; PYK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; PDHc, pyruvate dehydrogenase complex; LDH, L-lactate dehydrogenase; ODC, oxaloacetate decarboxylase; CS, citrate synthase; MDH, malate dehydrogenase; MQO, malate:quinone oxidoreductase; ME, malic enzyme; AK, aspartokinase; TCA, tricarboxylic acid; dashed line, concerted-feedback inhibition by lysine and threonine. Metabolites examined in this study are shown in blue. Enzymes subjected to activity measurement are shown in beige.

Fig. 2. Profiles of glucose metabolism in the parent strain P, *pyc*-deleted mutant strain PΔPC, and *ppc*-deleted mutant strain PΔPEPC. Each strain was cultured in a 2 L jar fermentor under conditions described in the Materials and Methods. (A) Growth, (B) residual glucose, and (C) to (H) metabolites indicated in each graph. Black circle, P; blue circle, PΔPC; red circle, PΔPEPC. Values are the means of three independent experiments with error bars for standard errors.

Fig. 3. Relative expression levels of the anaplerotic enzymes in the parent strain P, *pyc*-deleted mutant strain PΔPC, and *ppc*-deleted mutant strain PΔPEPC. (A) Relative expression levels of *pyc* mRNA (encoding PC) and *ppc* mRNA (encoding PEPC). Each strain was cultured in a 2 L jar fermentor until early stationary phase under L-lysine-producing conditions, as described in the Materials and Methods. The levels of gene transcripts were measured in triplicate and normalized to 16S rRNA transcript levels. Relative quantities of the indicated mRNAs (10^{-5}) are

555 shown. The level of 16S rRNA transcript was defined as 1.0. Values are means $\pm$ standard errors.
556 (B) Western blotting of PC in strains P, P Δ PC, and P Δ PEPC. The bands from three biological
557 replicate experiments are shown with replicate numbers 1 to 3. Highlighted bands indicate PC.
558 Each strain was cultured as in (A).



Supplementation of OAA

Logarithmic phase

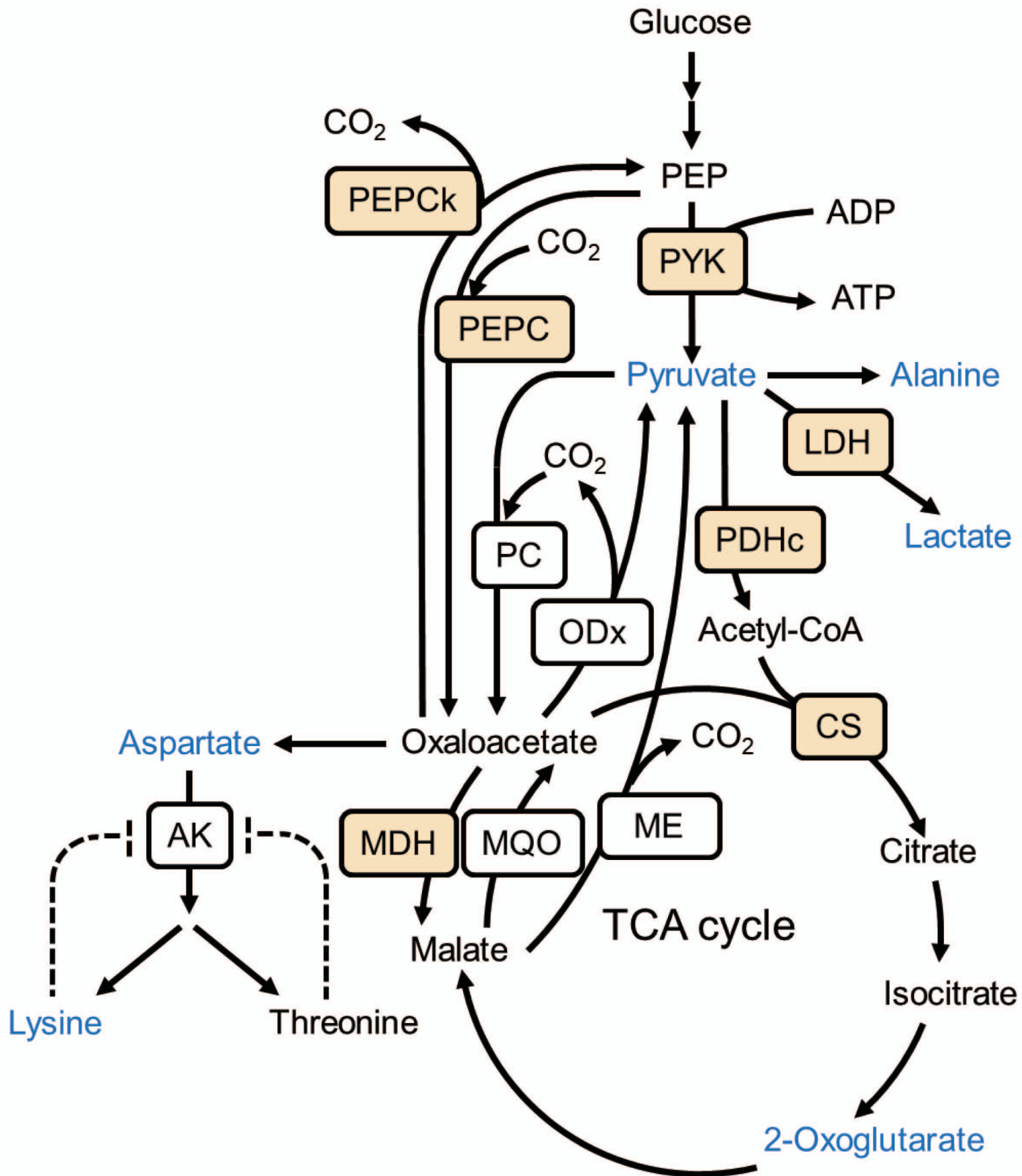
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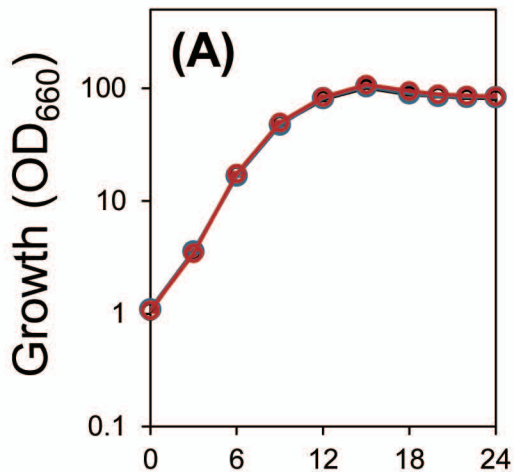
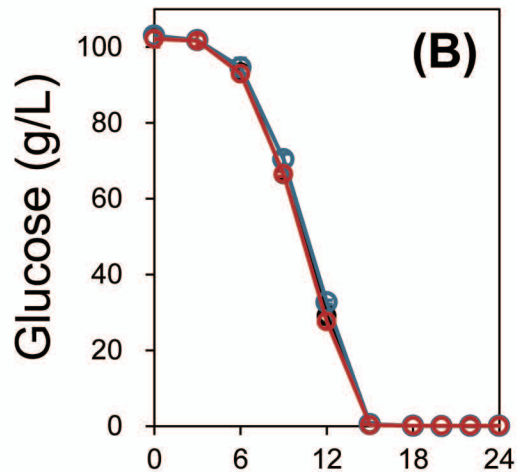
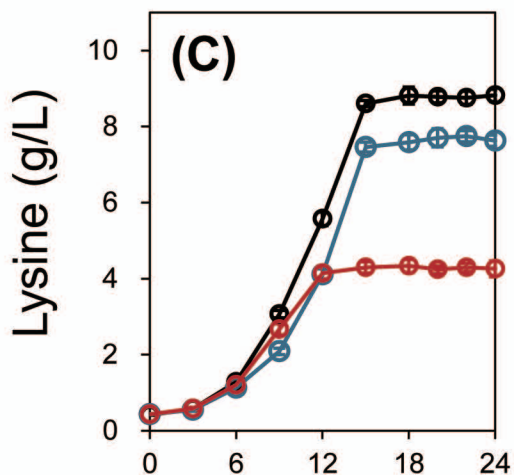
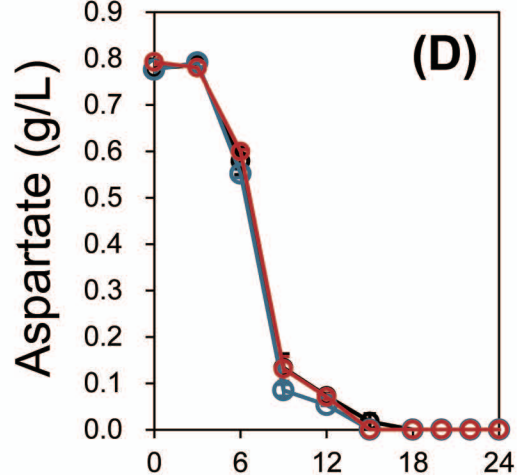
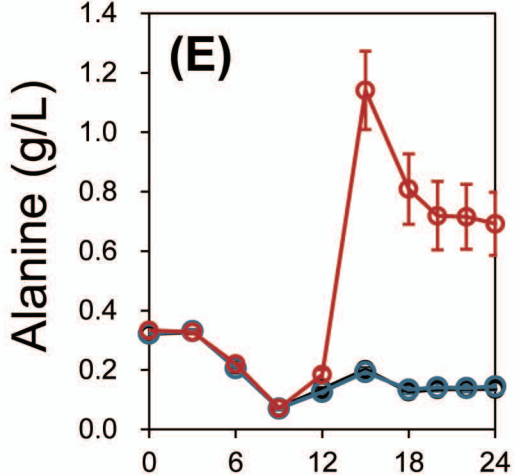
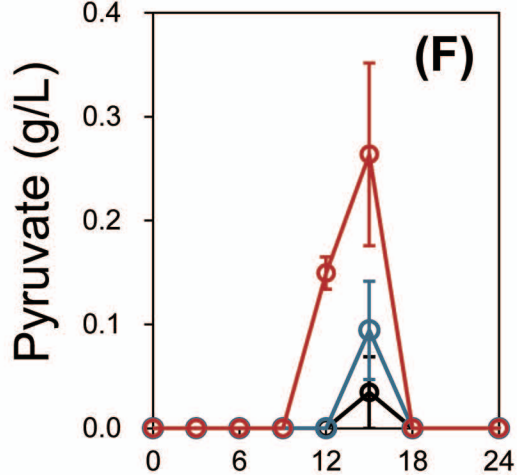
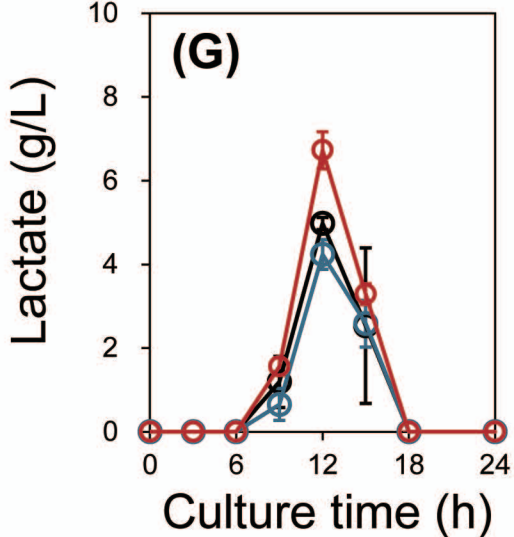
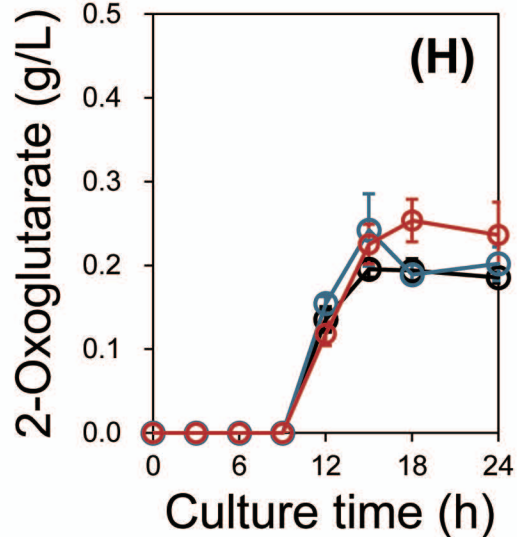
PEPC

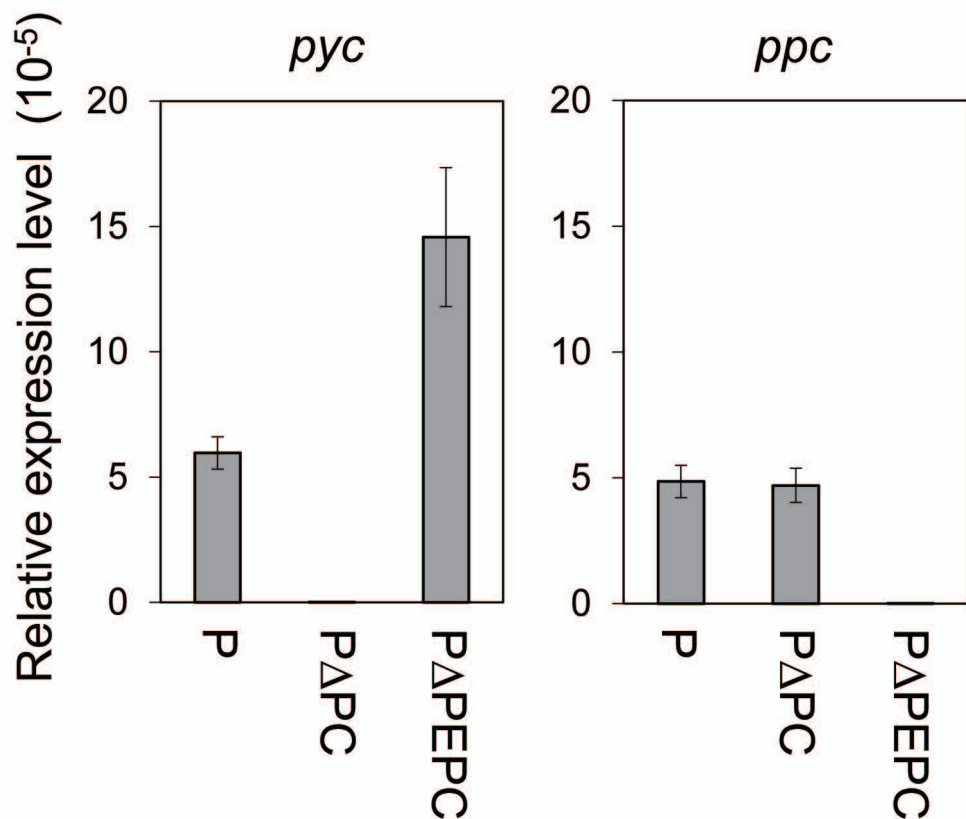
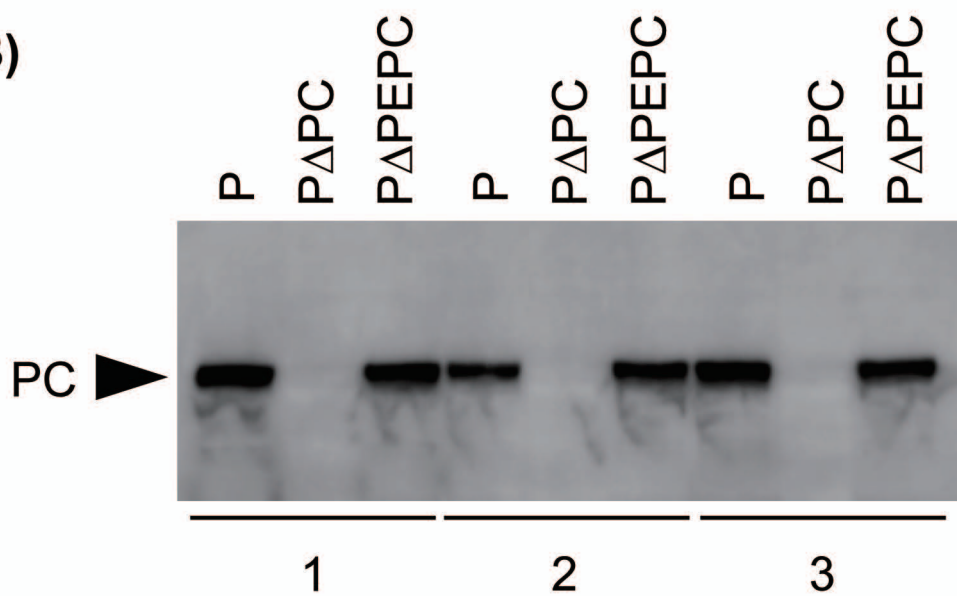
Stationary phase

PC

PEPC





(A)**(B)**

1 **Table 1. Bacterial strains and plasmids used in this study.**

Strain or plasmid	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>F</i> ⁻ , Φ 80d <i>lacZ</i> Δ <i>M15</i> , Δ (<i>lacZYA-argF</i>) <i>U169</i> , <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>r_K⁻ m_K⁺</i>), <i>phoA</i> , <i>supE44</i> , λ ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	National BioResource Project (NIG ^a , Mishima, Japan)
<i>Corynebacterium glutamicum</i>		
ATCC13032	Wild-type, ATCC13032	ATCC ^b
P	Feedback-inhibition resistant AK (T311I)	(34)
P Δ PC	T311I-AK, Δ <i>pyc</i>	This study
P Δ PEPC	T311I-AK, Δ <i>ppc</i>	This study
Plasmid		
pBS4S	Integration vector, <i>sacB</i> , Km ^r	(33)
pBS4S Δ <i>pyc</i>	Plasmid for introducing <i>pyc</i> -deficient mutation	This study
pBS4S Δ <i>ppc</i>	Plasmid for introducing <i>ppc</i> -deficient mutation	This study

2 ^a NIG, National Institute of Genetics,

3 ^b ATCC, American Type Culture Collection

1 **Table 2. Primers used in this study.**

Name	Sequence (5' to 3')
Primers used for Δpyc mutant construction	
pyc_df1	CAGTGAATTCGAGCTGAGGTAAGATGTCTGCAGGTGGAAG
pyc_dr1	CTCTGGGGTTGGGCCAATAAAAG
pyc_df2	GGCCCAACCCCGAGGCCAGACGATAAGGGTATGCGCAATG
pyc_dr2	GATCCCCGGGTACCGCAGAGTTGGTGAGCAGGAATCGTG
Primers used for Δppc mutant construction	
ppc_1	CAGTGAATTCGAGCTGAGTTGCGCAGCGCAGTGGAAAG
ppc_2	GTGGCCGATGTGCTGCGGAACCGCATTAGAGCAGTGGATTG
ppc_3	CAGCACATCGGCCACAGCTTCTG
ppc_4	GATCCCCGGGTACCGCGATGACATCAGGTTCCCTCGGTCAAATC
Primers used for qRT-PCR	
RT-16S_df2	GCGTCGTCTGTGAAATCCCG
RT-16S_dr2	CGCCATTGGTGTTCCTCCTG
RT-pyc_df	TGATGATGAGCAGGGACGCA
RT-pyc_dr	TGGCAGTGGCAGATCCTTGA
RT-ppc_df2	CTGGCAGCATGACACGAGAC
RT-ppc_dr2	ACATCGGATCCAGGCCTTCC

Table 3. Measured enzyme activities in strains P, PΔPC, and PΔPEPC.

Enzyme	Specific activity (nmol min ⁻¹ (mg protein) ⁻¹)								
	P			P ΔPC			P ΔPEPC		
PEPC	146	±	3	146	±	4	†1	±	0.1
PEPCK	138	±	13	124	±	5	126	±	9
PYK	1260	±	65	1163	±	113	1193	±	46
PDHc	49.2	±	1.2	49.5	±	3.0	64.3	±	5.9
CS	428	±	14	441	±	7	462	±	18
LdhA	449	±	19	391	±	38	420	±	24
MDH	835	±	29	832	±	27	804	±	67

^aSee legend to Figure 1 for abbreviated names of enzymes.

^bValues are means ± standard errors (n = 3).

† Significant differences compared with parent strain (t-test, P < 0.05).