

# Boosting Anaplerotic Reactions by Pyruvate Kinase Gene Deletion and Phosphoenolpyruvate Carboxylase Desensitization for Glutamic Acid and Lysine Production in *Corynebacterium glutamicum*

Atsushi Yokota, Kazunori Sawada, and Masaru Wada

**Abstract** In the 1980s, Shiio and coworkers demonstrated using random mutagenesis that the following three phenotypes were effective for boosting lysine production by *Corynebacterium glutamicum*: (1) low-activity-level citrate synthase ( $CS^L$ ), (2) phosphoenolpyruvate carboxylase (PEPC) resistant to feedback inhibition by aspartic acid ( $PEPC^R$ ), and (3) pyruvate kinase (PYK) deficiency. Here, we reevaluated these phenotypes and their interrelationship in lysine production using recombinant DNA techniques.

The *pyk* deletion and  $PEPC^R$  (D299N in *ppc*) independently showed marginal effects on lysine production, but both phenotypes synergistically increased lysine yield, demonstrating the importance of PEPC as an anaplerotic enzyme in lysine production. Similar effects were also found for glutamic acid production.  $CS^L$  (S252C in *glcA*) further increased lysine yield. Thus, using molecular techniques, the combination of these three phenotypes was reconfirmed to be effective for lysine production. However, a simple  $CS^L$  mutant showed instabilities in growth and lysine yield.

Surprisingly, the *pyk* deletion was found to increase biomass production in wild-type *C. glutamicum* ATCC13032 under biotin-sufficient conditions. The mutant showed a 37% increase in growth (based on  $OD_{660}$ ) compared with the ATCC13032 strain in a complex medium containing 100 g/L glucose. Metabolome analysis revealed the intracellular accumulation of excess precursor metabolites. Thus, their conversion into biomass was considered to relieve the metabolic

---

A. Yokota (✉), K. Sawada, and M. Wada  
Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University,  
Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan  
e-mail: [yokota@chem.agr.hokudai.ac.jp](mailto:yokota@chem.agr.hokudai.ac.jp)

distortion in the *pyk*-deleted mutant. Detailed physiological studies of various *pyk*-deleted mutants also suggested that malate:quinone oxidoreductase (MQO) is important to control both the intracellular oxaloacetic acid (OAA) level and respiration rate. These findings may facilitate the rational use of *C. glutamicum* in fermentation industries.

**Keywords** Anaplerotic pathway, Citrate synthase, *Corynebacterium glutamicum*, Feedback inhibition, Glutamic acid, Lysine, Malate:quinone oxidoreductase, Phosphoenolpyruvate carboxylase, Pyruvate kinase, Respiration

## Contents

- 1 Contribution of Anaplerotic Reactions to Lysine Yield: Phosphoenolpyruvate Carboxylase vs. Pyruvate Carboxylase
  - 1.1 Introduction
  - 1.2 Phosphoenolpyruvate Carboxylase Reaction for Lysine Production
  - 1.3 Pyruvate Carboxylase as an Important Anaplerotic Enzyme for Lysine Production
  - 1.4 Refocusing on Phosphoenolpyruvate Carboxylase for Lysine Production
- 2 Effects of Pyruvate Kinase Mutation on Glutamic Acid and Lysine Production in the Presence or Absence of Phosphoenolpyruvate Carboxylase-Desensitizing Mutation
  - 2.1 Introduction
  - 2.2 Effects of *pyk* Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Glutamic Acid Production
  - 2.3 Effects of *pyk* Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Lysine Production
  - 2.4 Effect of Citrate Synthase Mutation on Lysine Production
  - 2.5 Decreased Phosphoenolpyruvate Carboxylase Activity Associated with *pyk* Deletion
  - 2.6 Interrelationship Among *pyk* Deletion, Phosphoenolpyruvate Carboxylase Resistant to Feedback Inhibition by Aspartic Acid, and Low-Activity-Level Citrate Synthase
  - 2.7 Conclusion
- 3 Enhanced Biomass Production in a *pyk*-Deleted Mutant of *Corynebacterium glutamicum* Cultured Under Biotin-Sufficient Conditions
  - 3.1 Introduction
  - 3.2 Enhanced Biomass Production in a *pyk*-Deleted Mutant Under Biotin-Sufficient Conditions
  - 3.3 Measurements of Anaplerotic Enzyme Activities in a *pyk*-Deleted Mutant
  - 3.4 Metabolome Analysis
  - 3.5 Excretion of Glycolytic Intermediates in *pyk*-Deleted Lysine Producers
- 4 Alterations of Malate:Quinone Oxidoreductase Activity in a *pyk*-Deleted Mutant and Elucidation of its Physiological Roles
  - 4.1 Introduction
  - 4.2 Malate:Quinone Oxidoreductase as the Main Site of Entry for Electrons into the Respiratory Chain in *Corynebacterium glutamicum*
  - 4.3 Malate:Quinone Oxidoreductase as a Controller of the Respiration Rate in *Corynebacterium glutamicum*
  - 4.4 Malate:Quinone Oxidoreductase as a Fine-tuner of Oxaloacetic Acid Concentration
- 5 Conclusions
- References

# 1 Contribution of Anaplerotic Reactions to Lysine Yield: Phosphoenolpyruvate Carboxylase vs. Pyruvate Carboxylase

## 1.1 Introduction

The anaplerotic reaction that replenishes oxaloacetic acid (OAA) is important for fermentative production of lysine because OAA, a precursor metabolite for lysine biosynthesis, is continuously withdrawn from the tricarboxylic acid (TCA) cycle during lysine production. In other words, an efficient anaplerotic reaction is required to increase lysine yield. Two anaplerotic reactions operate in *Corynebacterium glutamicum*: the formation of OAA from phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPC) and that from pyruvic acid by pyruvate carboxylase (PC). The contributions of these two reactions to lysine production have been investigated in detail for over 3 decades. Here, a brief history of these studies is provided.

## 1.2 Phosphoenolpyruvate Carboxylase Reaction for Lysine Production

In the 1980s, Shiio and coworkers were the first to shed light on the PEPC reaction for lysine production [1]. Using repeated random mutagenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment, they demonstrated enhanced lysine yields in *Brevibacterium flavum* (later reclassified as *C. glutamicum*) mutants having three distinct phenotypes: (1) low-activity-level citrate synthase ( $CS^L$ ), (2) PEPC that is resistant to feedback inhibition by aspartic acid ( $PEPC^R$ ), and (3) a pyruvate kinase (PYK) defect, in either a feedback-inhibition-resistant aspartokinase ( $AK^R$ ) background or a null-activity homoserine dehydrogenase background, suggesting the contribution of PEPC to catalyzing an anaplerotic reaction in lysine production. A PYK-defective mutant, KL-18, derived from an  $AK^R$ -type lysine producer, No. 2-190, exhibiting both  $CS^L$  and  $PEPC^R$ , is a representative example of the importance of PEPC as an anaplerotic enzyme for lysine production ([2], Table 1). As shown in Fig. 1,  $PEPC^R$  may contribute to improved lysine production, presumably through a smooth supply of OAA from PEP. A mutation causing PYK deficiency may block the metabolism of PEP to pyruvic acid, thereby directing the conversion of PEP into OAA by  $PEPC^R$ . On the other hand,  $CS^L$  is expected to decrease the consumption of OAA, enabling more OAA and pyruvic acid to be used for lysine biosynthesis. However, as these studies were conducted using mutants derived randomly by repeated NTG treatment, the precise contribution of each mutation to lysine production and the interrelationship among these mutations remain to be elucidated.

**Table 1** Effects of PYK mutation on lysine production in various *C. glutamicum* strains

Strain	Relevant mutation	Lys productivity	Reference
<i>Brevibacterium flavum</i>			
No. 2-190	CS <sup>L</sup> , PEPC <sup>R</sup> , AK <sup>R</sup>	38 g/L (as Lys·HCl)	Shiio et al. [2]
KL-18	PK-defective mutant of No. 2-190 <sup>a</sup>	43 g/L (as Lys·HCl)	
<i>Corynebacterium lactofermentum</i>			
ATCC21799	Lys producer, AEC <sup>R</sup> <sup>b</sup>	26.3 g/L	Gubler et al. [7]
L124	<i>pyk</i> -gene disruptant of ATCC21799	15.0 g/L	
<i>Corynebacterium glutamicum</i>			
ATCC21253	Lys producer, <i>hom</i> , <i>leu</i> <sup>c</sup>	20 g/L	Park et al. [8]
SM575	<i>pyk</i> -gene disruptant of ATCC21253	14 g/L	
<i>Corynebacterium glutamicum</i>			
ATCC13032 lysC <sup>fbr</sup>	<i>lysC</i> <sup>fbr</sup> <sup>d</sup>	10 mM (from 80 mM Glc.)	Becker et al. [9]
ATCC13032 lysC <sup>fbr</sup> ΔPYK	<i>pyk</i> -gene deletion of ATCC13032 lysC <sup>fbr</sup>	6 mM	

<sup>a</sup>Derived by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment

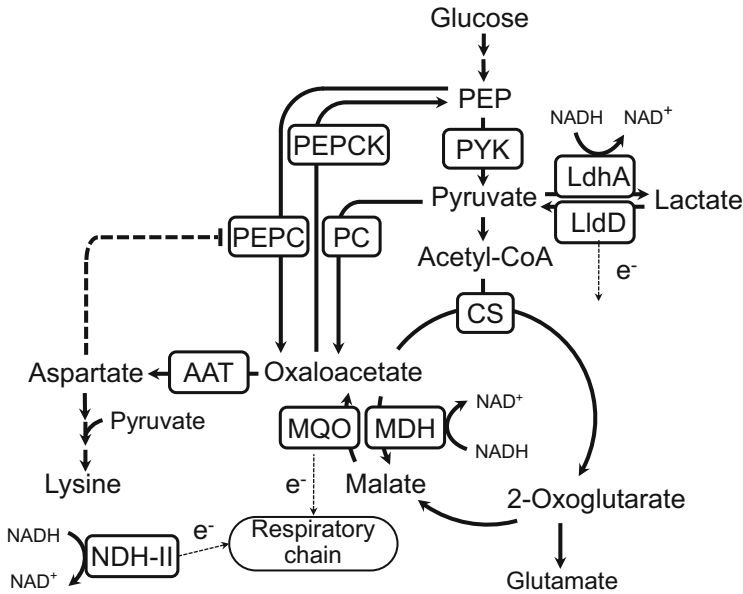
<sup>b</sup>AEC<sup>R</sup>, S-(2-aminoethyl)-L-cysteine resistance

<sup>c</sup>*hom*, *leu*, auxotrophic for homoserine and leucine, respectively

<sup>d</sup>*lysC*<sup>fbr</sup>, feedback-desensitized aspartokinase gene with T311I mutation

### 1.3 Pyruvate Carboxylase as an Important Anaplerotic Enzyme for Lysine Production

From the early 1990s, the development of genetic engineering enabled more precise metabolic analysis in *C. glutamicum*. Moreover in 1997, the activity of PC, another anaplerotic enzyme, was detected for the first time in this species using a permeabilized cell preparation [3]. This promoted identification of the anaplerotic reaction that contributes to lysine production in this bacterium. Specifically, for lysine production, PC but not PEPC was found to be important as a major anaplerotic enzyme supplying OAA; knockout of the PEPC gene did not affect lysine production [4], overexpression of the PC gene enhanced it, while inactivation of the PC gene led to a decrease in lysine yield [5]. Moreover, the positive effects of PYK-defective mutation on lysine production as reported by Shiio and coworkers [2, 6] were not observed, and even negative effects were detected in three independent studies in which *pyk*-gene knockout mutants were generated by genetic engineering [7–9]. As listed in Table 1, introduction of a *pyk* knockout mutation to various lysine producers having either AK<sup>R</sup> or homoserine auxotrophy led to decreased lysine productivity, to approximately 50% of those of the corresponding



**Fig. 1** Metabolic pathways focusing on anaplerotic reactions and respiration-related reactions in *Corynebacterium glutamicum*. PEP phosphoenolpyruvate, PYK pyruvate kinase, PEPC phosphoenolpyruvate carboxylase, PEPCCK phosphoenolpyruvate carboxykinase, PC pyruvate carboxylase, AAT aspartate aminotransferase, CS citrate synthase, LdhA lactate dehydrogenase, LldD quinone-dependent lactate dehydrogenase, MQO malate:quinone oxidoreductase, MDH malate dehydrogenase, NDH-II, NADH dehydrogenase-II, e<sup>-</sup> electron. The dashed line denotes feedback inhibition

parents. Under these conditions, PC has generally been recognized as an important anaplerotic enzyme for lysine production; the P458S mutation in *pyc* (enhanced PC activity) [10] has often been employed to construct lysine producers. Until recently, these contradictions concerning the effect of PYK mutation on lysine production were ignored. However, these discrepancies strongly suggested the importance of the coexistence of PYK defect and PEPC<sup>R</sup> for the enhancement of lysine production, as reported by Shiio and coworkers in their work using random mutagenesis [2, 6].

### 1.4 Refocusing on Phosphoenolpyruvate Carboxylase for Lysine Production

Although the PEPC reaction had been overlooked regarding lysine production, recently, PEPC was again demonstrated to be important for lysine production by a study in which its feedback inhibition by aspartic acid was deregulated [11]. Incidentally, a recent molecular technique study also demonstrated the effectiveness of

decreased CS activities on lysine production in *C. glutamicum* [12]. Therefore, the interrelationships among mutations conferring the aforementioned phenotypes described by Shiio and coworkers, namely, CS<sup>L</sup>, PEPC<sup>R</sup>, and PYK defect, on lysine production need to be reevaluated using modern recombinant DNA techniques.

## 2 Effects of Pyruvate Kinase Mutation on Glutamic Acid and Lysine Production in the Presence or Absence of Phosphoenolpyruvate Carboxylase-Desensitizing Mutation

### 2.1 Introduction

As described in the previous section, *pyk* knockout or deletion [7–9] and PEPC<sup>R</sup> [11] generated by recombinant DNA techniques were independently identified to have negative and positive effects on lysine production, respectively. However, the combined effect of these mutations on lysine production had not been clarified. In addition, no reports have described the effect of either *pyk* deletion or PEPC<sup>R</sup> on glutamic acid production. Thus, we have investigated the effects of *pyk* deletion on glutamic acid and lysine production in the presence or absence of the PEPC<sup>R</sup> mutation. In addition, the impact of CS<sup>L</sup> on lysine production, which Shiio and coworkers [13] suggested to have a promoting effect, was also evaluated using recombinant DNA techniques.

### 2.2 Effects of *pyk* Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Glutamic Acid Production

We derived a *pyk*-deleted mutant (strain D1) from wild-type *C. glutamicum* ATCC13032 using the double-crossover chromosome replacement technique and investigated its glutamic acid productivity under biotin-limited conditions [14]. When cultured in a 2-L jar fermentor using Medium F4 (complex medium with 100 g/L glucose and 3 µg/L biotin), strain D1 produced 25% more glutamic acid (32.0 g/L) than did ATCC 13032 (25.6 g/L) (Table 2). This was the first demonstration of the primary effects of *pyk* deletion in a defined *C. glutamicum* mutant.

An amino acid residue involved in PEPC<sup>R</sup> in *C. glutamicum* ATCC13032 was next identified based on the PEPC sequence information of *C. glutamicum* No. 70 [15]. A single amino acid substitution in PEPC, D299N, was found to relieve the feedback inhibition by aspartic acid. A simple mutant, strain R1, having this D299N substitution in PEPC was constructed from ATCC13032 using the same double-crossover chromosome replacement technique as in the case of strain D1.

**Table 2** Effects of *pyk* deletion on glutamic acid and lysine production in *C. glutamicum*

Strain	Relevant mutation	Glutamic acid (g/L)	Aspartic acid (g/L)	Lysine (g/L)	Reference
<i>Glutamic acid producer</i>					
ATCC13032	Wild-type	25.6	0.44	– <sup>a</sup>	Sawada et al. [14]
D1	$\Delta pyk$	32.0	2.3	–	
R1	PEPC <sup>R</sup>	31.0	1.2	–	Wada et al. [16]
DR1	$\Delta pyk$ , PEPC <sup>R</sup>	36.9	4.4	–	
<i>Lysine producer</i>					
P	AK <sup>R</sup>	–	–	9.36	Yanase et al.[17]
D2	AK <sup>R</sup> , $\Delta pyk$	–	–	10.1	
R2	AK <sup>R</sup> , PEPC <sup>R</sup>	–	–	10.8	
DR2	AK <sup>R</sup> , $\Delta pyk$ , PEPC <sup>R</sup>	–	–	12.9 <sup>b</sup>	
DRL2	AK <sup>R</sup> , $\Delta pyk$ , PEPC <sup>R</sup> , CS <sup>L</sup>	–	–	15.7 <sup>b</sup>	

PEPC<sup>R</sup>, desensitized phosphoenolpyruvate carboxylase resulting from the D299N mutation in the *ppc* gene

AK<sup>R</sup>, desensitized aspartokinase resulting from the T311I mutation in the *lysC* gene

CS<sup>L</sup>, reduced-activity citrate synthase resulting from the S252C mutation in the *glTA* gene

<sup>a</sup>Not applicable

<sup>b</sup>Significant differences ( $P < 0.05$ ) compared with strain P by *t*-test

Strain R1 produced significantly more (21% more) glutamic acid (31.0 g/L) than did ATCC13032 in a jar fermentor culture under biotin-limited conditions in Medium F4 (Table 2), indicating a positive effect of PEPC<sup>R</sup> on glutamic acid production. This effect was also investigated in combination with *pyk* deletion [16]. For this purpose, strain DR1, having both a *pyk* deletion and PEPC<sup>R</sup>, was constructed using strain D1 as the parent. Glutamic acid production by strain DR1 was elevated up to 36.9 g/L (Table 2), which was 44% higher than that by ATCC13032 and significantly higher than those by D1 and R1. Thus, these two mutations were found to exhibit a synergistic effect on glutamic acid production in *C. glutamicum*.

### 2.3 Effects of *pyk* Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Lysine Production

To clarify the effects of *pyk* deletion and PEPC<sup>R</sup> on lysine production, these mutations were introduced into lysine-producing *C. glutamicum* singly or in combination [17]. The *C. glutamicum* ATCC13032 mutant having AK<sup>R</sup> conferred by T311I amino acid substitution [10] was used as the parent strain (strain P). Strain P produced 9.36 g/L lysine from 100 g/L glucose in a jar fermentor culture. Under

these conditions, while the simple mutant D2 with *pyk* deletion or R2 with PEPC<sup>R</sup> (D299N) showed marginally increased lysine yields (10.1 and 10.8 g/L, respectively; Table 2), the mutant strain DR2 having both mutations showed a synergistic increase in lysine production (38% higher, 12.9 g/L; Table 2).

## 2.4 *Effect of Citrate Synthase Mutation on Lysine Production*

The importance of CS<sup>L</sup> in lysine production was previously suggested by Shii and coworkers [13]. However, no defined CS-defective mutant had been reported until recently, when *C. glutamicum* mutants with CS<sup>L</sup> created by a molecular approach were demonstrated to be effective for boosting lysine production [12]. We also identified a single amino acid substitution (S252C) responsible for CS<sup>L</sup> in *C. glutamicum* No. 70 [15]. This mutation was then introduced into the lysine-producing mutant strain DR2 having both *pyk* deletion and PEPC<sup>R</sup> mutations. The resulting strain, DRL2, showed a greater increase in lysine yield than did strain DR2 (22% higher, 15.7 g/L; Table 2). Thus, the CS<sup>L</sup> mutation was confirmed to be effective for lysine production, and these three mutations, *pyk* deletion, PEPC<sup>R</sup>, and CS<sup>L</sup>, were found to enhance lysine yield coordinately in *C. glutamicum* [17].

## 2.5 *Decreased Phosphoenolpyruvate Carboxykinase Activity Associated with *pyk* Deletion*

In both glutamic acid-producing and lysine-producing strains, alterations of the enzyme activities of the anaplerotic pathway were measured. In all of the *pyk*-deleted strains evaluated, D1, DR1, D2, and DR2, an increase in PEPC and a significant decrease in phosphoenolpyruvate carboxykinase (PEPCK) activities were detected compared with those in the corresponding *pyk* wild-type strains. As an example, changes in enzyme activities in strain D1 during culture for glutamic acid production in Medium F4 under biotin-limited conditions are shown in Table 3. The strain C1, whose *pyk* had been complemented by plasmid, showed similar PEPC and PEPCK activities to those in the wild-type strain, suggesting that the observed enzyme activity changes were caused by the *pyk* deletion. PEPCK activities in strains D2 and DR2 were 17% and 54% of those in the parental strain P and strain R2, respectively [17].

Since the enhanced PEPC activity and reduced PEPCK activity may boost anaplerotic flux from PEP to OAA, the *pyk*-deleted strains seemed to avoid PEP overaccumulation resulting from the absence of the PYK reaction. Aspartic acid formation in the culture medium under biotin-limited conditions (Table 2) also indicated increased OAA availability in the *pyk*-deleted mutants, as aspartic acid is synthesized via a one-step aminotransferase reaction from OAA (Fig. 1); the *pyk*-



**Table 3** Activities of enzymes involved in phosphoenolpyruvate/oxaloacetate metabolism and electron transfer to the respiratory chain

Enzyme	Specific activity [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ] <sup>a</sup>			Ratio (D1/wild-type)
	Wild-type	Strain D1	Strain C1	
<i>Medium F4<sup>b</sup></i>				
PYK	872 ± 56	<10	1209 ± 220	– <sup>c</sup>
PEPC	143 ± 15	175 ± 1.0	132 ± 3.0	1.22 <sup>d</sup>
PEPCK	53.5 ± 4.5	22.6 ± 4.7	52.9 ± 9.0	0.422 <sup>d</sup>
MQO	384 ± 55	417 ± 89	303 ± 63	1.09
MDH	638	672	650	1.05
<i>Medium F5<sup>c</sup></i>				
PYK	931 ± 197	<10	1057 ± 199	–
PEPC	103 ± 7.6	157 ± 7.9	136 ± 33	1.52 <sup>d</sup>
PEPCK	186 ± 14	22.5 ± 2.9	156 ± 5.0	0.121 <sup>d</sup>
MQO	698 ± 99	260 ± 39	770 ± 75	0.372 <sup>d</sup>
MDH	1743 ± 360	1713 ± 162	1684 ± 236	0.983
LldD	61.5 ± 14	35.4 ± 17	53.9 ± 22	0.576
SDH	64.0 ± 6.0	53.7 ± 16	53.8 ± 8.7	0.839
NDH-II	451 ± 35	252 ± 44	467 ± 10	0.559 <sup>d</sup>

<sup>a</sup>Values are mean ± SD ( $n = 3$ ) except for MDH ( $n = 2$ )

<sup>b</sup>Complex medium containing 3 µg/L biotin. Data from a report by Sawada et al. [14]

<sup>c</sup>Not applicable

<sup>d</sup>Significant differences ( $P < 0.05$ ) by  $t$ -test

<sup>e</sup>Complex medium containing 60 µg/L biotin. Data from a report by Sawada et al. [20]

deleted mutants, strains D1 and DR1, produced 2.3 and 4.4 g/L aspartic acid, respectively, while the wild-type strain produced only 0.44 g/L.

## 2.6 Interrelationship Among *pyk* Deletion, Phosphoenolpyruvate Carboxylase Resistant to Feedback Inhibition by Aspartic Acid, and Low-Activity-Level Citrate Synthase

Notably, CS<sup>L</sup> and PEPC<sup>R</sup> mutations were simultaneously introduced when a prototrophic revertant, No. 15, was derived from a CS-defective glutamic acid auxotroph, *B. flavum* No. 214, by Shioo and coworkers [18]. Furthermore, the PYK-defective mutation was also simultaneously introduced when an *S*-(2-aminoethyl)-L-cysteine (AEC)-resistant lysine-producing mutant, No. 1-231, was derived from strain No. 15 harboring both CS<sup>L</sup> and PEPC<sup>R</sup> [1]. These findings suggested causal relationships between these mutations as the probability of two mutations occurring simultaneously is extremely low.

It was speculated that PEPC<sup>R</sup> may relieve the stress caused by CS<sup>L</sup>, such as the possible accumulation of PEP. In addition, the PYK-defective mutation seemed to be necessary to increase the intracellular lysine concentration by an enhanced OAA

supply to competitively cancel out the toxic effect of a high concentration of AEC, a lysine analogue. PEPC<sup>R</sup> may also stabilize the effects of PYK-defective mutation by enabling the smooth conversion of PEP into OAA, thus avoiding the accumulation of toxic PEP.

The fact that the previously reported CS<sup>L</sup> mutants [12] were derived from a strain harboring the P458S mutation in *pyc* (enhanced PC activity, [10]) supported our speculation, as this mutation may relieve the metabolic constraints, for example, the possible accumulation of pyruvic acid, caused by CS<sup>L</sup>. We also experienced difficulty in introducing the CS<sup>L</sup> mutation (S252C) into strain P to obtain the simple CS<sup>L</sup> mutant (unpublished results). The obtained CS<sup>L</sup> mutant exhibited unstable growth and lysine productivity (lower than or comparable to that of strain P). Although this issue was not examined, it is possible that the CS<sup>L</sup> was compensated for by the expression of another enzyme possessing CS activity (e.g., methylcitrate synthase) [19], possibly leading to the observed instability. These findings suggest that the CS<sup>L</sup> mutant strains are stable only under conditions in which the anaplerotic reactions (PEPC or PC) have been enhanced.

## 2.7 Conclusion

All three mutations focused on by Shiio and coworkers during the 1980s in random mutagenesis studies, *pyk* deletion, PEPC<sup>R</sup>, and CS<sup>L</sup>, have been shown to have positive effects on lysine production using modern recombinant DNA techniques as well. The positive effect of *pyk* deletion on lysine production was more evident against a PEPC<sup>R</sup> background. Under lysine-producing (biotin-sufficient) conditions, PC (Fig. 1), but not PEPC, was regarded as the major anaplerotic enzyme supplying OAA. However, our results also showed the importance of the PEPC reaction as an alternative anaplerotic reaction when its feedback inhibition is deregulated. Thus, our results highlight the potential for *pyk* deletion in combination with PEPC<sup>R</sup> to enhance the anaplerotic reaction to improve lysine and glutamic acid production in *C. glutamicum*.

## 3 Enhanced Biomass Production in a *pyk*-Deleted Mutant of *Corynebacterium glutamicum* Cultured Under Biotin-Sufficient Conditions

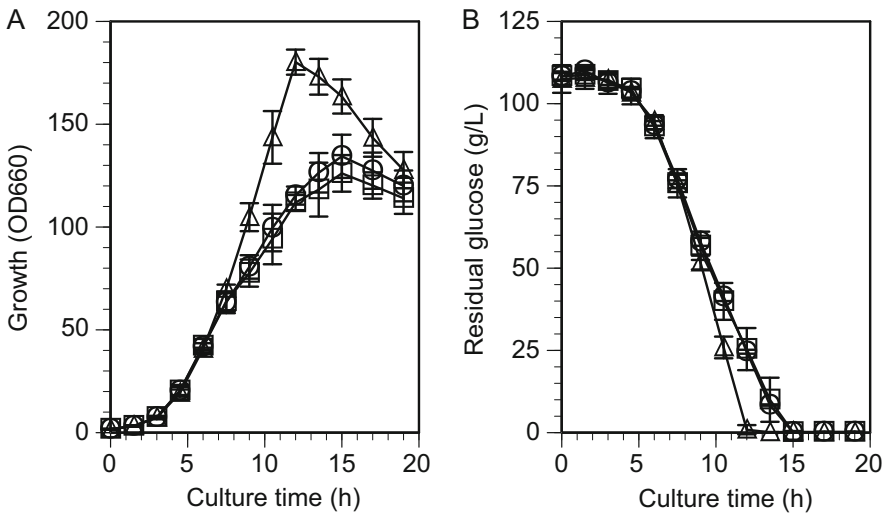
### 3.1 Introduction

The previous section described evaluation of the fermentation characteristics of a simple *pyk*-deleted mutant, strain D1, under biotin-limited conditions, which revealed 25% higher glutamic acid yield than that in the wild-type strain

[14]. This section summarizes the results of evaluating strain D1 under biotin-sufficient conditions. Surprisingly, strain D1 showed increased biomass production, which has not been described as a phenotype of *C. glutamicum* mutants during the long history of studies on this species.

### 3.2 Enhanced Biomass Production in a *pyk*-Deleted Mutant Under Biotin-Sufficient Conditions

Enhanced biomass production in a *pyk*-deleted mutant under biotin-sufficient conditions was evaluated in both complex Medium F5 and minimal CGXII medium, each containing 60 µg/L biotin [20]. Interestingly, strain D1 showed a 37% increase in growth in Medium F5 (based on OD<sub>660</sub>; Fig. 2A) compared with the wild-type ATCC13032. An increased rate of specific glucose consumption (consumed glucose (g) [dry cell weight (g)]<sup>-1</sup> h<sup>-1</sup>) was also observed (Fig. 2B) (35% higher than that in the parent). In addition, increased biomass production was observed in the minimal CGXII medium, although the increase (16%, based on OD<sub>660</sub>) was smaller than that observed in Medium F5. The *pyk*-complemented strain, C1, showed the same growth level and glucose consumption rate as those of ATCC13032 (Figs. 2A, B), indicating that the observed phenotypes had been caused by the *pyk* deletion.



**Fig. 2** Profiles of growth and glucose consumption of the wild-type *C. glutamicum* ATCC13032 strain, *pyk*-deleted mutant (D1), and *pyk*-complemented strain (C1) cultured in Medium F5. Reproduction of (A) and (B) from the report by Sawada et al. [20]. (A) Growth and (B) glucose consumption. Symbols: *circle*, wild-type strain; *triangle*, D1 strain; *square*, C1 strain. Values are means of three independent experiments. Bars represent the standard deviations

### **3.3 *Measurements of Anaplerotic Enzyme Activities in a *pyk*-Deleted Mutant***

Both PEPC and PEPCK activities were measured in cells cultured in Medium F5 under biotin-sufficient conditions (Table 3). Strain D1 showed a 52% increase in PEPC activity and an 88% decrease in PEPCK activity compared with ATCC13032, which were similar to the changes observed in Medium F4 under biotin-limited conditions, although the changes were more pronounced in Medium F5 than in Medium F4. These simultaneous changes in PEPC and PEPCK activities seemed to represent an important strategy to avoid PEP overaccumulation caused by *pyk* deletion, irrespective of the biotin concentration. The *pyk*-complemented strain C1 also showed enzyme activities similar to those of the wild-type strain (Table 3).

### **3.4 *Metabolome Analysis***

Clarification of the mechanism underlying the increased biomass production appeared to be difficult. Eventually, we implemented a metabolome analysis using cells cultured in CGXII medium, which provided valuable information to explain the enhanced growth in the mutant [20]. As shown in Table 4, there was accumulation of many intermediate metabolites located upstream of the PYK reaction, in both the glycolytic pathway and the pentose phosphate pathway, some of which were precursor metabolites for biomass production. On the other hand, the levels of intermediate metabolites located downstream of the PYK reaction, including the TCA cycle metabolites, were decreased. The intermediate concentration ratios of D1 to ATCC13032 tended to increase at 11 h (early stationary phase) compared with those at 6 h (logarithmic growth phase) (Table 4), suggesting increased metabolic distortion toward early stationary phase culture in strain D1. The accumulated precursor metabolites seemed to be converted into biomass, which probably relieved the metabolic distortion in strain D1. Metabolic changes in strain D1 under biotin-sufficient conditions, deduced from the glucose consumption rate, changes in enzyme activity, transcriptional analysis, and metabolome analysis, are summarized in Fig. 3 [20].

### **3.5 *Excretion of Glycolytic Intermediates in *pyk*-Deleted Lysine Producers***

Notably, previously reported simple *pyk*-deleted lysine producers (Table 1) not only showed lower lysine yields than those of their parents, but also produced glycolytic intermediate-related compounds such as dihydroxyacetone in combination with

**Table 4** Ratios of metabolite concentrations in D1 strain cells to those in wild-type strain cells cultured in CGXII medium

Metabolite	Ratio	
	(D6h/W6h) <sup>a</sup>	(D11h/W11h) <sup>b</sup>
<i>Glycolytic pathway</i>		
Glucose 6-phosphate <sup>c</sup>	2.63	2.59
Fructose 6-phosphate <sup>c</sup>	2.28	1.79
Fructose 1,6-diphosphate	1.58	1.41
Dihydroxyacetone phosphate <sup>c</sup>	1.22	1.70
3-Phosphoglyceric acid <sup>c</sup>	1.93	3.27
Phosphoenolpyruvate <sup>c</sup>	2.11	5.59
Pyruvic acid <sup>c</sup>	0.899	0.214
<i>TCA cycle</i>		
Acetyl CoA <sup>c</sup>	0.663	0.542
Citric acid	0.523	0.676
2-Oxoglutaric acid <sup>c</sup>	1.17	0.816
Succinic acid	0.708	0.522
Fumaric acid	1.03	0.719
Malic acid	0.844	0.656
<i>Pentose phosphate pathway</i>		
6-Phosphogluconic acid	1.83	8.23
Ribulose 5-phosphate	1.79	4.32
Ribose 5-phosphate <sup>c</sup>	1.55	2.57
Sedoheptulose 7-phosphate	2.51	6.31

The data are from Table 5 in the report by Sawada et al. [20]

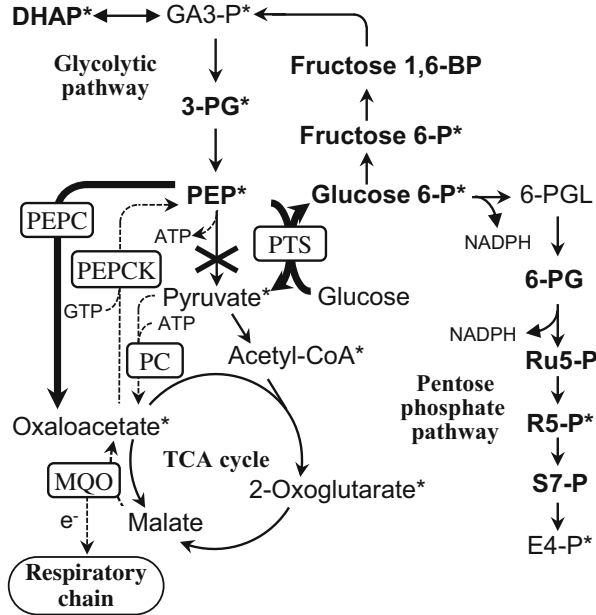
<sup>a</sup>Ratio of metabolite concentrations in strain D1 cultured for 6 h to those in the wild-type strain cultured for 6 h

<sup>b</sup>Ratio of metabolite concentrations in strain D1 cultured for 11 h to those in the wild-type strain cultured for 11 h

<sup>c</sup>Precursor metabolites essential for biomass synthesis

either glycerol or glyceraldehyde [7–9]. These C3 metabolites may be derived from dihydroxyacetone phosphate or glyceraldehyde phosphate. For example, *pyk*-gene-disrupted *Corynebacterium lactofermentum* L124 produced both 3.5 g/L dihydroxyacetone and 14.7 g/L glyceraldehyde along with 15 g/L lysine, while its parent strain *C. lactofermentum* ATCC21799 produced 26.3 g/L lysine, without these C3 metabolites arising as by-products [7].

Under these conditions, our metabolome analysis strongly suggested that, in simple *pyk*-deleted lysine-producing mutants, the accumulation of glycolytic intermediates induced C3 metabolites as by-products, which reduced the lysine yield. On the other hand, in lysine producers such as strain DR2, having both *pyk* deletion and PEPC<sup>R</sup>, glycolytic intermediates seemed to be smoothly converted to OAA by the deregulated PEPC, leading to improved lysine production without C3 metabolites arising as by-products.



**Fig. 3** Schematic model of carbon flux in *C. glutamicum* strain D1. The cross represents *pyk* deletion. Reproduction of Fig. 3 from the report by Sawada et al. [20]. The *thick arrow line* indicates increased flux and the *dotted arrow line* decreased flux, as deduced from enzyme activity measurements, transcriptional analysis, or rate analysis. Metabolites that increased in abundance as detected by metabolome analysis of strain D1 are shown in boldface. Asterisk indicates a precursor metabolite for biomass synthesis. *Glucose 6-P* glucose 6-phosphate, *PTS*, *PEP* carbohydrate phosphotransferase system, *fructose 6-P* fructose 6-phosphate, *fructose1,6-BP* fructose1,6-bisphosphate, *GA3-P* glyceraldehyde 3-phosphate, *DHAP* dihydroxyacetone phosphate, *3-PG* glycerate 3-phosphate, *6-PGL* 6-phosphogluconate, *6-PG* 6-phosphogluconate, *Ru5-P* ribulose 5-phosphate, *R5-P* ribose 5-phosphate, *S7-P* sedoheptulose 7-phosphate, *E4-P* erythrose 4-phosphate. Other abbreviations are the same as shown in the legend of Fig. 1

**Table 5** Fold changes in the activities of MDH, MQO, and respiration in ATPase and *pyk* mutants of *C. glutamicum* compared with those in their respective parental strain

	Fold change mutant strain/parent strain			
	<i>C. glutamicum</i> A-1	<i>C. glutamicum</i> D1		<i>C. glutamicum</i> D2
	ATPase-defect	$\Delta pyk$		AK <sup>R</sup> , $\Delta pyk$
	Medium G3 <sup>a</sup>	Medium F4	Medium F5	Medium L1 <sup>b</sup>
MDH	2.0 ↑	1.05 →	0.98 →	0.96 →
MQO	2.6 ↑	1.09 →	0.37 ↓	0.38 ↓
Respiration	1.4 ↑	1.00 →	0.43 ↓	0.65 ↓

Symbols: upward arrow, increased; rightward arrow, no change; down ward arrow, decreased

<sup>a</sup>Semisynthetic medium containing 60  $\mu\text{g/L}$  biotin [22]

<sup>b</sup>Lysine production medium containing 300  $\mu\text{g/L}$  biotin [17]

## **4 Alterations of Malate:Quinone Oxidoreductase Activity in a *pyk*-Deleted Mutant and Elucidation of its Physiological Roles**

### **4.1 Introduction**

Malate:quinone oxidoreductase (MQO) is a unique TCA cycle enzyme that catalyzes the conversion of malate to OAA with concomitant electron transfer to menaquinone in the respiratory chain [21]. During the characterization of *pyk*-deleted mutants, we found another interesting role of MQO in the regulation of carbon metabolism in *C. glutamicum*, which is described in this section.

### **4.2 Malate:Quinone Oxidoreductase as the Main Site of Entry for Electrons into the Respiratory Chain in *Corynebacterium glutamicum***

In *C. glutamicum*, it has been reported that MQO constitutes an NADH reoxidation system in coupling with malate dehydrogenase (MDH) that catalyzes the reverse reaction with concomitant oxidation of NADH. In our previous study [22], measurements of the activities of known enzymes involved in NADH reoxidation other than MQO and MDH, quinone-dependent lactate dehydrogenase (LldD) and lactate dehydrogenase (LdhA), both constituting a similar coupling system to MQO/MDH, and NADH dehydrogenase-II (NDH-II), identified the MQO/MDH coupling system as a major gate of electron transfer from NADH to the respiratory chain, judging from the far higher MQO activity than those of LldD and NDH-II. In line with these observations, an H<sup>+</sup>-ATPase-defective mutant of *C. glutamicum*, strain A-1, which showed enhanced glucose consumption and respiration, exhibited elevations in both MQO/MDH activities and their gene expression levels compared with those in the parent strain, ATCC13032 ([22]; Table 5).

### **4.3 Malate:Quinone Oxidoreductase as a Controller of the Respiration Rate in *Corynebacterium glutamicum***

The finding that MQO is involved in OAA metabolism prompted us to measure MQO activity in the simple *pyk*-deleted mutant, strain D1, during culture under biotin-sufficient conditions in Medium F5 [20]. In terms of the results, a dramatic decrease in MQO activity down to 37% of that in the parent ATCC13032 was found, with a concomitant decrease in the respiration rate to 43% of that in the

parent (Tables 3 and 5). These changes recovered to the parental levels in the *pyk*-complemented strain C1 (Table 3). The decreased MQO activity was regulated at the transcriptional level [20]. Similar simultaneous decreases in both MQO activity and the respiration rate were also observed in the *pyk*-deleted lysine producer strain D2 (38% MQO activity/65% respiration rate compared with the parent strain P, Table 5; [17]). In both cases, no significant change in activity was observed in MDH (Tables 3 and 5). These positive correlations between MQO activities and respiration rates observed in both of the *pyk*-deleted mutants (strains D1 and D2) and the aforementioned H<sup>+</sup>-ATPase-defective mutant (strain A1) led us to conclude that MQO may be involved in respiration control in *C. glutamicum*. Measurements of respiration-related enzymes, namely MQO, LldD, SDH, and NDH-II, in the wild-type ATCC13032 cultured in Medium F5 again confirmed MQO as the major enzyme contributing to respiration (Table 3).

#### **4.4 Malate:Quinone Oxidoreductase as a Fine-tuner of Oxaloacetic Acid Concentration**

Apart from the roles played by MQO in respiration control in *C. glutamicum*, its possible role in carbon metabolism as a TCA cycle enzyme needs to be considered. Our study revealed that, in contrast to the case under biotin-sufficient conditions, such coordinated decreases in MQO activity and respiration rate were not observed in strain D1 when cultured under biotin-limited conditions in Medium F4 during glutamic acid production [20]. In *pyk*-deleted mutants, strains D1 and D2, cultured under biotin-sufficient conditions, development of an excessive OAA supply was apparent, which probably led to the decreased MQO activity to relieve OAA overaccumulation (Fig. 1). However, under biotin-limited conditions in Medium F4, OAA overaccumulation did not seem to take place as TCA cycle intermediates including OAA were consumed as precursors of glutamic acid biosynthesis, thereby probably rendering a decrease in MQO activity unnecessary. These observations led us to propose an additional role of MQO in carbon metabolism as a modulator or safety valve to fine-tune the OAA concentration, as well as its role in controlling the respiration rate (redox balance) in *C. glutamicum* [20]. The regulation of MQO gene expression in response to these metabolic constraints needs to be clarified in the future to obtain a clearer understanding of the roles of MQO.

## **5 Conclusions**

Our studies on *pyk*-deleted mutants clarified the conditions under which the PEPC reaction is a useful anaplerotic reaction to supply OAA for lysine and glutamic acid production in *C. glutamicum*. Furthermore, detailed physiological studies suggested



MQO to be important in controlling both carbon flow at the OAA node and respiration rate/redox balance. These findings may contribute to the rational use of this bacterium in fermentation industries.

**Acknowledgments** We thank the staff at the Research Institute for Bioscience Products & Fine Chemicals, Ajinomoto Co., Inc., for their collaboration with this work. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (KAKENHI No. 23580096, to M.W.).

## References

1. Ozaki H, Shiio I (1983) Production of lysine by pyruvate kinase mutants of *Brevibacterium flavum*. *Agric Biol Chem* 47:1569–1576
2. Shiio I, Yokota A, Sugimoto S (1987) Effect of pyruvate kinase deficiency on L-lysine productivities of mutants with feedback-resistant aspartokinases. *Agric Biol Chem* 51:2485–2493
3. Peters-Wendisch PG, Wendisch VF, Paul S et al (1997) Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*. *Microbiology* 143:1095–1103
4. Gubler M, Park SM, Jetten M et al (1994) Effects of phosphoenol pyruvate carboxylase deficiency on metabolism and lysine production in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* 40:857–863
5. Peters-Wendisch PG, Schiel B, Wendisch VF et al (2001) Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. *J Mol Microbiol Biotechnol* 3:295–300
6. Shiio I, Yoshino H, Sugimoto S (1990) Isolation and properties of lysine-producing mutants with feedback-resistant aspartokinase derived from a *Brevibacterium flavum* strain with citrate synthase- and pyruvate kinase-defects and feedback-resistant phosphoenolpyruvate carboxylase. *Agric Biol Chem* 54:3275–3282
7. Gubler M, Jetten M, Lee SH et al (1994) Cloning of the pyruvate kinase gene (*pyk*) of *Corynebacterium glutamicum* and site-specific inactivation of *pyk* in a lysine-producing *Corynebacterium lactofermentum* strain. *Appl Environ Microbiol* 60:2494–2500
8. Park SM, Sinskey AJ, Stephanopoulos G (1997) Metabolic and physiological studies of *Corynebacterium glutamicum* mutants. *Biotechnol Bioeng* 55:864–879
9. Becker J, Klopprogge C, Wittmann C (2008) Metabolic responses to pyruvate kinase deletion in lysine producing *Corynebacterium glutamicum*. *Microb Cell Fact* 7:8
10. Ohnishi J, Mitsuhashi S, Hayashi M et al (2002) A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. *Appl Microbiol Biotechnol* 58:217–223
11. Chen Z, Bommareddy RR, Frank D et al (2014) Dereglulation of feedback inhibition of phosphoenolpyruvate carboxylase for improved lysine production in *Corynebacterium glutamicum*. *Appl Environ Microbiol* 80:1388–1393
12. Ooyen J, Noack S, Bott M et al (2012) Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity. *Biotechnol Bioeng* 109:2070–2081
13. Yokota A, Shiio I (1988) Effects of reduced citrate synthase activity and feedback-resistant phosphoenolpyruvate carboxylase on lysine productivities of *Brevibacterium flavum* mutants. *Agric Biol Chem* 52:455–463
14. Sawada K, Zen-in S, Wada M et al (2010) Metabolic changes in a pyruvate kinase gene deletion mutant of *Corynebacterium glutamicum* ATCC 13032. *Metab Eng* 12:401–407

15. Mori M, Shiiro I (1984) Production of aspartic acid and enzymatic alteration in pyruvate kinase mutants of *Brevibacterium flavum*. *Agric Biol Chem* 48:1189–1197
16. Wada M, Sawada K, Ogura K et al (2016) Effects of phosphoenolpyruvate carboxylase desensitization on glutamic acid production in *Corynebacterium glutamicum* ATCC 13032. *J Biosci Bioeng* 121:172–177
17. Yanase M, Aikoh T, Sawada K et al (2016) Pyruvate kinase deletion as an effective phenotype to enhance lysine production in *Corynebacterium glutamicum* ATCC13032: redirecting the carbon flow to a precursor metabolite. *J Biosci Bioeng* 122:160–167
18. Shiiro I, Ozaki H, Ujigawa-Takeda K (1982) Production of aspartic acid and lysine by citrate synthase mutants of *Brevibacterium flavum*. *Agric Biol Chem* 46:101–107
19. Radmacher E, Eggeling L (2007) The three tricarboxylate synthase activities of *Corynebacterium glutamicum* and increase of L-lysine synthesis. *Appl Microbiol Biotechnol* 76:587–595
20. Sawada K, Wada M, Hagiwara T et al (2015) Effect of pyruvate kinase gene deletion on the physiology of *Corynebacterium glutamicum* ATCC13032 under biotin-sufficient non-glutamate-producing conditions: enhanced biomass production. *Metab Eng Commun* 2:67–75
21. Molenaar D, van der Rest ME, Driessens A et al (2000) Functions of the membrane-associated and cytoplasmic malate dehydrogenases in the citric acid cycle of *Corynebacterium glutamicum*. *J Bacteriol* 182:6884–6891
22. Sawada K, Kato Y, Imai K et al (2012) Mechanism of increased respiration in an H<sup>+</sup>-ATPase-defective mutant of *Corynebacterium glutamicum*. *J Biosci Bioeng* 113:467–473