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Pyruvate kinase deletion as an effective phenotype to enhance lysine production in *Corynebacterium glutamicum* ATCC13032: Redirecting the carbon flow to a precursor metabolite

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Various attempts have been made to enhance lysine production in *Corynebacterium glutamicum*. Pyruvate kinase (PYK) defect is one of the strategies used to enhance the supply of oxaloacetic acid (OAA), a precursor metabolite for lysine biosynthesis. However, inconsistent effects of this mutation have been reported: positive effects of PYK defect in mutants having phosphoenolpyruvate carboxylase (PEPC) desensitized to feedback inhibition by aspartic acid, while negative effects in simple PYK gene (*pyk*) knockout mutants. To address these discrepancies, the effects of *pyk* deletion on lysine yield were investigated with or without the D299N mutation in *ppc* rendering PEPC desensitization. *C. glutamicum* ATCC13032 mutant strain P with a feedback inhibition-desensitized aspartokinase was used as the parent strain, producing 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 the PEPC-desensitization mutation showed marginally increased lysine yield (~1.1-fold, not significant), the mutant DR2 strain having both mutations showed synergistically increased lysine productivity (1.38-fold, 12.9 g/L). Therefore, the *pyk* deletion is effective under a PEPC-desensitized background, which ensures enhanced supply of OAA, thus clarifying the discrepancies. A citrate synthase defective mutation (S252C in *gltA*) further increased the lysine yield in strain DR2 (1.68-fold, 15.7 g/L). Thus, these three mutations coordinately enhanced the lysine yield. Both the malate:quinone oxidoreductase activity and respiration rate were significantly reduced in strains D2 and DR2. Overall, these results provide valuable knowledge for engineering the anaplerotic reaction to increase lysine yield in *C. glutamicum*.

[**Key words:** *Corynebacterium glutamicum*; Lysine; Phosphoenolpyruvate carboxylase; Pyruvate kinase; Citrate synthase; Feedback inhibition]

Since its discovery in 1958, numerous studies have been conducted to understand and improve lysine production in *Corynebacterium glutamicum* due to its increasing demand as an animal feed additive (1). The results obtained from the past five decades can be summarized as follows: (i) the primary requirement for lysine production is release of feedback inhibition of aspartokinase (AK), a key enzyme for biosynthesis of the aspartic acid family of amino acids, by lysine plus threonine (Fig. 1) (2,3); (ii) an enhanced supply of precursor metabolites such as oxaloacetic acid (OAA) is effective to improve lysine production (Fig. 1) (2,3); and (iii) enhanced NADPH recycling—e.g., through the pentose phosphate pathway—further increases lysine yield, because large amounts of the reducing equivalent NADPH are required for lysine biosynthesis (3,4).

Within category ii, a series of pioneering studies was performed by Shiio and colleagues (5,6) during the 1980s using mutants of *Brevibacterium flavum* No. 2247 (later reclassified as *C. glutamicum*). They suggested the importance of decreased citrate synthase (CS) activity, the release of feedback inhibition of phosphoenolpyruvate carboxylase (PEPC) by aspartic acid and a pyruvate kinase (PYK) defective mutation to improve lysine productivity, presumably through an enhanced supply of precursor metabolites, including OAA (Fig. 1) (5,6). However, because these studies were conducted using mutants derived randomly by repeated *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) treatment, the precise contribution and inter-relationship of each phenotype for lysine production remain to be clarified.

From the early 1990s, the advent of a genetic engineering technique enabled more precise metabolic analysis in *C. glutamicum*. Contrary to what many assumed, under lysine production (biotin-sufficient) conditions, pyruvate carboxylase (PC) (Fig. 1), but not PEPC, was found to function as the major anaplerotic enzyme supplying OAA; knockout of the PEPC gene did not affect lysine production (7), while over-expression of the PC gene enhanced lysine production (8). However, PEPC has recently been reported to be important for lysine production when its feedback inhibition by aspartic acid is deregulated (9). Regarding the CS defect, recent studies have also demonstrated that *C. glutamicum* mutants with decreased CS activity created by a genetic

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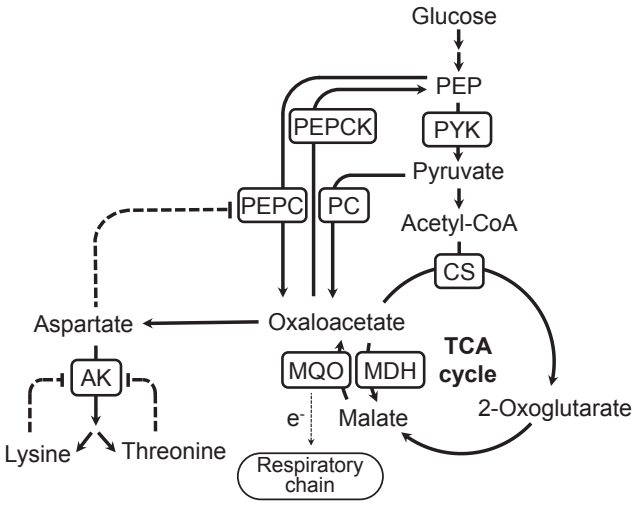

FIG. 1. Metabolic pathways related to the phosphoenolpyruvate/oxaloacetate reactions leading to lysine biosynthesis in *Corynebacterium glutamicum*. PEP, phosphoenolpyruvate; PYK, pyruvate kinase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; CS, citrate synthase; MQO, malate:quinone oxidoreductase; MDH, malate dehydrogenase; AK, aspartokinase; e^-, electron; ———|, feedback inhibition.

engineering approach are effective for lysine production (10). Therefore, both PEPC desensitization and decreased CS activity were proven to be effective for lysine production, not only by studies using classical random mutagenesis, but also by those employing modern recombinant DNA techniques.

However, there have been contradictory results concerning the effect of PYK mutation on lysine production. While Shiio and colleagues reported positive effects on lysine production using conventional PYK-defective mutants with both low-activity CS and feedback-insensitive PEPC (11,12), no effect or negative effects were reported in simple PYK gene (*pyk*) knockout mutants generated by genetic engineering methods (13–15). These discrepancies might suggest the importance of CS and/or PEPC mutations in conventional PYK-defective mutants for the enhancement of lysine production, but the details are yet to be elucidated.

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Source or reference
Corynebacterium glutamucum		
ATCC13032	Wild-type, ATCC13032	ATCC
ATCC14067	Wild-type, formaly, *Brevibacterium flavum* No. 2247, ATCC14067	ATCC
No. 70	Aspartic acid producer, *Brevibacterium flavum* No. 70	18
D1	Δ*pyk*	21
R1	Feedback-inhibition desensitized PEPC (D299N)	20
DR1	Δ*pyk*, D299N-PEPC	20
P	Feedback-inhibition desensitized AK (T311I)	This study
D2	Δ*pyk*, T311I-AK	This study
R2	D299N-PEPC, T311I-AK	This study
DR2	Δ*pyk*, D299N-PEPC, T311I-AK	This study
DRL2	Δ*pyk*, D299N-PEPC, T311I-AK, S252C-CS	This study
Escherichia coli		
JM109	*endA*⁻, *hsdR*⁻, *recA*⁻	Takara Bio
Plasmid		
pBS4S	Integration vector, *sacB*, Km^r	17
pBS4SlysC	pBS4S carrying wild-type *lysC*	This study
pBS4SlysC*	pBS4S carrying T311I-*lysC*	This study
pBS4SgltA	pBS4S carrying wild-type *gltA*	This study
pBS4SgltA*	pBS4S carrying S252C-*gltA*	This study

In the present study, to conclude the debate as to the effect of PYK defect on lysine production, we investigated the effect of PYK mutation on lysine production in the presence or absence of the PEPC-desensitized mutation. The PYK mutation may induce overaccumulation of PEP in the cell (16). However, this might not simply result in enhanced supply of OAA via the PEPC reaction, because PEPC activity is regulated by feedback inhibition via aspartic acid. For efficient conversion from PEP to OAA, this feedback inhibition must be deregulated to achieve stable enhancement of lysine production potentially. Another aim of this study was to investigate the combined effect of these three mutations, i.e., *pyk* deletion, PEPC desensitization and the CS defect, on lysine production to understand the inter-relationship of each mutation on lysine production. Although PEPC desensitization (9) and the CS defect (10) were identified independently to be effective for lysine production, interactions of these mutations with the PYK-defective mutation in terms of lysine productivity have not been clarified. The results obtained in this study not only reevaluate pioneering work performed by Shiio and colleagues using modern molecular techniques but also provide a scientific basis for enhanced lysine production through boosting OAA levels via the PEPC reaction in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains and plasmids The bacterial strains used in this study are listed in Table 1. *Escherichia coli* JM109 was used for plasmid construction. Plasmid pBS4S harboring both kanamycin resistance and *sacB* (17) was used to introduce both the AK-desensitized (T311I in *lysC*) and CS-reduced (S252C in *gltA*) mutations in *C. glutamicum* ATCC13032 using the double-crossover replacement technique. The pBS4S plasmid is non-replicative in *C. glutamicum*; thus, only strains containing the plasmid DNA fragment in their chromosome show kanamycin-resistance and sucrose-sensitive phenotypes. The aspartic acid-producing *C. glutamicum* strain No. 70 (formerly *B. flavum* No. 70) (18) was used to identify the mutation point in the CS gene that resulted in reduced enzyme activity.

Media Complete medium, Medium 7, was described previously (19). The sucrose-containing medium, Medium S10 (20), was used to confirm the completion of double-crossover replacement during strain construction. For lysine production in a 2-L jar fermentor, Medium S2 (19) and Medium L1 were used as seed medium and fermentation medium, respectively. Medium L1 contained (per liter) 100 g glucose, 40 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 8 mg $MnSO_4 \cdot 4–5H_2O$, 200 μg thiamine·HCl, 300 μg biotin, and 32.0 mL soybean-meal hydrolysate (Mieki, total nitrogen, 30.4 g/L). The pH was adjusted to 7.0 using 28% (w/w) ammonia solution. *E. coli* JM109 was cultured in Luria-Bertani medium, to which kanamycin (50 mg/L) was added for the culture of plasmid-harboring cells.

Construction of the AK-desensitized mutants P, D2, R2, and DR2 A DNA fragment encompassing *lysC-asd* from ATCC13032 containing an *Sph*I site was amplified using primers lysC-asd-F-Sp and lysC-asd-R-Sp (Table 2) and PrimeSTAR DNA polymerase (Takara Bio Inc., Ohtsu, Japan). PCR was performed using an initial denaturation at 98°C for 2 min, followed by 30 cycles of denaturation for

TABLE 2. Primers used in this study.

Primer	Sequence
lysC-asd-F-Sp	5′-atgcGCATGC[a]gacaggacaagcactggttg-3′
lysC-asd-R-Sp	5′-atgcGCATGC[a]gtcggtagacccagtttttc-3′
lysC-T311I-F	5′-gacatcaT[b]cttcacctgccctcgttcc-3′
lysC-T311I-R	5′-ggtgaagA[b]tgatgtcggtggtgccgtc-3′
lysC-F1	5′-cacggaaacgcacgcattgt-3′
gltA-F1	5′- tagcgtgttaaccggaccag -3′
gltA-R1	5′-gaccggtagctcaatctgtg-3′
gltA-F-Ba	5′-ggttGGATCC[c]tagcgtgttaaccggaccag -3′
gltA-R-Ba	5′-ggttGGATCC[c]gaccggtagctcaatctgtg -3′
gltA-S252C-F	5′-gatcggttG[d]cgcacaggccaacatgttt -3′
gltA-S252C-R	5′-cctgtgcgcaaC[d]cgatcatacgaacggt -3′
gltA-S252C-allele-F	5′-cgttcgtatgatcgtttg-3′
gltA-S252C-allele-R	5′-attcgttgccggtgtaga-3′

[a] GCATGC, *Sph*I site.
[b] Capital letters represent the introduced point mutation T311I.
[c] GGATCC, *Bam*HI site.
[d] Capital letters represent the introduced point mutation S252C.