Applied Biochemistry and Biotechnology (2020) 191:955–967
https://doi.org/10.1007/s12010-020-03231-y

CRISPR-Cpf1-Assisted Engineering of *Corynebacterium glutamicum* SNK118 for Enhanced L-Ornithine Production by NADP-Dependent Glyceraldehyde-3-Phosphate Dehydrogenase and NADH-Dependent Glutamate Dehydrogenase

Jinjun Dong[1] · Baojun Kan[1] · Hui Liu[1] · Milin Zhan[1] · Shuxian Wang[1] · Guochao Xu[1] · Ruizhi Han[1] · Ye Ni[1,2]

Received: 30 October 2019 / Accepted: 8 January 2020 /
Published online: 16 January 2020

Abstract

Here, *Corynebacterium glutamicum* SNK118 was metabolically engineered for L-ornithine production through CRISPR-Cpf1-based genome manipulation and plasmid-based heterologous overexpression. Genes *argF*, *argR*, and *ncgl2228* were deleted to block the degradation of L-ornithine, eliminate the global transcriptional repression, and alleviate the competitive branch pathway, respectively. Overexpression of *CsgapC* (NADP-dependent glyceraldehyde 3-phosphate dehydrogenases gene from *Clostridium saccharobutylicum* DSM 13864) and *BsrocG* (NADH-dependent glutamate dehydrogenase gene from *Bacillus subtilis* HB-1) resulted markedly increased ornithine biosynthesis. Eventually, the engineered strain KBJ11 (SNK118Δ*argR*Δ*argF*Δ*ncgl2228*/pXMJ19-*CsgapC*-*BsrocG*) was constructed for L-ornithine overproduction. In fed-batch fermentation, L-ornithine of 88.26 g/L with productivity of 1.23 g/L/h (over 72 h) and yield of 0.414 g/g glucose was achieved by strain KBJ11 in a 10-L bioreactor. Our result represents the highest titer and yield of L-ornithine production by microbial fermentation. This study suggests that heterologous expression of *CsgapC* and *BsrocG* could promote L-ornithine production by *C. glutamicum* strains.

Keywords *Corynebacterium glutamicum* · L-ornithine · CRISPR-Cpf1 · NADPH pool · Glyceraldehyde-3-phosphate dehydrogenase

Jinjun Dong and Baojun Kan contributed equally to this work.

✉ Ye Ni
yni@jiangnan.edu.cn

[1] Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122 Jiangsu, China

[2] Key Laboratory of Guangxi Biorefinery, Nanning 530003 Guangxi, China

Introduction

L-ornithine, as an intermediate metabolite of urea cycle, is an alkaline non-essential amino acid with a variety of physiological and biochemical functions. L-ornithine is beneficial for body recovery from diseases such as trauma, burns, infections, and even cancer. It also has a therapeutic effect on liver diseases [1] and wound healing [2]. At present, L-ornithine can be produced by enzymatic hydrolysis of arginine by arginase, aerobic fermentation by recombinant microorganisms, including *E. coli* [3], *Corynebacterium crenatum* [4], and *Corynebacterium glutamicum* strains [5–8]. In *C. glutamicum*, L-glutamate can be converted to L-ornithine through four enzymes encoded by an operon *argCJBD*, accompanying 2 mol consumption of NADPH for 1 mol of L-ornithine [9].

C. glutamicum, an environmentally friendly and non-pathogenic Gram-positive soil bacteria, has been widely applied in industrial biotechnology processes for producing various organic acids and amino acids including 2,3-butanediol [10], succinic acid [11, 12], L-valine [13], L-lysine [14, 15], L-isoleucine [16], L-arginine [17–19], and L-ornithine [7, 8]. With the rapid development of molecular biology and synthetic biology, CRISPR system quickly became the most popular gene editing tool in the fields of human biology, agriculture, and microbiology [20–25]. Now, CRISPR-Cpf1 system has emerged as a new and highly efficient gene editing tool in *C. glutamicum* [26]. There have been few reports on the application of CRISPR-Cpf1 system in the metabolic engineering of *C. glutamicum*.

In recent years, there have been a number of reports on engineering of *C. glutamicum* for producing L-ornithine. Jiang et al. developed a *C. glutamicum* strain, which allowed 24.1-g/L L-ornithine in a 5-L bioreactor by metabolic modulation and adaptive evolution [6]. Zhang et al. systematically constructed *C. glutamicum* strain SO1 for L-ornithine synthesis by inactivation of *argF*, *ncgl1221*, *argR*, *putP*, *ncgl2228*, attenuation of 2-oxoglutarate dehydrogenase, and overexpression of *gldH* and *lysE*. Recently, systematic manipulation of both central metabolic pathways and hexose monophosphate pathway was adopted by the same group to obtain *C. glutamicum* strain SO16 for producing 32.3 g/L of -ornithine [8, 27]. Shu et al. combined the approaches of abolishing competing branch pathways and constructing a linear transacetylation pathway, resulting in a *C. crenatum* strain capable of producing 40.4-g/L -ornithine in a 5-L bioreactor [4]. However, the performance of above strains might not meet the requirements of industrial production. Kim et al. created a -ornithine-producing *C. glutamicum* strain by inactivating *argF*, *proB*, and *argR*, enhancing the pentose phosphate pathway, and overexpressing *argCJBD*. In fed-batch fermentation, 51.5-g/L ornithine with a yield of 0.24-g/g glucose and productivity of 1.29g/L/h were achieved [7]. In terms of strengthening NADPH pool, redirecting the carbon flux towards the pentose phosphate pathway could lead to a loss of carbon flux due to released carbon dioxide.

C. glutamicum SNK 118 is an industrial L-arginine-producing strain, which has been engineered for enhanced L-arginine production [19]. In present work, *C. glutamicum* SNK 118 was metabolically engineered by deleting *argF*, *argR*, and *ncgl2228* genes using CRISPR-Cpf1 knockout system, and gene overexpression for improved carbon flux and NADPH supply. Consequently, a high L-ornithine-producing *C. glutamicum* strain KBJ11 was constructed and evaluated in fed-batch fermentation.

Materials and Methods

Bacterial Strains and Growth Condition

A high -arginine-producing strain *C. glutamicum* SNK118 [19] preserved in the laboratory was used as the initial strain for the production of L-ornithine. Genomic DNA of *Clostridium acetobutylicum* 1.7, *Clostridium beijerinckii* DSM 1739, *Clostridium saccharobutylicum* DSM 13864, and *Bacillus subtilis* HB-1 were used as PCR templates. *E. coli* JM109 and *E. coli* BL21 (DE3) were used in gene cloning and expression studies. All *Clostridium* sp. strains were inoculated in Reinforced Clostridial Medium (10.0-g/L meat extracts beef, 3.0-g/L yeast extract, 1.0-g/L soluble starch, 5.0-g/L, glucose, 10.0-g/L peptone, 3.0-g/L sodium chloride, 0.5 -g/L cysteine hydrochloride, and 3.0-g/L sodium acetate) at room temperature [28]. For *E. coli* and *B. subtilis* strains, Luria-Bertani (LB) broth was applied, containing appropriate antibiotics for selection. When required, 34 μg/ml of chloramphenicol (Cm) or 50 μg/mL of kanamycin (Km) was added.

C. glutamicum strains were cultivated in brain heart infusion (BHI) medium (18.5-g/L brain heart infusion with 91 g/L sorbitol and 0.3 g/L L-His) or BHI plates (18 g/L agar) at 30 °C for genetic disruption and complementation. When pJYS3 series were to be cured, the *C. glutamicum* strains were cultured overnight in kanamycin-free medium at 34 °C [26]. When required, 10 μg/mL of chloramphenicol (Cm) or 30 μg/mL of kanamycin (Km) was added.

Flask Fermentation

In shake flask fermentation, single clone of the strains was streaked on BHI agar plates for 24 h of incubation. Subsequently, a colony was inoculated and grown on BHI agar-slant in a test tube at 30 °C for 18 h. Then the culture was transferred into a 250-mL shaker flask containing 30 mL of the seed medium. Each liter of the seed medium consisted of 30-g glucose, 25-g corn steep liquor, 5-g $(NH_4)_2SO_4$, 0.8-g carbamide, 0.5-g $MgSO_4{\cdot}7H_2O$, 1.5-g KH_2PO_4, 0.5-g $K_2HPO_4{\cdot}3H_2O$, 0.3-g L-arginine, 0.3-g L-His, and 50-μg biotin. After 16 h of cultivation at 30 °C and 120 rpm, 2.5 mL of seed culture was inoculated in a 500-mL shake flask containing 30-mL fermentation medium. Each liter of the fermentation medium contained 80-g glucose, 20-g corn steep liquor, 40-g $(NH_4)_2SO_4$, 0.8-g carbamide, 1-g $MgSO_4{\cdot}7H_2O$, 1.5-g KH_2PO_4, 0.5-g $K_2HPO_4{\cdot}3H_2O$, 0.3-g L-arginine, 0.3-g L-His, 100-μg biotin, 200-μg thiamine, 22.38-mg $MnSO_4{\cdot}H_2O$, 18.3-mg $FeSO_4{\cdot}7H_2O$, and 30-g $CaCO_3$. The flask fermentation was performed in triplicate at 30 °C and 120 rpm, and 400-μL samples were collected timely for further analysis. During fermentation, glucose was fed from an 750 g/L of glucose stock solution when the residual glucose titer was below 10 g/L of glucose, and appropriate amount of $NH_3{\cdot}H_2O$ was added to maintain the pH at 6.8–7.0 according to phenol red indicator. All strains constructed in this work are listed in Table 1.

Fed-Batch Fermentation

Fed-batch fermentation of *C. glutamicum* KBJ11 was carried out in 10-L bioreactors with 5.5-L working volume. Each liter of the medium consisted of 80-g glucose, 20-g corn steep liquor, 40-g $(NH_4)_2SO_4$, 0.8-g carbamide, 1-g $MgSO_4{\cdot}7H_2O$, 1.5-g KH_2PO_4, 0.5-g $K_2HPO_4{\cdot}3H_2O$, 0.3-g L-arginine, 0.3-g L-His, 100-μg biotin, 200-μg thiamine, 22.38-mg $MnSO_4{\cdot}H_2O$, 18.3-mg $FeSO_4{\cdot}7H_2O$, and 10-mg chloramphenicol. The aeration and temperature were maintained at 1–1.5 vvm and 30 °C, respectively. Dissolved oxygen level was controlled at 20–30% by adjusting the agitation speed, and the pH was maintained at 6.8 by addition of 15% ammonia solution. When

Table 1 Strains and plasmids used in this study

Strain/plasmid	Description	Sources
Strains		
E. coli JM109	Host strain for cloning	Takara
E. coli BL21(DE3)	Host strain for expression	Takara
SNK118	L-Arginine-producing *C. glutamicum* mutant strain	Industrial
JML04	*C. glutamicum* SNK118Δ*argR*	This study
KBJ01	SNK118Δ*argF*Δ*argR*	This study
KBJ02	SNK118Δ*argR*Δ*argF* harboring pXMJ19	This study
KBJ03	SNK118Δ*argR*Δ*argF* harboring pXMJ19-*CbgapC*	This study
KBJ04	SNK118Δ*argR*Δ*argF* harboring pXMJ19-*CagapC*	This study
KBJ05	SNK118Δ*argR*Δ*argF* harboring pXMJ19-*CsgapC*	This study
KBJ06	SNK118Δ*argR*Δ*argF* harboring pXMJ19-*BsrocG*	This study
KBJ07	SNK118Δ*argR*Δ*argF*Δ*ncgl2228*	This study
KBJ08	SNK118Δ*argR*Δ*argF*Δ*ncgl2228* harboring pXMJ19	This study
KBJ09	SNK118Δ*argR*Δ*argF*Δ*ncgl2228* harboring pXMJ19-*CsgapC*	This study
KBJ10	SNK118Δ*argR*Δ*argF*Δ*ncgl2228* harboring pXMJ19-*BsrocG*	This study
KBJ11	SNK118Δ*argR*Δ*argF*Δ*ncgl2228* harboring pXMJ19-*CsgapC-tac-BsrocG*	This study
Plasmids		
pXMJ19	Cm^r, Shuttle vector for expression of proteins (Ptac, lacIq)	[40]
pXMJ19-*CbgapC*	pXMJ19 carrying *CbgapC* gene from *Clostridium beijerinckii* DSM 173	This study
pXMJ19-*CagapC*	pXMJ19 carrying *CagapC* gene from *Clostridium acetobutylicum* 1.7	This study
pXMJ19-*CsgapC*	pXMJ19 carrying *CsgapC* gene from *Clostridium saccharobutylicum* DSM 13864	This study
pXMJ19-*BsrocG*	pXMJ19 carrying *rocG* gene from *Bacillus subtilis* HB-1	This study
pXMJ19-*CsgapC-BsrocG*	pXMJ19 carrying *Clostridium saccharobutylicum* DSM 13864 *CsgapC* and *Bacillus subtilis* HB-1 *BsrocG*	This study
pJYS3_Δ*crtYf*	Kanamycin resistance (kan^R), shuttle vector	[26]
pJYS3_Δ*argF*Δ*argR*	kan^R, shuttle vector,harboring Δ*argF*Δ*argR* fragment	This study
pJYS3_Δ*ncgl2228*	kan^R, shuttle vector,harboring Δ*ncgl2228* fragment	This study

OD_{660} reached 20, 0.8-mM IPTG (final concentration) was added for induction. When the residual glucose was below 10 g/L, concentrated glucose (750 g/L) was added. Fermentation was performed in at least three independent experiments.

Enzyme Activity Assay

Glyceraldehyde-3-phosphate dehydrogenase activity was assayed spectrophotometrically by measuring the reduction of NADP (or NAD) at a wavelength of 340 nm and 30 °C [29]. The standard reaction mixture contained 300 μL of 100-mM Tris-HCl (pH 8.5), 60 μL of 10-mM H_3PO_4, 60 μL of 10-mM NADP (or NAD), 60 μL of 10-mM glyceraldehyde 3-phosphate, 50-μL β-mercaptoethanoland, and 70-μL crude extract. Catalytic reaction was initiated by the addition of cell-free extracts. One unit of glyceraldehyde-3-phosphate dehydrogenase activity was defined as

the amount of enzyme required for catalyzing the reduction of 1-μmol NAD (or NADP) in 1 min under the above conditions.

Glutamate dehydrogenase activity was assayed spectrophotometrically as described previously [30]. The standard reaction mixture contained 580 μL of 55-mM Tris-HCl (pH 7.5) containing 2% glycerol, 10-mM NaCl, 100-mM NH_4Cl, 10-mM α-ketoglutarate, 0.2-mM NADPH (or NADH), and 20-μL crude extract. Catalytic reaction was initiated by addition of the cell-free extracts. Enzyme activity was calculated by monitoring the decrease in absorbance at 340 nm. One unit of glutamate dehydrogenase activity was defined as the amount of enzyme catalyzing the oxidation of 1-μmol NADH (or NADPH) in 1 min under the above conditions.

Protein concentration was calculated by Bradford method using bovine serum albumin as a standard. Extinction coefficient of 6220 M^{-1} cm^{-1} was used.

Primers, Construction of Plasmids and Strains, and Gene Knockout

All plasmids constructed in this study are listed in Table 1. *E. coli*/*C. glutamicum* shuttle plasmid pXMJ19 was used for gene expression under *tac* promoter with optimized ribosome binding site (RBS) in *C. glutamicum*. CRISPR-Cpf1-assisted genome editing tool was generously provided by Prof. Sheng Yang [26]. The all-in-one plasmid pJYS3_Δ*crtYf* consisting of FnCpf1, CRISPR RNA, and homologous arms was used for gene deletions in *C. glutamicum*. Genetic manipulation for deletion of *argF*, *argR*, and *ncgl2228* in the genome of *C. glutamicum* was carried out as described in previous study. All DNA fragments were amplified by PCR from genomic DNA of corresponding microorganism with sequence-specific primers. Primer design was performed using CE Design V1.04 Software. DNA fragment and vector assembly were performed following standard procedure of Gibson [31]. Transformation of *E. coli* and *C. glutamicum* strains via electroporation was carried out following standard procedures [32, 33]. Genotypes of recombinant strains were determined by PCR and DNA sequencing.

Analytical Procedures

Cell growth was monitored by measuring OD_{660} using a spectrophotometer (UV-1800PC; Mapada Instruments, Shanghai, China). Both glucose and glutamate levels were measured using an SBA-40C biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, Shandong, China).

Amino acid concentrations were analyzed by Hitachi Amino Acid Analyzer (L-8900; HITACHI, Japan). BBCAs concentrations were determined by an Agilent HPLC (1260; Agilent Technologies, Germany) following a previous procedure [34]. L-ornithine concentrations were determined by colorimetry using ninhydrin at 515 nm, as described previously [35]. All assays were performed in three individual experiences.

Results and Discussion

C. glutamicum SNK118 Is a Promising Strain for L-Ornithine Production

L-ornithine is a precursor for L-arginine synthesis. *C. glutamicum* SNK118, an L-arginine producer with sequenced genome (accession No. PRJNA428026) [19], was used as chassis

strain for the production of L-ornithine. Gene *argF*, encoding ornithine carbamoyltransferase, was deleted to interrupt the degradation of L-ornithine to L-citrulline and L-arginine as shown in Fig. 1. Also, *argR* of *arg* operon, which is close to *argF*, was inactivated simultaneously for unnecessary interference. The resulted strain, KBJ01 (SNK118Δ*argF*Δ*argR*), became an L-arginine auxotroph, whereas the gene deletions did not affect cell growth owing to seed medium supplemented with 0.3-g/L L-arginine for cure (Fig. 2a). As a result, the production of L-ornithine by KBJ01 reached 28.42 g/L after 84 h in shaker-flask, which is 46-fold higher than that produced by JML04 (SNK118Δ*argR*) (0.62 g/L) (Fig. 2a), and 1.54-fold of that produced by an engineered strain originated from *C. glutamicum* S9114 (18.4 g/L) [36]. Thus, application of CRISPR-Cpf1 for abolition of *argF* is effective for L-ornithine accumulation. Moreover, KBJ01 produced 1.33 g/L of branched chain amino acids (BCAAs) in the fermentation supernatant (Fig. 2b). Therefore, preliminary results suggest that KBJ01 is a potential L-ornithine-producing strain.

Screening of Glyceraldehyde-3-Phosphate Dehydrogenases for Enhanced NADPH Supply

Fermentative production of amino acids often requires a large amount of NADPH [37]. The pathway flux of L-ornithine biosynthesis requires 2 mol of NADPH per mole of L-ornithine from glucose substrate in *C. glutamicum*. As reported, substitute of endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase with NADP-dependent glyceraldehyde 3-phosphate dehydrogenase from *Streptococcus mutans* improved both NADPH abundance and L-lysine production in *C. glutamicum* [38]. Thus, insufficient intracellular NADPH content has been recognized as a limiting factor in L-ornithine biosynthesis. Here, to enhance NADPH supply for L-ornithine biosynthesis, additional NADPH supply generated through glycolytic

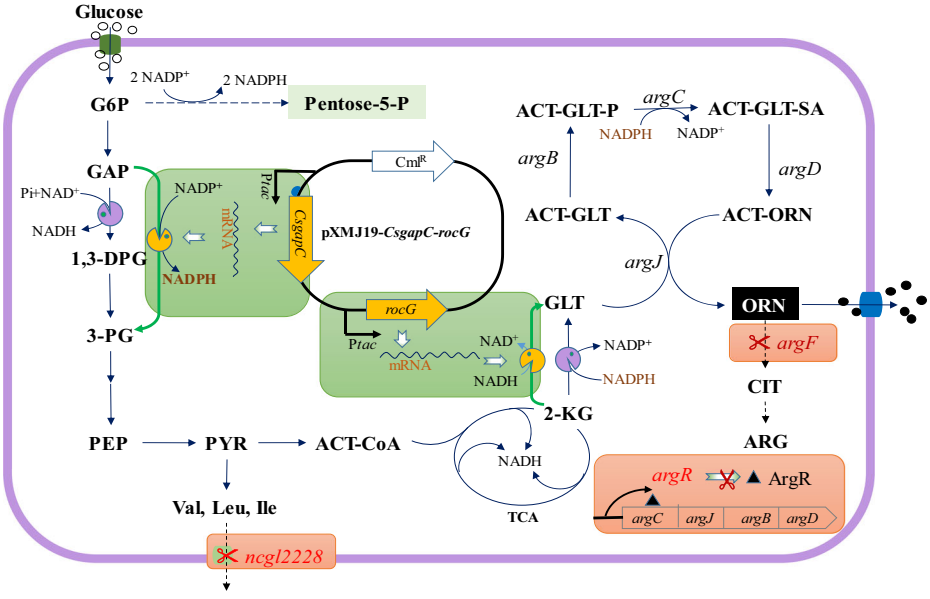

Fig. 1 Major metabolic pathways associated with L-ornithine biosynthesis in *C. glutamicum* and metabolic engineering approaches adopted for enhanced L-ornithine production in this study

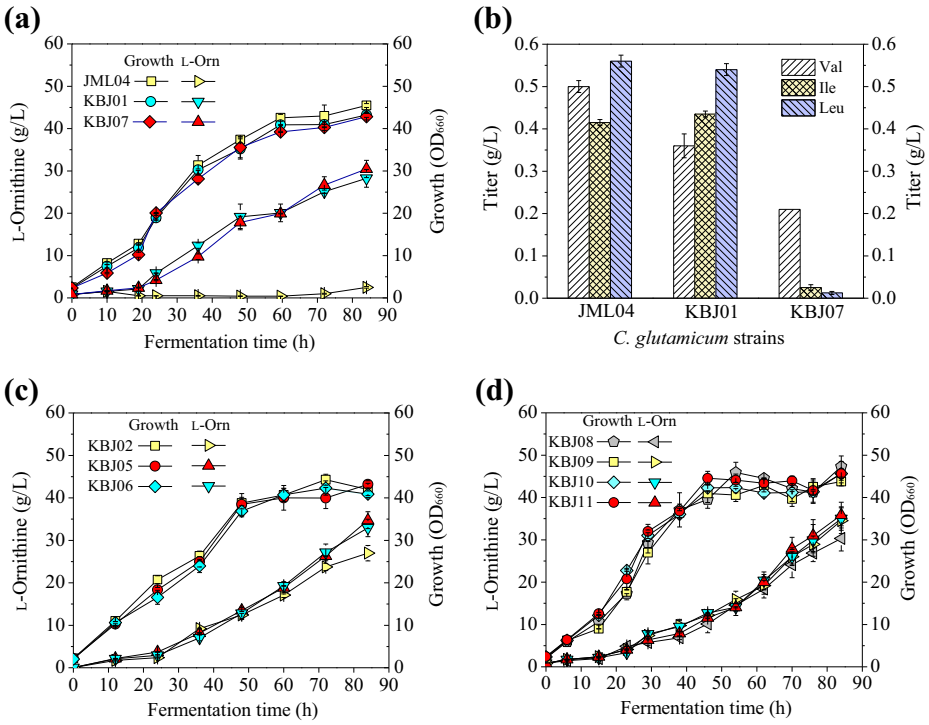

Fig. 2 L-ornithine production by recombinant *C. glutamicum* strains. Time course of cell growth and L-ornithine production by JML04, KBJ01, KBJ07 (**a**); KBJ02, KBJ05, KBJ06 (**c**); KBJ08, KBJ09, KBJ10, KBJ11 (**d**); and BCAAs byproducts produced by JML04, KBJ01, KBJ07 (**b**)

pathway of *C. glutamicum* was attempted, using NADP-dependent glyceraldehyde 3-phosphate dehydrogenases (GapCs) from different microorganisms. Three *gapC* genes from *Clostridium acetobutylicum* 1.7, *Clostridium beijerinckii* DSM 1739, and *Clostridium saccharobutylicum* DSM 13864 were selected.

Three *gapC* genes from various microbes were cloned into shuttle vector pXMJ19 and expressed with optimized RBS under tac promoter in *E. coli* BL21 (DE3). Then, activity of recombinant glyceraldehyde-3-phosphate dehydrogenase in cell extracts was determined as summarized in Table 2. The recombinant CsgapC exhibited a significantly higher specific activity (5263 mU/mg) than that of CagapC (2000 mU/mg) at 30 °C, whereas CbgapC showed no catalytic activity. Then, different recombinant plasmids were introduced into SNK118Δ*argF*Δ*argR* strain individually to evaluate their roles in providing an alternative source of NADPH. It has been reported that functional expression of *CagapC*, which encodes NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, resulted in greater availability of NADPH in *C. glutamicum* [9]. Nevertheless, only CsgapC displayed an enzymatic activity of 74 mU/mg (Table 2) in the presence of glyceraldehyde-3-phosphate and NADP as substrates. It is speculated that differences in species may lead to the discrepancy. These results suggest that heterologous expression of *CsgapC* gene could render an alternative source of NADPH.

Expression of *rocG* for Reduced NADPH Consumption

In previous report, mining natural NADH-utilizing dehydrogenases enabled improved lysine synthesis in *C. glutamicum* [14]. Glutamate dehydrogenase from *C. glutamicum* preferably utilizes NADPH as cofactor. Overexpression of *rocG* from *Bacillus subtilis*, which encodes a NADH-dependent glutamate dehydrogenase, could improve the production of L-ornithine to 14.8 g/L in *C. glutamicum* strain [9]. Furthermore, to reduce the consumption of NADPH, *BsrocG* was introduced into strain KBJ02 (SNK118Δ*argF*Δ*argR*/pXMJ19) to allow NADH consumption and NADPH accumulation for L-ornithine synthesis (Fig. 1). As shown in Table 2, the specific activities of recombinant *BsrocG* was determined to be 152 mU/mg using ammonia, NADH, and 2-ketoglutarate as substrates in strain KBJ02, while no activity was detected with NADPH as cofactor. Consistent with previous report [9], *BsrocG* was functionally expressed in KBJ02, which could have positive effect on enhancing NADPH level.

Expression of *CsgapC* or *BsrocG* for Improved L-Ornithine Synthesis

To evaluate the function of *CsgapC* and *BsrocG* on L-ornithine production by strain KBJ01, flask fermentation using strains KBJ05 (KBJ01/pXMJ19-*CsgapC*) and KBJ06 (KBJ01/pXMJ19-*BsrocG*) was performed. Empty plasmid pXMJ19 was introduced into KBJ01 to construct KBJ02 as a control. As a result, the accumulation of L-ornithine by KBJ05 and KBJ06 reached 34.75 g/L and 32.93 g/L respectively after 84 h of cultivation, which was 28.8% and 22.1% higher than that (26.98 g/L) obtained with KBJ02 (Table 3), presumably due to improved NADPH supply. Considering the possible influence of recombinant plasmids, cell growth, and ornithine biosynthesis of stains (KBJ02, KBJ05, and KBJ06) was evaluated in flask fermentation. In fact, no difference was observed in the growth of these strains (Fig. 2c). It has been reported that replacing NAD-utilizing glyceraldehyde 3-phosphate dehydrogenase from *E. coli* with NADP-utilizing enzyme from *C. acetobutylicum* could facilitate NADPH-dependent pathways and resulted in a significantly higher productivity [39]. Notably, recombinant expression of *CsgapC* replenished novel targets for promoting the biosynthesis of NADPH-dependent metabolites. Besides, compared with enhancing oxidative pentose phosphate pathway [7, 8], this approach is not accompanied by a loss of carbon flux.

Reducing BCAAs Transportation Exerts a Positive Effect on L-Ornithine Synthesis

Obviously, byproducts of branched chain amino acids are disadvantageous for ornithine production in *C. glutamicum* since they could compete the common precursor pyruvate. In

Table 2 Specific activities of recombinant GapCs and RocG in *E. coli* and *C. glutamicum*

Strain	Enzyme activity (mU/mg)					
	CaGapC		CsGapC		BsRocG	
	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NADH	NADPH
E. coli	< 5	2000 ± 100	< 5	5263 ± 300	2108 ± 150	< 5
KBJ02	< 5	< 5	< 5	74 ± 3	152 ± 8	<5

Cells were collected after 60 h of fermentation at 30 °C. Enzyme activities were measured as described in the "Materials and Methods." SDs were calculated based on three biologically independent experiments

Table 3 L-ornithine production by engineered *C. glutamicum* strains

Strains	Overexpression		Deletion			Ornithine titer (g/L)	Yield (g/g glucose)
	CsgapC	*BsrocG*	*argR*	*argF*	*ncgl2228*		
JML04	−	−	+	−	−	0.62 ± 0.05	0.010 ± 0.001
KBJ01	−	−	+	+	−	28.24 ± 2.10	0.240 ± 0.031
KBJ02	−	−	+	+	−	26.98 ± 3.10	0.237 ± 0.025
KBJ05	+	−	+	+	−	34.75 ± 1.97	0.274 ± 0.030
KBJ06	−	+	+	+	−	32.93 ± 2.56	0.271 ± 0.009
KBJ07	−	−	+	+	+	30.46 ± 2.05	0.260 ± 0.017
KBJ08	−	−	+	+	+	30.34 ± 2.98	0.249 ± 0.038
KBJ09	+	−	+	+	+	34.52 ± 3.08	0.270 ± 0.031
KBJ10	−	+	+	+	+	35.01 ± 2.76	0.278 ± 0.022
KBJ11	+	+	+	+	+	35.85 ± 3.05	0.280 ± 0.034
KBJ11*	+	+	+	+	+	88.26 ± 2.04	0.414 ± 0.025

+ indicates modification; − indicates no modification

KBJ02 and KBJ08 were used as control (harboring plasmid pXMJ19)

KBJ11* was cultivated in a 10-L bioreactor. All other strains were cultivated in 500-mL shake flasks

Data represent mean values and standard deviations based on three independent experiments

C. glutamicum, gene *ncgl2228* encodes a branched-chain amino acid permease. Attenuation of *ncgl2228* in *C. glutamicum* S9114 resulted in enhanced biosynthesis of L-ornithine [27]. Considering the unwanted accumulation of BCAAs (mainly L-valine, L-isoleucine, and L-leucine), strain KBJ07 (SNK118Δ*argR*Δ*argF*Δ*ncgl2228*) was constructed by deleting *ncgl2228* in KBJ01. As expected, KBJ07 allowed production of 30.46 g/L L-ornithine with a yield of 0.26 g/g glucose after 84 h, which is 7.9% and 7% higher than that of the control strain KBJ01 (SNK118Δ*argF*Δ*argR*) (Table 3), respectively. Meanwhile, the accumulations of L-valine, L-isoleucine, and L-leucine were decreased from 0.34, 0.44, and 0.55 g/L to 0.21, 0.02, and 0.01 g/L (Fig. 2b). Compared with KBJ01, strain KBJ07 exhibited a slightly lower L-ornithine production before 60 h, whereas 7.9% higher L-ornithine titer at 84 h (Fig. 2a). Additionally, there was no appreciable difference on cell growth and glucose consumption (data not shown) between two strains. Therefore, removing permease of BCAAs could positively affect the carbon fluxes balance as well as L-ornithine production.

Effect of Gene Overexpression and Deletions on L-Ornithine Synthesis

Above results demonstrated that *ncgl2228* deletion and heterologous expression of *CsgapC* or *BsrocG* were favorable to L-ornithine production by *C. glutamicum*. Hence, KBJ09 (SNK118Δ*argR*Δ*argF*Δ*ncgl2228*/pXMJ19-*CsgapC*), KBJ10 (SNK118Δ*argR*Δ*argF*Δ*ncgl2228*/pXMJ19-*BsrocG*), and KBJ11 (SNK118Δ*argR*Δ*argF*Δ*ncgl2228*/pXMJ19-*CsgapC-tac-BsrocG*) were constructed. After fermentation in shake flasks for 84 h, strain KBJ11 allowed the highest L-ornithine titer of 35.85 g/L with a yield of 0.28-g/g glucose (Table 3), which was improved appreciably compared with those of KBJ08 (30.34 g/L and 0.249 g/g). Meanwhile, KBJ09 and KBJ10 could produce 34.52- and 35.01-g/L L-ornithine with yields of 0.27 and 0.278 g/g glucose, respectively. Also the growth curves and glucose consumption (data is not displayed) were similar among strains KBJ08, KBJ09, KBJ10, and KBJ11 (Fig. 2d), suggesting that heterologous gene expression did not affect cell growth of *C. glutamicum*.

Above results showed that strain KBJ11 was capable of efficient synthesis of L-ornithine, demonstrating that these modification strategies have positive and synergistic effects on strengthening L-ornithine production of *C. glutamicum*, which is discrepant with the negligible effect of simultaneous expression of *gapC* and *rocG* on L-ornithine production in previous report [9]. Overexpression of *CsgapC*-encoding NADP-utilizing glyceraldehyde-3-phosphate dehydrogenase could drive more metabolic flux of carbon into glycolytic pathway along with improved NADPH pool, thereby producing more precursors such as pyruvate. Also, obstruction of competitive pathway (*ncgl2228*) might be a thrift of energy and precursors for L-ornithine synthesis. Besides, *BsrocG*-encoding NAD-utilizing glutamate dehydrogenase was overexpressed under a strong *tac* promoter for increased internal glutamate and NADPH levels. Notably, the -ornithine titer produced by strain KBJ11 was 27% higher than that of KBJ01 (SNK118Δ*argF*Δ*argR*).

Fed-Batch Fermentation Using Engineered *C. glutamicum* Strain KBJ11 in a 10-L Bioreactor

To further investigate the performance of strain KBJ11, fed-batch fermentation in a 10-L bioreactor was carried out (Fig. 3). Initial accumulation of L-ornithine (3.7 g/L) was observed at 8 h. Then, a rapid increase in L-ornithine production occurred in the next 30 h along with fast cell growth. Notably, the highest L-ornithine concentration of 88.26 g/L was reached after 72 h of fermentation, which was about 2.5 times as much as that in flask fermentation. L-ornithine yield of 0.414-g/g glucose and volumetric productivity of 1.23 g/L/h were achieved by KBJ11 during fed-batch fermentation in a 10-L bioreactor. Kim et al. reported a high L-ornithine production of 51.5 g/L in fed-batch fermentation in a 6.6-L fermenter, in which yield of 0.24-g/g glucose and productivity of 1.29 g/L/h were attained [7]. Compared with this study, the lower L-ornithine yield (0.24 g/g) could be largely due to a loss of carbon flux towards pentose phosphate pathway. In this study, the maximum OD_{660} value reached 63.7 at 68 h without delay in cell growth. Moreover, BCAAs and other amino acids were not detected, which was undoubtedly beneficial for enhanced L-ornithine biosynthesis. The results demonstrate the great potential of the engineered strain KBJ11 in industrial L-ornithine production.

In future study, other genetic modification and process control strategies, such as replacing native promoter of glucose uptake genes and ornithine transporter genes with strong promoters

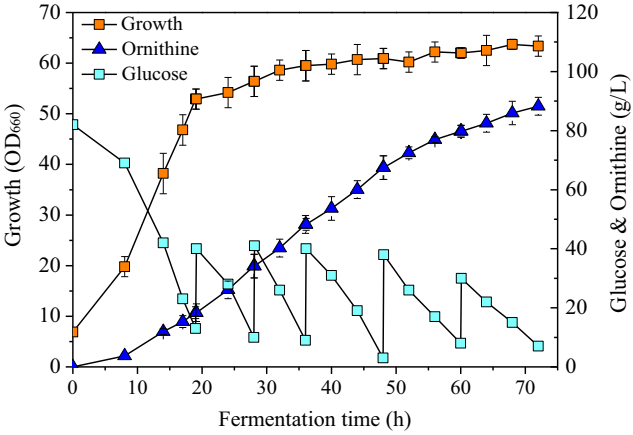

Fig. 3 Fed-batch fermentation of *C. glutamicum* KBJ11 for L-ornithine production in a 10-L bioreactor

(like EF-Tu, sod), as well as pH and dissolved oxygen regulations should be investigated to further improve the L-ornithine production.

Conclusions

Here, an efficient ornithine-producing *C. glutamicum* strain KBJ11was constructed. Genes *argF*, *argR*, and *ncgl2228* were deleted to cut off the conversion of ornithine to citrulline, block the global transcriptional repressor, and eliminate competitive branch pathway. Besides, co-overexpression of *CsgapC* and *BsrocG* rendered enhanced ornithine synthesis. In a 10-L bioreactor, L-ornithine of 88.26 g/L and yield of 0.414-g/g glucose were achieved by strain KBJ11. The titer and yield of L-ornithine present the highest levels in microbial fermentation. *C. glutamicum* SNK118 is a promising industrial strain that could be metabolically engineered for producing L-arginine, L-ornithine, and other amino acids.

Acknowledgments This work was supported by National Natural Science Foundation of China (31601463), National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-07), National Key R&D Program (2018YFA0901700), Top-notch Academic Programs Project of Jiangsu Higher Education Institutions, and the Program of Introducing Talents of Discipline to Universities (111-2-06).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Salvatore, F., Cimino, F., d'Ayello-Caracciolo, M., & Cittadini, D. (1964). Mechanism of the protection by l-ornithine-l-aspartate mixture and by l-arginine in ammonia intoxication. *Archives of Biochemistry and Biophysics, 107*(3), 499–503. https://doi.org/10.1016/0003-9861(64)90307-8.
2. Shi, H. P., Fishel, R. S., Efron, D. T., Williams, J. Z., Fishel, M. H., & Barbul, A. (2002). Effect of supplemental ornithine on wound healing. *Journal of Surgical Research, 106*(2), 299–302. https://doi.org/10.1006/jsre.2002.6471.
3. Lee, Y. J., & Cho, J. Y. (2006). Genetic manipulation of a primary metabolic pathway for L-ornithine production in *Escherichia coli*. *Biotechnology Letters, 28*(22), 1849–1856. https://doi.org/10.1007/s10529-006-9163-y.
4. Shu, Q. F., Xu, M. J., Li, J., Yang, T. W., Zhang, X., Xu, Z. H., & Rao, Z. M. (2018). Improved L-ornithine production in *Corynebacterium crenatum* by introducing an artificial linear transacetylation pathway. *Journal of Industrial Microbiology & Biotechnology, 45*(6), 393–404. https://doi.org/10.1007/s10295-018-2037-1.
5. Hwang, G. H., & Cho, J. Y. (2014). Enhancement of L-ornithine production by disruption of three genes encoding putative oxidoreductases in *Corynebacterium glutamicum*. *Journal of Industrial Microbiology & Biotechnology, 41*(3), 573–578. https://doi.org/10.1007/s10295-013-1398-8.
6. Jiang, L. Y., Chen, S. G., Zhang, Y. Y., & Liu, J. Z. (2013). Metabolic evolution of *Corynebacterium glutamicum* for increased production of L-ornithine. *BMC Biotechnology, 13*(1), 47–57. https://doi.org/10.1186/1472-6750-13-47.
7. Kim, S. Y., Lee, J., & Lee, S. Y. (2015). Metabolic engineering of *Corynebacterium glutamicum* for the production of L-ornithine. *Biotechnology and Bioengineering, 112*(2), 416–421. https://doi.org/10.1002/bit.25440.
8. Zhang, B., Yu, M., Wei, W. P., & Ye, B. C. (2018). Optimization of L-ornithine production in recombinant *Corynebacterium glutamicum* S9114 by *cg3035* overexpression and manipulating the central metabolic pathway. *Microbial Cell Factories, 17*(1), 91. https://doi.org/10.1186/s12934-018-0940-9.

9. Jiang, L. Y., Zhang, Y. Y., Li, Z., & Liu, J. Z. (2013). Metabolic engineering of *Corynebacterium glutamicum* for increasing the production of L-ornithine by increasing NADPH availability. *Journal of Industrial Microbiology & Biotechnology, 40*(10), 1143–1151. https://doi.org/10.1007/s10295-013-1306-2.
10. Yang, J., Kim, B., Kim, H., Kweon, Y., & Lee, J. (2015). Industrial production of 2,3-butanediol from the engineered Corynebacterium glutamicum. *Applied Biochemistry and Biotechnology, 176*(8), 2303–2313. https://doi.org/10.1007/s12010-015-1719-7.
11. Chen, T., Zhu, N., & Xia, H. (2014). Aerobic production of succinate from arabinose by metabolically engineered *Corynebacterium glutamicum*. *Bioresource Technology, 151*, 411–414. https://doi.org/10.1016/j.biortech.2013.10.017.
12. Liu, W., Zheng, P., Yu, P., & Yang, Q. (2015). A two-stage process for succinate production using genetically engineered *Corynebacterium acetoacidophilum*. *Process Biochemistry, 50*(11), 1692–1700. https://doi.org/10.1016/j.procbio.2015.07.017.
13. Chen, C., Li, Y. Y., Hu, J. Y., Dong, X. Y., & Wang, X. Y. (2015). Metabolic engineering of *Corynebacterium glutamicum* ATCC13869 for L-valine production. *Metabolic Engineering, 29*, 66–75. https://doi.org/10.1016/j.ymben.2015.03.004.
14. Wu, W. J., Zhang, Y., Liu, D. H., & Chen, Z. (2018). Efficient mining of natural NADH-utilizing dehydrogenases enables systematic cofactor engineering of lysine synthesis pathway of *Corynebacterium glutamicum*. *Metabolic Engineering, 52*, 77–86. https://doi.org/10.1016/j.ymben.2018.11.006.
15. Xu, J. Z., Wu, Z. H., Gao, S. J., & Zhang, W. G. (2018). Rational modification of tricarboxylic acid cycle for improving L-lysine production in *Corynebacterium glutamicum*. *Microbial Cell Factories, 17*(1), 105. https://doi.org/10.1186/s12934-018-0958-z.
16. Wang, J., Wen, B., Wang, J., Xu, Q. Y., Zhang, C. L., Chen, N., & Xie, X. X. (2013). Enhancing l-isoleucine production by *thrABC* overexpression combined with *alaT* deletion in *Corynebacterium glutamicum*. *Applied Biochemistry and Biotechnology, 171*(1), 20–30. https://doi.org/10.1007/s12010-013-0321-0.
17. Park, S. H., Kim, H. U., Kim, T. Y., Park, J. S., Kim, S. S., & Lee, S. Y. (2014). Metabolic engineering of *Corynebacterium glutamicum* for L-arginine production. *Nature Communications, 5*, 4618. https://doi.org/10.1038/ncomms5618.
18. Xu, M. J., Qin, J. R., Rao, Z. M., You, H. Y., Zhang, X., Yang, T. W., Wang, X. Y., & Xu, Z. H. (2016). Effect of polyhydroxybutyrate (PHB) storage on L-arginine production in recombinant *Corynebacterium crenatum* using coenzyme regulation. *Microbial Cell Factories, 15*, 15. https://doi.org/10.1186/s12934-016-0414-x.
19. Zhan, M. L., Kan, B. J., Dong, J. J., Xu, G. C., Han, R. Z., & Ni, Y. (2019). Metabolic engineering of *Corynebacterium glutamicum* for improved L-arginine synthesis by enhancing NADPH supply. *Journal of Industrial Microbiology & Biotechnology, 46*(1), 45–54. https://doi.org/10.1007/s10295-018-2103-8.
20. Long, L., Guo, D. D., Gao, W., Yang, W. W., Hou, L. P., Ma, X. N., Miao, Y. C., Botella, J. R., & Song, C. P. (2018). Optimization of CRISPR/Cas9 genome editing in cotton by improved sgRNA expression. *Plant Methods, 14*(1), 85–89. https://doi.org/10.1186/s13007-018-0353-0.
21. Guo, J. G., Li, K., Jin, L. F., Xu, R., Miao, K. T., Yang, F. B., Qi, C. Y., Zhang, L., Botella, J. R., Wang, R., & Miao, Y. C. (2018). A simple and cost-effective method for screening of CRISPR/Cas9-induced homozygous/biallelic mutants. *Plant Methods, 14*, 40. https://doi.org/10.1186/s13007-018-0305-8.
22. Wang, L. L., Rubio, M. C., Xin, X., Zhang, B. L., Fan, Q. L., Wang, Q., Ning, G. G., Becana, M., & Duanmu, D. Q. (2019). CRISPR/Cas9 knockout of leghemoglobin genes in *Lotus japonicus* uncovers their synergistic roles in symbiotic nitrogen fixation. *New Phytologist, 224*(2), 818–832. https://doi.org/10.1111/nph.16077.
23. Gao, W., Long, L., Tian, X. Q., Xu, F. C., Liu, J., Singh, P. K., Botella, J. R., & Song, C. P. (2017). Genome editing in cotton with the CRISPR/Cas9 system. *Frontiers in Plant Science, 8*, 1364. https://doi.org/10.3389/fpls.2017.01364.
24. Pyne, M. E., Mooyoung, M., Chung, D. A., & Chou, C. P. (2015). Coupling the CRISPR/Cas9 system with lambda red recombineering enables simplified chromosomal gene replacement in *Escherichia coli*. *Applied and Environmental Microbiology, 81*(15), 5103–5114. https://doi.org/10.1128/AEM.01248-15.
25. Wang, H. Y., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell, 153*(4), 910–918. https://doi.org/10.1016/j.cell.2013.04.025.
26. Jiang, Y., Qian, F. H., Yang, J. J., Liu, Y. M., Dong, F., Xu, C. M., Sun, B. B., Chen, B., Xu, X. S., Li, Y., Wang, R. X., & Yang, S. (2017). CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nature Communications, 8*, 15179. https://doi.org/10.1038/ncomms15179.
27. Zhang, B., Ren, L. Q., Yu, M., Zhou, Y., & Ye, B. C. (2018). Enhanced l-ornithine production by systematic manipulation of l-ornithine metabolism in engineered *Corynebacterium glutamicum* S9114. *Bioresource Technology, 250*, 60–68. https://doi.org/10.1016/j.biortech.2017.11.017.

28. Dong, J. J., Han, R. Z., Xu, G. C., Gong, L., Xing, W. R., & Ni, Y. (2018). Detoxification of furfural residues hydrolysate for butanol fermentation by *Clostridium saccharobutylicum* DSM 13864. *Bioresource Technology, 259*, 40–45. https://doi.org/10.1016/j.biortech.2018.02.098.
29. Iddar, A., Valverde, F., Serrano, A., & Soukri, A. (2002). Expression, purification, and characterization of recombinant nonphosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*. *Protein Expression and Purification, 25*(3), 519–526. https://doi.org/10.1016/S1046-5928(02)00032-3.
30. Belitsky, B. R., & Sonenshein, A. L. (1998). Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. *Journal of Bacteriology, 180*(23), 6298–6305.
31. Gibson, D. G., Chuang, R. Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods, 6*(5), 343–345. https://doi.org/10.1038/nmeth.1318.
32. Van der Rest, M. E., Lange, C., & Molenaar, D. (1999). A heat shock following electroporation induces highly effcient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Applied Microbiology and Biotechnology, 52*(4), 541–545. https://doi.org/10.1007/s002530051557.
33. Xu, D. Q., Tan, Y. Z., Huan, X. J., Hu, X. Q., & Wang, X. Y. (2010). Construction of a novel shuttle vector for use in *Brevibacterium flavum*, an industrial amino acid producer. *Journal of Microbiological Methods, 80*(1), 86–92. https://doi.org/10.1016/j.mimet.2009.11.003.
34. Chernobrovkin, M. G., Anan'eva, I. A., Shapovalova, E. N., & Shpigun, O. A. (2004). Determination of amino acid enantiomers in pharmaceuticals by reversed-phase high-performance liquid chromatography. *Journal of Analytical Chemistry, 59*(1), 55–63. https://doi.org/10.1023/B:JANC.0000011669.08932.d8.
35. Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. *Archives of Biochemistry and Biophysics, 67*(1), 10–15. https://doi.org/10.1016/0003-9861(57)90241-2.
36. Zhang, B., Yu, M., Zhou, Y., Li, Y. X., & Ye, B. C. (2017). Systematic pathway engineering of *Corynebacterium glutamicum* S9114 for L-ornithine production. *Microbial Cell Factories, 16*(1), 158. https://doi.org/10.1186/s12934-017-0776-8.
37. Becker, J., Zelder, O., Häfner, S., Schröder, H., & Wittmann, C. (2011). From zero to hero—design-based systems metabolic engineering of L-lysine production. *Metabolic Engineering, 13*(2), 159–168. https://doi.org/10.1016/j.ymben.2011.01.003.
38. Takeno, S., Hori, K., Ohtani, S., Mimura, A., Mitsuhashi, S., & Ikeda, M. (2016). L-lysine production independent of the oxidative pentose phosphate pathway by *Corynebacterium glutamicum* with the *Streptococcus mutans gapN* gene. *Metabolic Engineering, 37*, 1–10. https://doi.org/10.1016/j.ymben.2016.03.007.
39. Martínez, I., Zhu, J. F., Lin, H., Bennett, G. N., & San, K. Y. (2008). Replacing Escherichia coli NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from *Clostridium acetobutylicum facilitates* NADPH dependent pathways. *Metabolic Engineering, 10*(6), 352–359. https://doi.org/10.1016/j.ymben.2008.09.001.
40. Jakoby, M., Ngouoto-Nkili, C. E., & Burkovski, A. (1999). Construction and application of new *Corynebacterium glutamicum* vectors. *Biotechnology Techniques, 13*(6), 437–441. https://doi.org/10.1023/A:1008968419217.