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ORIGINAL ARTICLE

Increasing L-lysine production in *Corynebacterium glutamicum* by engineering amino acid transporters

Jing Xiao[1] · Datao Wang[1] · Lei Wang[2] · Yanjun Jiang[1] · Le Xue[1] · Songsen Sui[4] · Jianbin Wang[4] · Chuanzhuang Guo[4] · Ruiming Wang[1] · Junqing Wang[1] · Nan Li[3] · Han Fan[1] · Maocui Lv[1]

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Abstract

Corynebacterium glutamicum has a long and successful history in the biotechnological production of L-lysine. Besides the adjustment of metabolic pathways, intracellular and extracellular transport systems are critical for the cellular metabolism of L-lysine or its by-products. Here, three amino acid transmembrane transporters, namely, GluE, BrnE/BrnF, and LysP, which are widely present in *C. glutamicum* strains, were each investigated by gene knockout. In comparison with that in the wild-type strain, the yield of L-lysine increased by 9.0%, 12.3%, and 10.0% after the deletion of the *gluE*, *brnE*/*brnF*, and *lysP* genes, respectively, in *C. glutamicum* 23,604. Moreover, the amount of by-product amino acids decreased significantly when the *gluE* and *brnE*/*brnF* genes were deleted. It was also demonstrated that there was no effect on the growth of the strain when the *gluE* or *lysP* gene was deleted, whereas the biomass of *C. glutamicum* WL1702 (Δ*brnE*/Δ*brnF*) in the fermentation medium was significantly reduced in comparison with that of the wild type. These results also provide useful information for enhancing the production of L-lysine or other amino acids by *C. glutamicum*.

Keywords *gluE* · *brnE*/*brnF* · *lysP* · Gene knockout · Amino acid transmembrane transporter · L-Lysine

Abbreviations

AcCoA	Acetyl coenzyme A
Ala	L-Alanine
Asn	L-Asparagine
Asp	L-Aspartate
AspSa	Aspartate semialdehyde
Chor	Chorismate
Cit	Citrate
Fum	Fumaric acid
G6P	Glucose 6-phosphate
GDL	Gluconolactone
Glc	Glucose
Gln	L-Glutamine
Glu	L-Glutamate
Gluc	D-Gluconic acid
Gluc6P	6-Phospho-D-gluconate
Glx	Glyoxylate
Gly	L-Glycine
Hcys	Homocysteine
His	L-Histidine
Hser	Homoserine
ICit	Isocitrate
Ile	L-Isoleucine
αKG	α-Ketoglutarate

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✉ Junqing Wang
wjqtt.6082@163.com

✉ Nan Li
linan1166@mail.tust.edu.cn

[1] State Key Laboratory of Biobased Material and Green Papermaking (LBMP), Qilu University of Technology), Jinan 250353, Shandong, People's Republic of China

[2] School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China

[3] College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, People's Republic of China

[4] Zhucheng Dongxiao Biotechnology Co., Ltd, Xinxing Town, Zhucheng 262200, Shandong, People's Republic of China

Kval	2-Ketovaline
Leu	L-Leucine
Lys	L-Lysine
Mal	Malic acid
OA	Oxaloacetic acid
Met	L-Methionine
Orn	Ornithine
PEP	Phosphoenolpyruvate
PG3	Glycerate 3-phosphate
Phe	L-Phenylalanine
Pro	L-Proline
PRPP	5-Phospho-D-ribosylpyrophosphate
Pyr	Pyruvate
R5P	Ribulose 5-phosphate
Ser	L-Serine
Suc	Succinate
SucCoA	Succinate coenzyme A
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
Val	L-Valine

Introduction

L-Lysine is an essential amino acid for mammals and can promote the development of mammals and strengthen the immune system in humans. L-Lysine has significant value in feed additives (Katheline 2013) and dietary supplements, as well as in cosmetics and pharmaceuticals (Koffas and Stephanopoulos 2005). With the widening of the range of applications of lysine, the demand for lysine is rapidly increasing every year. The fermentative production of lysine by *Corynebacterium glutamicum* was developed in 1958 by Kyowa Hakko Kogyo Co., Ltd. (Ikeda 2016). *Corynebacterium glutamicum* is an aerobic nonpathogenic Gram-positive soil bacterium. As an excellent producer, it is utilized for the industrial production of amino acids, including L-glutamate and L-lysine (Shukuo et al. 2004; Kalinowski et al. 2003). Moreover, the main producers of L-lysine are almost all auxotrophic engineered strains of *C. glutamicum*, particularly in terms of changes in metabolic flux (Wittmann and Becker 2007).

In the fermentative production of lysine, many by-products, such as glutamic acid, methionine, leucine, isoleucine, and valine, are formed. Reducing the accumulation of by-products is an effective approach to reducing production costs, because the downstream purification and recovery process might thereby be simplified (Hou et al. 2012a). The influence of the transport of certain amino acid by-products in the cell will cause the concentration of the by-product in the cell to increase, thereby inhibiting the formation of the by-product (Chen et al. 2015). Furthermore, decreasing the formation of certain by-products may improve the yield of the target product, because the carbon source might thus be utilized more exclusively for the biosynthesis of the target product (Chen et al. 2015). The extracellular export of amino acids from *C. glutamicum* is very important, and the proteins involved in the export of amino acids have been thoroughly characterized at the biochemical and genetic level (Marin and Krämer 2007). For example, intracellular L-methionine, L-leucine, L-isoleucine, and L-valine can be exported from the cell via a two-component export system consisting of the proteins BrnE and BrnF, which are encoded by the genes *brnE* and *brnF*, respectively (Kennerknecht et al. 2002; Lange et al. 2012). Some experiments have indicated that overexpressing the export system BrnE/BrnF could increase the production of L-methionine and L-isoleucine by *C. glutamicum* (Nicole et al. 2002; Qin et al. 2014). Furthermore, a mutant in which both the above genes were deleted no longer exported L-isoleucine. Moreover, the *NCgl1221* gene, which encodes a mechanosensitive channel homolog, is involved in the mechanism of secretion of L-glutamate (Nakamura et al. 2007). The expression, localization, construction, and functions of *NCgl1221* have been identified and reported. This gene has also been termed *gluE* (Yamashita et al. 2013; Yao et al. 2009; Yoshitaka et al. 2012). The amino acid transporter LysE is responsible for the transport of L-lysine and L-arginine in *C. glutamicum*, and the expression of *lysE* increases the production of lysine (Vrljic et al. 1997). In this study, the two genes of *gluE* and *brnE/brnF* were knocked out by the principle of two homologous single exchanges to inactivate the extracellular transporters GluE and BrnE/BrnF, thereby exploring the effect on the production of L-lysine. Besides, in *C. glutamicum* the uptake of L-lysine is catalyzed by a lysine/alanine exchange carrier encoded by the *lysP* gene (Bröer and Krämer 1990). It has also been demonstrated that the excretion of lysine is independent of the lysine uptake system (Seep-Feldhaus et al. 2010).

The functions and transport mechanisms of the three abovementioned amino acid transporters have been extensively studied. The effects of inactivating these three transporters on the respective target amino acids have also been reported (Marin and Krämer 2007). The transfer of intracellular glutamate to the outside of the cell is controlled by the NCgl1221 protein encoded by the *gluE* gene, which is a mechanically sensitive channel homologue and an important glutamate transporter (Yao et al. 2009). The intracellular methionine and branched amino acids are transported to the outside of the cell through a transport system composed of BrnE and BrnF (Qin et al. 2014). In addition, reducing the accumulation of by-products may increase the yield of the target product, because the carbon source will be more concentrated for the synthesis of the target product (Chen et al. 2015). However, there has thus far been no report on the effect of inactivating these three transporters in *C.*

glutamicum on the yield of L-lysine. Because the transport systems of bacterial cells are usually related to the excretion as well as the reuptake of products, which makes the engineering of transporters a useful strategy for the improvement of strains, we investigated the effect of inactivating the three abovementioned transmembrane transporters on the production of extracellular L-lysine (Fig. 1). We hypothesized that deleting the *gluE* gene would not affect the growth of the strain and, because the concentration of extracellular L-glutamate was not significantly decreased, the carbon source could not be employed to accumulate intracellular L-glutamate and hence could not be utilized more exclusively for the production of L-lysine.

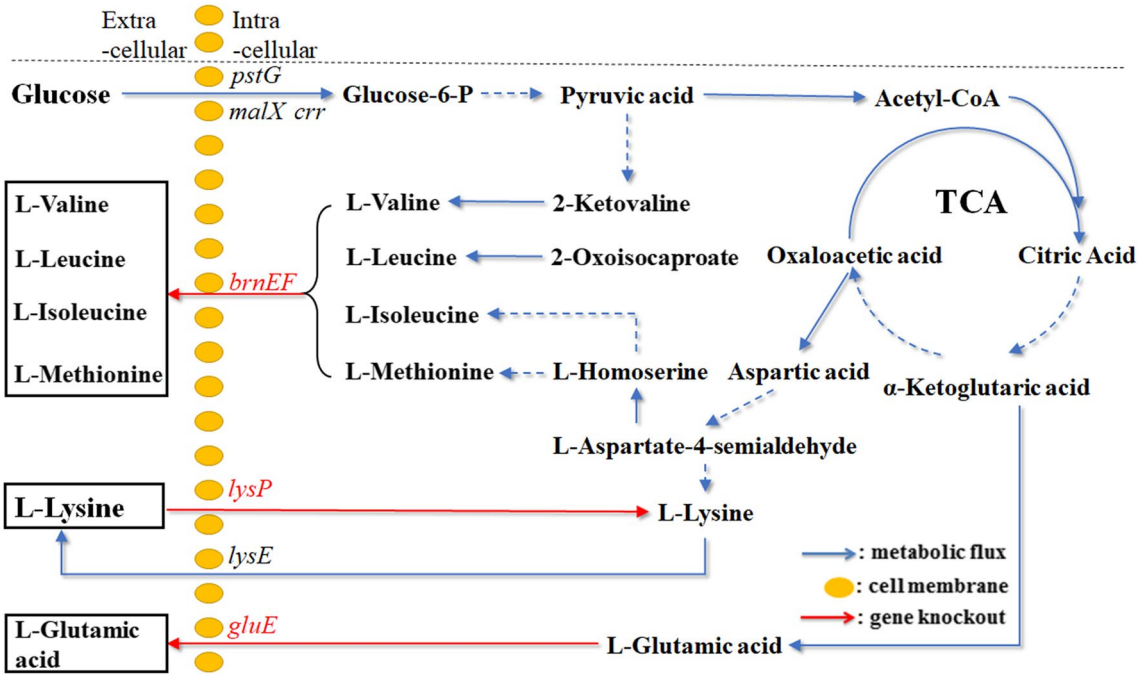

Fig. 1 Pathway engineering for production of L-lysine in recombinant *C. glutamicum*

Table 1 Strains, plasmids and primers used in this study

Strain/plasmid	Relevant genotype	Source
Strains		
C. glutamicum 23604	Wild-type	CICC
C. glutamicum WL1701	CICC23604, *Δ gluE*	This study
C. glutamicum WL1702	CICC23604, *ΔbrnE/ΔbrnF*	This study
C. glutamicum WL1703	CICC23604, *ΔlysP*	This study
C. glutamicum WL1704	CICC23604, *ΔbrnE/ΔbrnF*, *Δ gluE*	This study
C. glutamicum WL1705	CICC23604, *ΔbrnE/ΔbrnF*, *Δ gluE*, *ΔlysP*	This study
C. glutamicum WL1706	CICC23604, *ΔbrnE/ΔbrnF*, *ΔlysP*	This study
Plasmid		
pK19mobsacB	*sacB*, *lacZa*, Kanr, mcs mobilizable vector, allows for selection of double crossover *C. glutamicum*	
pK19mobsacB-*Δ gluE*	pK19mobsacB containing internal deletion of 1205 bp fragment of *gluE*	This study
pK19mobsacB-*ΔbrnE/ΔbrnF*	pK19mobsacB containing internal deletion of 1130 bp fragment of *brnE/brnF*	This study
pK19mobsacB-*ΔlysP*	pK19mobsacB containing internal deletion of 1134 bp fragment of *lysP*	This study

Materials and methods

Strains, plasmids, primers, and culture conditions

All the strains, plasmids, and primers used in this study are listed in Table 1. The nonreplicable integration plasmid pK19mobsacB was used to construct *C. glutamicum* deletion vectors. *Escherichia coli* DH5 cells were used as hosts for gene cloning and were routinely grown in Luria–Bertani (LB) medium at 37 °C. *Corynebacterium glutamicum* 23604 was used as the parent strain for generating mutants and was cultivated aerobically at 30 °C with LBG medium (LB medium supplemented with 5 g L^{-1} glucose). The fermentation medium used for production of L-lysine by *C. glutamicum* consisted of (per liter): 20 g glucose, 30 g corn pulp, 20 g $(NH_4)_2SO_4$, 10 g CH_3COONa, 5 g urea, 1.34 g L-alanine, 2 g KH_2PO_4, 1.35 g $MgSO_4{\cdot}7H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 0.04 g $MnSO_4{\cdot}H_2O$, 0.008 g nicotinamide, 0.001 g biotin, 0.04 g leucine. The basic medium is used for evaluate the synthetic ability of all amino acids by *C. glutamicum* consisted of (per liter): 5 g glucose, 1 g $(NH_4)_2SO_4$, 1 g sodium citrate, 0.2 g $MgSO_4{\cdot}7H_2O$, 4 g K_2HPO_4, 6 g KH_2PO_4 and adjusted to pH 7.2 with NaOH. When appropriate, kanamycin was added at a final concentration of 50 mg L^{-1} (*E. coli*) or 25 mg L^{-1} (*C. glutamicum*).

Construction of deletion vectors

The genome was extracted from *C. glutamicum* 23604 using an Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The polymerase chain reaction (PCR) primers used in this study are listed in Supplementary Table 1. In the first round of PCR, the chromosomal DNA of *C. glutamicum* 23604 was used as the template, and the primer pairs A1/A2, B1/B2, and C1/C2 were used to amplify the upstream regions of the *gluE*, *brnE*/*brnF*, and *lysP* genes, respectively. The primer pairs A3/A4, B3/B4, and C3/C4 were used to amplify the downstream regions of the respective genes. For the above six fragments, the conditions used for PCR amplification were as follows: pre-degeneration at 95 ℃ for 5 min, then 30 cycles of degeneration at 94 ℃ for 30 s, annealing at 57 ℃ for 30 s, and pre-extension at 72 ℃ for 90 s, followed by extension at 72 ℃ for 10 min and holding at 4 ℃. Then, gel purification was used to purify the PCR products using a gel extraction kit (Sangon Biotech, Shanghai, China). In the second round of overlap PCR, the products of the first round of PCR were used as templates in a molar ratio of 1:1, and A1/A4, B1/B4, and C1/C4 were used as primer pairs. The conditions used for the second PCR amplification were as follows: (1) pre-degeneration at 95 ℃ for 5 min, then five cycles of degeneration at 94 ℃ for 30 s, annealing at 57 ℃ for 30 s, and pre-extension at 72 ℃ for 90 s, followed by extension at 72 ℃ for 2 min; and (2) pre-degeneration at 95 ℃ for 5 min, then 30 cycles of degeneration at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, and pre-extension at 72 ℃ for 2.5 min, followed by extension at 72 ℃ for 10 min and holding at 4 ℃. Fragments of a fusion gene, namely, a 1205 bp fragment of *gluE*, a 1130 bp fragment of *brnE*/*brnF*, and a 1134 bp fragment of *lysP*, were thus obtained. The plasmid pK19mobsacB was digested using the restriction enzyme *Eco*RI (Thermo Fisher Scientific, United States). Via seamless cloning using a One Step Cloning Kit (Vazyme Biotech, Nanjing, China), the fragments obtained by overlap PCR were inserted into the plasmid pK19mobsacB, and then the recombinant plasmids were transfected into competent *E. coli* cells. Finally, colony PCR was used to screen for positive clones, and the identity of the fusion fragments was confirmed by sequencing.

Transformation and isolation of engineered strains

Competent *C. glutamicum* 23604 cells were prepared in advance. A single colony of *C. glutamicum* 23604 was inoculated into liquid LBG medium and cultured overnight. Then a 2% inoculant was inoculated into neutral complex medium (NCM) containing Tween 80 (0.1%) and glycine (3%) to give a value of the optical density at 600 nm (OD_{600}) of 0.3. When the OD_{600} value reached 0.9, the cells were chilled on ice for 15 min and harvested by centrifugation at 4 ℃ and 4000 *g* for 10 min. Then the cell pellets were washed four times with 20 mL ice-cold 10% glycerol. The cells were resuspended in 0.2 mL 10% glycerol, and 80 µL competent cells were selected. Next, the three different recombinant plasmids were extracted and concentrated. An 8 µL aliquot of DNA was added to the competent cells, which were transferred to a 1 mm electroporation cuvette (Bio-Rad Laboratories, Hercules, California, United States), of which the parameters were set at 1.8 kV and 5 ms. In comparison with the study by Ruan et al. (2015), there was a slight difference in the composition of NCM. After electroporation was carried out, a heat shock was applied immediately for 6 min.

Two rounds of positive selection for homologous recombination were performed (Fig. 2). Kanamycin resistance was first used to select for the integration of the plasmid into the chromosome. Resistant clones were selected and tested by PCR analysis with the primer pairs A1/A4, B1/B4, or C1/C4 using the chromosomal DNA as a template. DNA fragments with lengths of 1205 and 2264 bp were obtained by the deletion of the *gluE* gene. Deletion of the *brnE*/*brnF* gene yielded fragments with lengths of 1130 and 2060 bp, and deletion of the *lysP* gene yielded fragments with lengths of 1128 and 1377 bp. Subsequently, clones that survived and grew in the presence of sucrose were selected, because

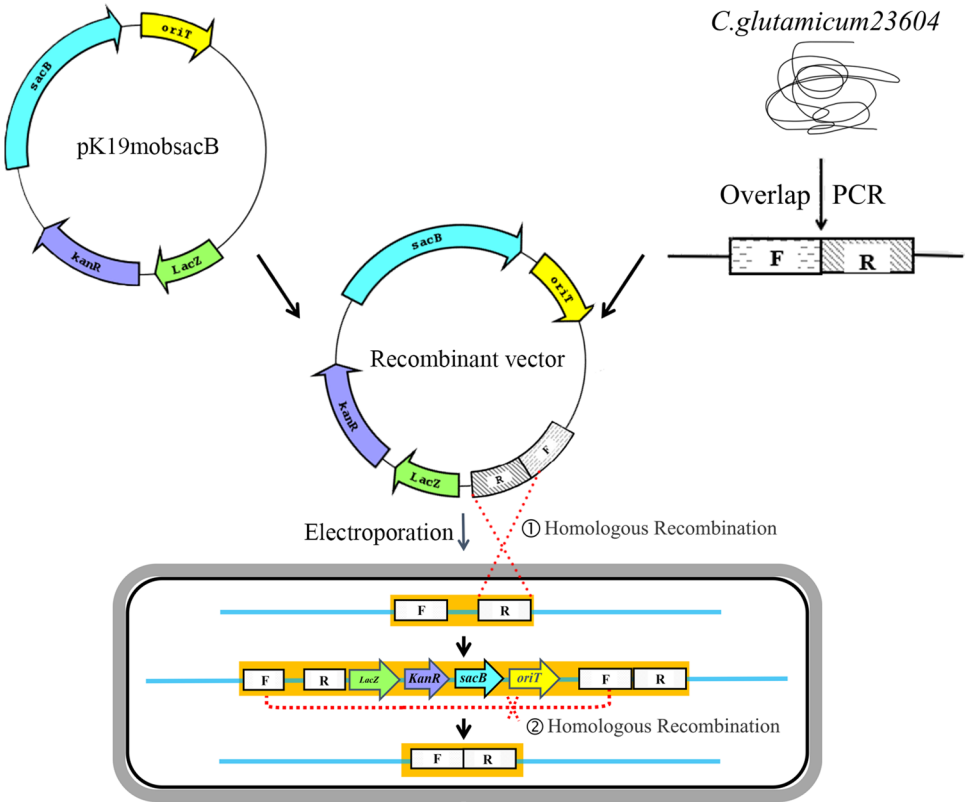

Fig. 2 Flowchart of construction of *C. glutamicum* engineered strains

it was assumed that they had lost the pK19mobsacB vector (Hou et al. 2012a). The second recombination resulted in either the desired deletion or the restoration of the wild-type characteristics. Clones selected using sucrose were tested by PCR analysis using A1/A4, B1/B4, or C1/C4 as primer pairs to identify clones that carried the desired deletion or had undergone allelic exchange. In the case of knockout of the *gluE* gene, only the 1205 bp PCR fragment was obtained. The 1130 and 1128 bp DNA fragments were amplified in the cases of knockout of the *brnE*/*brnF* and *lysP* genes, respectively.

Culture conditions used for L-lysine production

Three colonies of the engineered and wild-type strains were inoculated into liquid LBG medium and cultured overnight at 200 rpm and 30 ℃ in 200 mL flasks. After 12 h, 5% of each overnight culture was transferred to 100 mL fermentation medium. The remaining portions of the *C. glutamicum* WL1701 and *C. glutamicum* WL1702 strains, in which the *gluE* and *brnE*/*brnF* genes, respectively, were knocked out, were centrifuged and inoculated into the basic medium to give the same initial OD_{600} value. During the fermentation process, samples were taken from the fermentation medium and basic medium at intervals of 4 h to measure the OD_{600} value and concentrations of glucose and L-lysine. In the case of the *C. glutamicum* WL1701 strain, the yield of L-glutamate was measured. Each experiment was repeated three times, and the average values of the measurements were taken as the experimental results.

Analysis of growth, glucose consumption, and L-lysine production by engineered strains

The biomass concentration in a 100 μL aliquot of bacterial culture was measured by a photometer at 600 nm after an appropriate dilution or by gravimetric analysis, as described previously (Hou et al. 2012b). The supernatant of the culture broth obtained by centrifugation was used for the determination of the concentrations of glucose, L-lysine and L-glutamate, which were measured enzymatically using a biosensor (SBA-40C, Biology Institute of Shandong Academy of Sciences, Jinan, China; https://www.bio-sensor.org) with a standard process described in the instruction manual. An automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) was used to determine the compositions and contents of various amino acids in the fermentation broth and basic broth of the three strains of recombinant bacteria with a standard pipeline method described in the instruction manual.

UHPLC-QE-MS non-target metabolomics detection

100 μL of samples (the supernatant of the basic medium culture broth at 96 h) were transferred to an EP tube. After the addition of 400 μL of extract solution (acetonitrile: methanol = 1: 1, containing isotopically-labeled internal standard mixture), the samples were vortexed for 30 s, sonicated for 10 min in ice-water bath, and incubated for 1 h at – 40 ℃ to precipitate proteins. Then the sample was centrifuged at 12,000 rpm for 15 min at 4 ℃. The resulting supernatant was transferred to a fresh glass vial for LC/MS analysis.

LC–MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 ammonia hydroxide in water (pH 9.75) (A) and acetonitrile (B). The analysis was carried with elution gradient as follows: 0–0.5 min, 95% B; 0.5–7.0 min, 95%—65% B; 7.0–8.0 min, 65%—40% B; 8.0–9.0 min, 40% B; 9.0–9.1 min, 40%—95% B; 9.1–12.0 min, 95% B. The column temperature was 30 ℃. The auto-sampler temperature was 4 ℃, and the injection volume was 2 μL.

The QE HFX mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as following: sheath gas flow rate as 50 Arb, Aux gas flow rate as 10 Arb, capillary temperature 320 ℃, full MS resolution as 60,000, MS/MS resolution as 7500, collision energy as 10/30/60 in NCE mode, spray Voltage as 3.5 kV (positive) or − 3.2 kV (negative), respectively.

The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration by BiotreeCo., Ltd. Then an in-house MS2 database (BiotreeDB) was applied in metabolite annotation. The cut-off for annotation was set at 0.3.

Statistical analysis

Statistical analyses were performed using GraphPad Prism4 software. A two-tailed Student's *t* test was used to test statistical significance. The symbol (*) denotes a *p* value of at least < 0.05. The symbol (**) denotes a *p* value of at least < 0.01. All data were obtained from three independent experiments performed in triplicate, and the results are presented as mean and standard error of the mean.

Results and discussion

Effects of *gluE* gene knockout on L-lysine production

Using the pK19mobsacB plasmid to construct knockout vectors, we generated strains derived from the wild-type strain *C. glutamicum* 23604 with three different genic deletions in their genomes. The resulting recombinant strains were denoted as *C. glutamicum* WL1701, *C. glutamicum* WL1702, and *C. glutamicum* WL1703, respectively. To construct the strain *C. glutamicum* WL1701, we knocked out *gluE* by homologous recombination between the chromosomal *gluE* gene and a truncated *gluE* gene. The plasmid pK19mobsacB-Δ*gluE*, which incorporated the upstream and downstream regions of *gluE*, was introduced into wild-type *C. glutamicum* 23604. After two rounds of positive selection for homologous recombination and testing by PCR analysis, we obtained the *gluE* gene knockout strain denoted as *C. glutamicum* WL1701 (Supplementary Fig. 1). In this mutant, the *gluE* gene was completely inactivated by selecting the position of homologous arms in the genome. In comparison with wild-type *C. glutamicum* 23604, the yield of L-lysine increased by 9.0% after the deletion of the *gluE* gene (Fig. 3a, $p < 0.01$, Supplementary Fig. 6). However, whereas wild-type *C. glutamicum* 23604 secreted 0.907 g L^{-1} L-glutamate after fermentation for 96 h, the yield of L-glutamate secreted by *C. glutamicum* WL1701 was 0.775 g L^{-1} and was thus reduced by only 17% (Fig. 3b, $p < 0.01$ Supplementary Fig. 3). Moreover, after fermentation on the basic medium, it was found that the glucose consumption curves of the recombinant bacteria were basically identical to the control bacteria (Supplementary Fig. 3). While, it can be seen from Fig. 4 that the yield of L-glutamate from the recombinant bacteria after fermentation was 69.7% lower than that from the control bacteri a (Supplementary Fig. 3, $p < 0.01$, Supplementary Fig. 3). However, these results show that it is different from the previous research by Nakamura et al. (2007), they observed a significant drop in glutamate production after the entire *gluE* gene was deleted: the yield was 7.1 times lower than the wild type. A possible reason for our results is that the wild-type strain *C. glutamicum* 23604 is a glutamate-secreting strain. Even though we deleted the *gluE* gene completely, the mutant also secreted L-glutamate. Furthermore, from the growth and glucose consumption curves (Fig. 3c, d), we can conclude that the

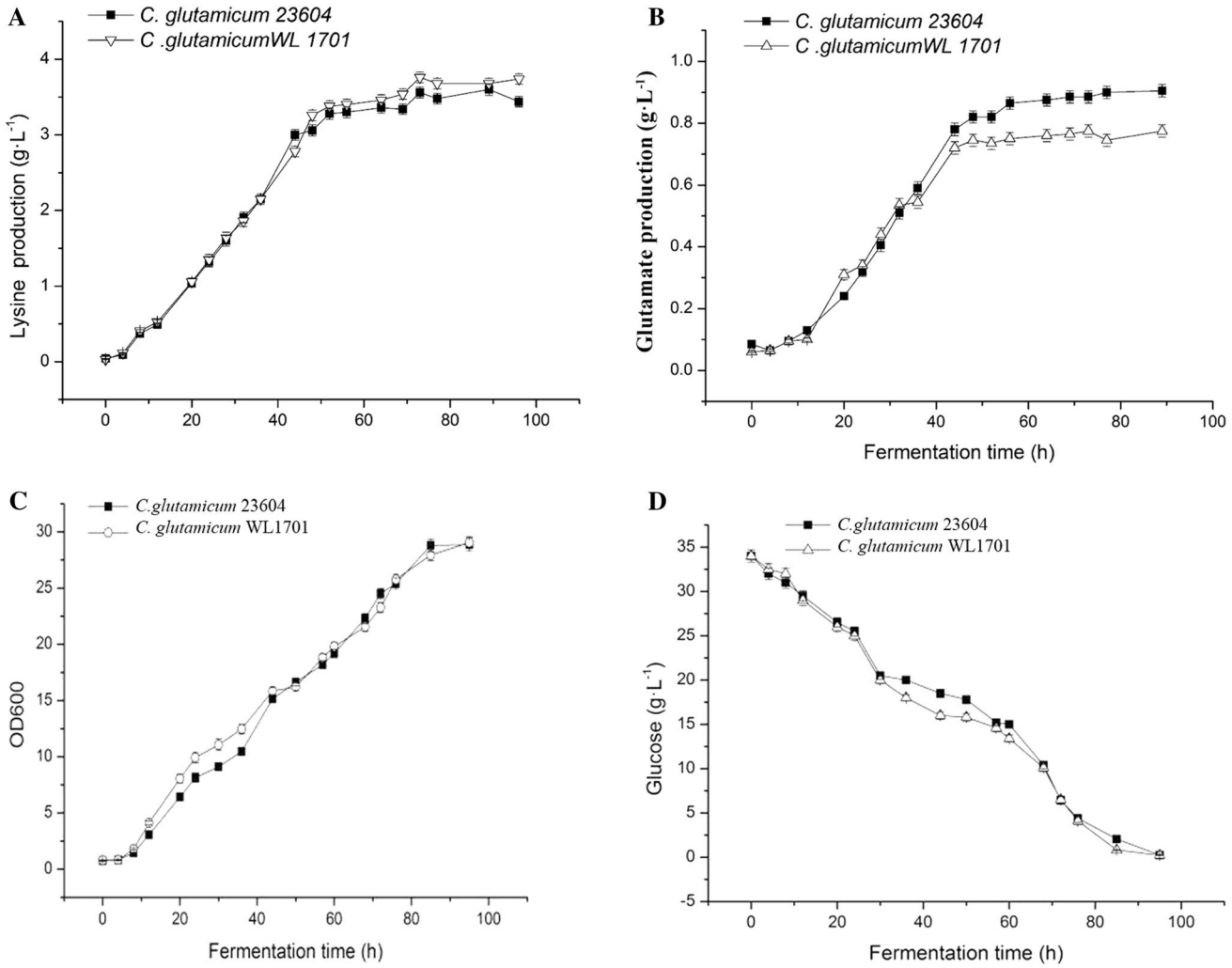

Fig. 3 Production of L-lysine by engineered strains of *C. glutamicum* WL1701 at fermentation medium. Samples were taken from the fermentation medium every 4 h to measure the L-glutamate, L-lysine, glucose and OD_{600} production of *C. glutamicum* 23604 and *C. glutamicum*WL1701, symbols: filled square strain *C. glutamicum* 23604, *hollow triangle* strain *C. glutamicum* WL1701. The standard errors are shown as bars

wild-type strain and mutant exhibited almost the same trends in growth and glucose consumption.

Effects of *brnE*/*brnF* gene knockout on L-lysine production

To obtain the *brnE*/*brnF* gene knockout strain *C. glutamicum* WL1702, we carried out two rounds of screening and testing by PCR analysis (Supplementary Fig. 4). In the fermentation medium, we found that the biomass and glucose consumption of the mutant significantly decreased in comparison with that of the wild-type strain after the same stable growth period (Fig. 5a). However, the yield of L-lysine was 12.3% higher than that from the wild-type *C. glutamicum* 23604 (Fig. 5b, $p < 0.01$ Supplementary Fig. 6), and the residual glucose of *C. glutamicum* WL1702 was close to zero earlier than that of the wild type during the fermentation (Fig. 5c).

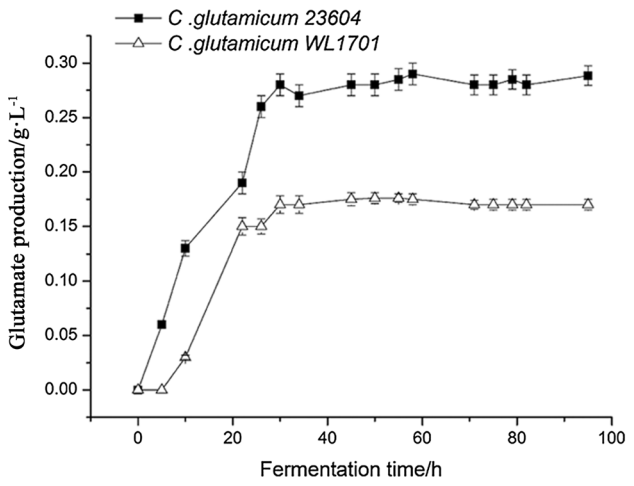

Fig. 4 Production of L-glutamate by engineered strains of *C. glutamicum* WL1701 at basic medium. Samples were taken from the fermentation medium every 4 h to measure the L-glutamate concentration of *C. glutamicum* 23604 and *C. glutamicum* WL1702, symbols: filled square strain *C. glutamicum* 23604, filled triangle strain *C. glutamicum* WL1702. The standard errors are shown as bars

From this result, we can conclude that after the deletion of *brnE*/*brnF* the yield of L-lysine produced by *C. glutamicum* WL1702 was higher than that produced by the wild type.

After fermentation on the basic medium and amino acid analysis, we found that the yield of L-lysine from *C. glutamicum* WL1702 increased by 14.2% in comparison with that from *C. glutamicum* 23604 (Fig. 6, $p < 0.05$, Supplementary Fig. 6). The yields of L-valine, L-leucine, and L-isoleucine from the wild type and *C. glutamicum* WL1702 were all 0 mg L^{-1}. However, the yield of L-methionine from *C. glutamicum* WL1702 was 0.331 mg L^{-1}, which represented a decrease of 63.7% in comparison with *C. glutamicum* 23604. The results showed that after the deletion of *brnE*/*brnF* the strain still had the ability to transport L-methionine. This is consistent with the findings of Christian et al. (2005) and indicates the presence of more than one additional L-methionine export system.

Effects of *lysP* gene knockout on L-lysine production

According to the methods used for screening *C. glutamicum* WL1701, a colony of *C. glutamicum* WL1703 was successfully obtained (Supplementary Fig. 5). After fermentation for 96 h, the yield of L-lysine was 4.84 g L^{-1}. In comparison with that from the wild-type *C. glutamicum* 23604, the yield of L-lysine increased by 10.0% (Fig. 7a, $p < 0.01$ Supplementary Fig. 6). The growth and glucose consumption curves of the mutant were similar to those of

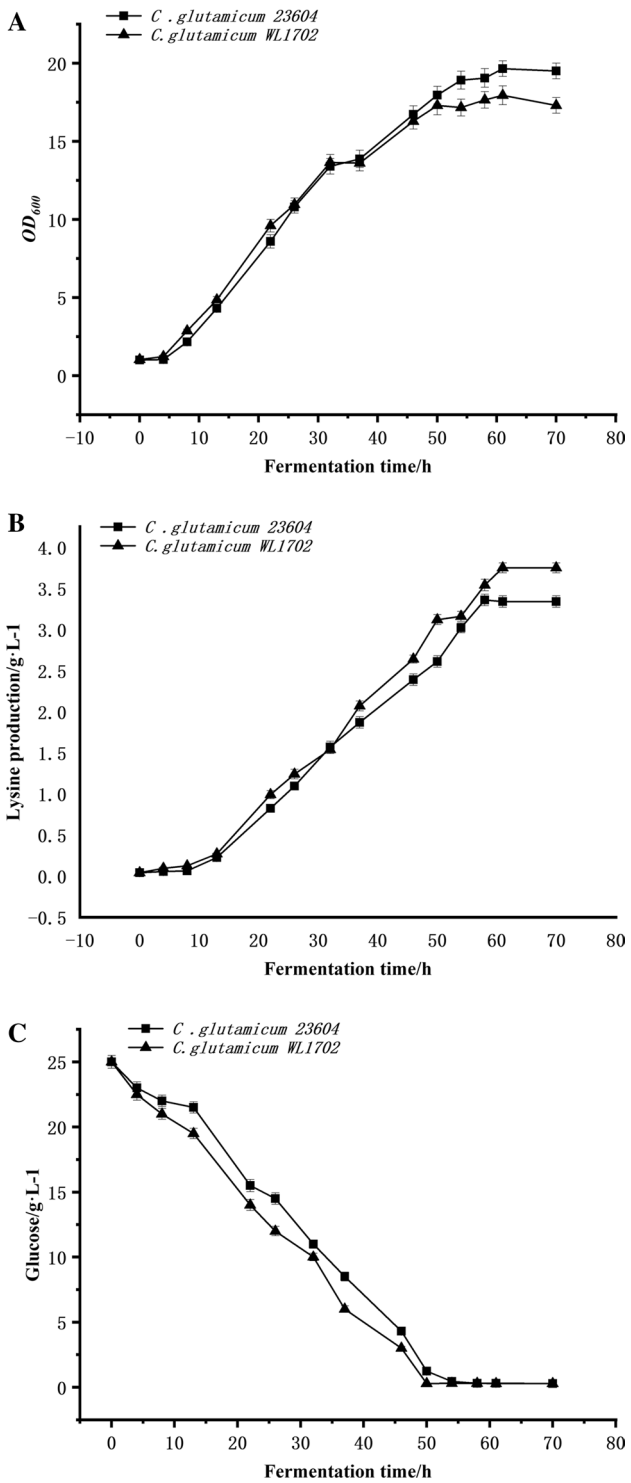

Fig. 5 Production of L-lysine by engineered strains of *C. glutamicum* WL1702 at fermentation medium. Samples were taken from the fermentation medium every 4 h to measure the L-lysine, glucose and OD_{600} of *C. glutamicum* 23604 and *C. glutamicum* WL1702, symbols: filled square strain *C. glutamicum* 23604, filled triangle strain *C. glutamicum* WL1702. The standard errors are shown as bars

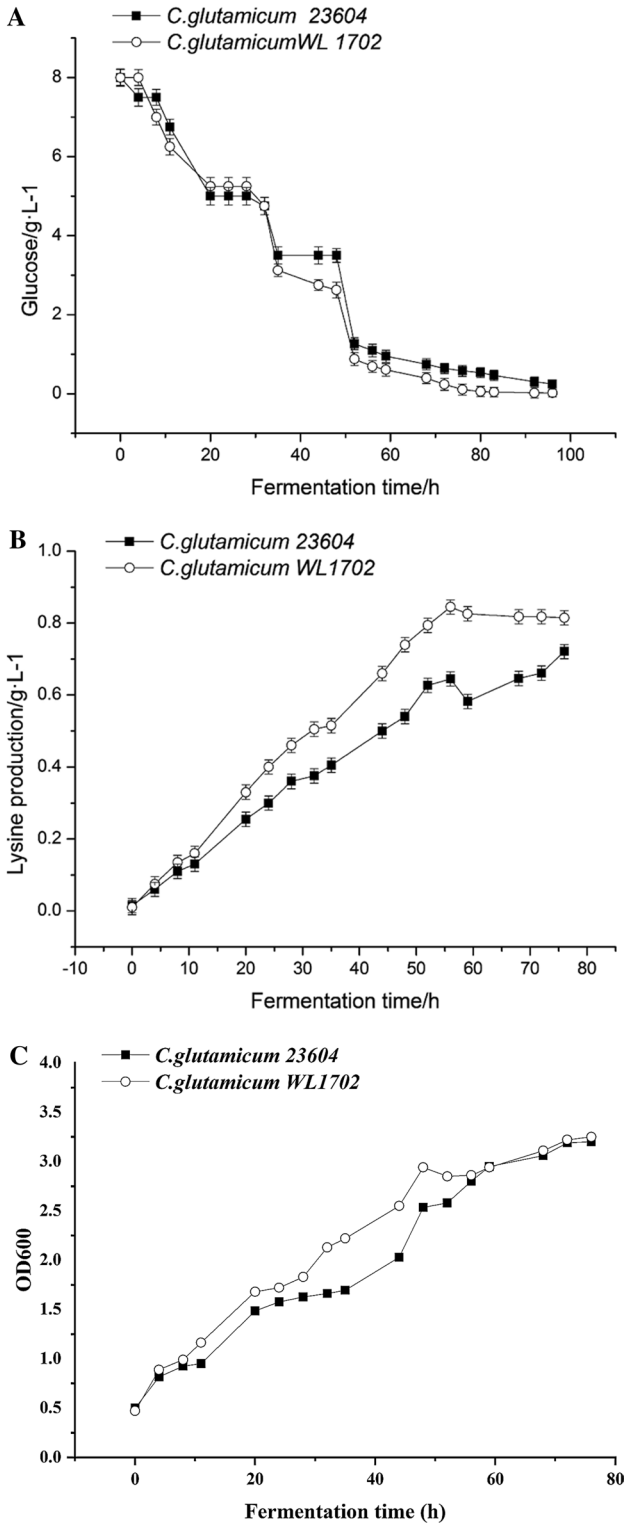

Fig. 6 Production of L-lysine by engineered strains of *C. glutamicum* WL1702 at basic medium. Samples were taken from the fermentation medium every 4 h to measure the L-lysine concentration of *C. glutamicum* 23604 and *C. glutamicum* WL1702, symbols: filled square strain *C. glutamicum* 23604, filled triangle strain *C. glutamicum* WL1702. The standard errors are shown as bars

the wild type (Fig. 7b, c). These results demonstrated that *lysP* knockout had no significant influence on the growth of the strain. From data obtained using the automatic amino acid analyzer (Table 2), we found that, although the yield of L-lysine increased, the yields and types of other amino acids were basically identical during in the fermentation with fermentation medium.

Overview of the *C. glutamicum* metabolomics data after gene knockout

In our metabolomics results, 28 core metabolites were identified and mapped to metabolic pathways in *C. glutamicum* (Fig. 8). These metabolites included amino acids, organic acids, carbohydrates, lipids and lipid-like molecules, purines/pyrimidines and other metabolites. In the fermentation medium, we found that some core metabolites of the mutant changed in comparison with that of the wild-type strain after fermentation for 96 h with the basic medium. The content of L-aspartic acid, a precursor of L-lysine, were increased after the deletion of the *gluE*, *brnE*/*brnF* and *lysP* genes, respectively, which may lead to the increase in L-lysine production. After the deletion of the *gluE* gene, the content of citrate and isocitrate (the precursors of L-lysine) were increased, it also led to the increase of some amino acids (such as L-tyrosine, L-alanine, L-isoleucine and L-methionine) production in other metabolic pathways. Meanwhile, the deletion of *brnE*/*brnF* and *lysP* genes may also affect TCA cycle. More dramatically, the deletion of the *gluE*, *brnE*/*brnF* and *lysP* genes also affects fatty acids and nucleotides metabolism, especially the increase of oleic acid, capric acid and stearic acid production.

Effects of double and triple knockout on L-lysine production

According to the methods used for screening *C. glutamicum* WL1701, a colony of *C. glutamicum* WL1704, *C. glutamicum* WL1706 was successfully obtained (Supplementary Fig. 7a–d). After the plasmid pK19mobsacB-*lysP* was electroporated into *C. glutamicum* WL1704, according to the method used to screen *C. glutamicum* WL1701, a colony of *C. glutamicum* WL1705 was successfully obtained (Supplementary Fig. 7e, f). The growth status and glucose consumption of double deletion bacteria *C. glutamicum* WL1704 and *C. glutamicum* WL1706 were basically the same as those of the control bacteria. The L-lysine production of the two double deletion bacteria was increased by 3% and 2% respectively compared

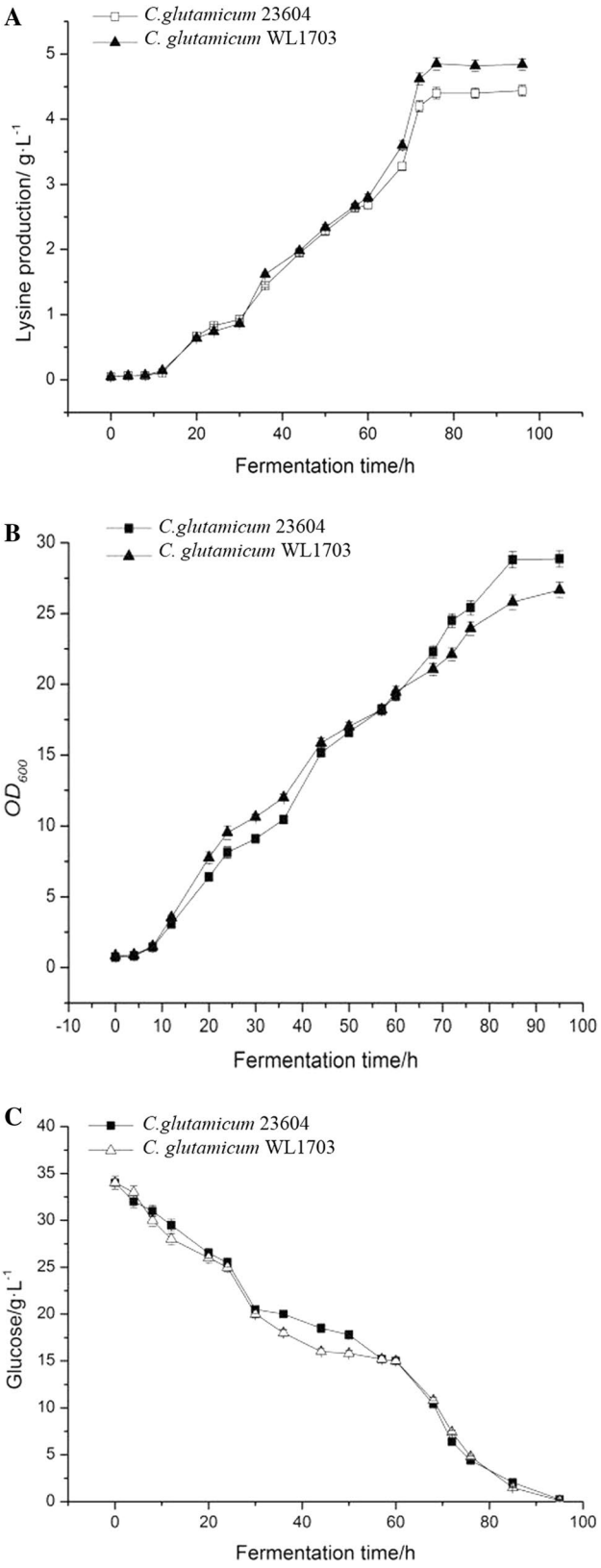

Fig. 7 Production of L-lysine by engineered strains of *C. glutamicum* WL1703 at fermentation medium. During the fermentation process, samples were taken from the fermentation medium every 4 h to measure the L-lysine, glucose and OD_{600} production of *C. glutamicum* 23604 and *C. glutamicum* WL1703, symbols: hollow square strain *C. glutamicum* 23604, filled triangle strain *C. glutamicum* WL1703. The standard errors are shown as bars

Table 2 Determination results of amino acid contents

Amino acid	*C. glutamicum* 23604 (g L^{-1})	*C. glutamicum* 23604 WL1703 (g L^{-1})
Asp	0.021 ± 0.002	0.021 ± 0.004
Glu	1.536 ± 0.007	1.554 ± 0.021
Gly	0.171 ± 0.009	0.183 ± 0.005
Ala	0.870 ± 0.048	0.942 ± 0.061
Cys	0.058 ± 0.003	0.053 ± 0.003
Val	0.382 ± 0.019	0.408 ± 0.006
Leu	0.540 ± 0.034	0.581 ± 0.029
Tyr	0.033 ± 0.002	0.056 ± 0.004
Lys	3.420 ± 0.019	3.790 ± 0.037

with the wild-type *C. glutamicum* 23604 (Supplementary Fig. 8). From the above results, it can be concluded that the L-lysine production of double-deleted bacteria is not as high as that of single-deleted bacteria. The simultaneous deletion of two genes in one strain did not increase the production of L-lysine. The three-deleted bacteria *C. glutamicum* WL1705 grew more slowly than the control bacteria, and the biomass in the stable phase was not as high as that of the control bacteria (Supplementary Fig. 9). The glucose consumption curves of the triple deletion bacteria and the control bacteria are basically the same (Supplementary Fig. 9). The lysine production of the triple deletion bacteria was 10.8% lower than that of the wild-type *C. glutamicum* 23604 (Supplementary Fig. 9). After the two membrane transporters were simultaneously inactivated, the production of L-lysine was not much different from the control bacteria, but there is a certain gap with the single deletion bacteria. The study also found that the simultaneous deletion of three genes will further reduce the production of lysine, the specific reasons need to be further analyzed in the future study.

In conclusion, we have studied the development of higher-yielding strains for the production of L-lysine and demonstrated the importance of amino acid transporters involved in L-lysine-related carbon flux pathways for increasing the yield of L-lysine. By knocking out the *gluE*, *brnE*/*brnF*, and *lysP* genes, we found that the three corresponding amino acid transporters have different effects on the production of L-lysine. The yield of L-lysine in

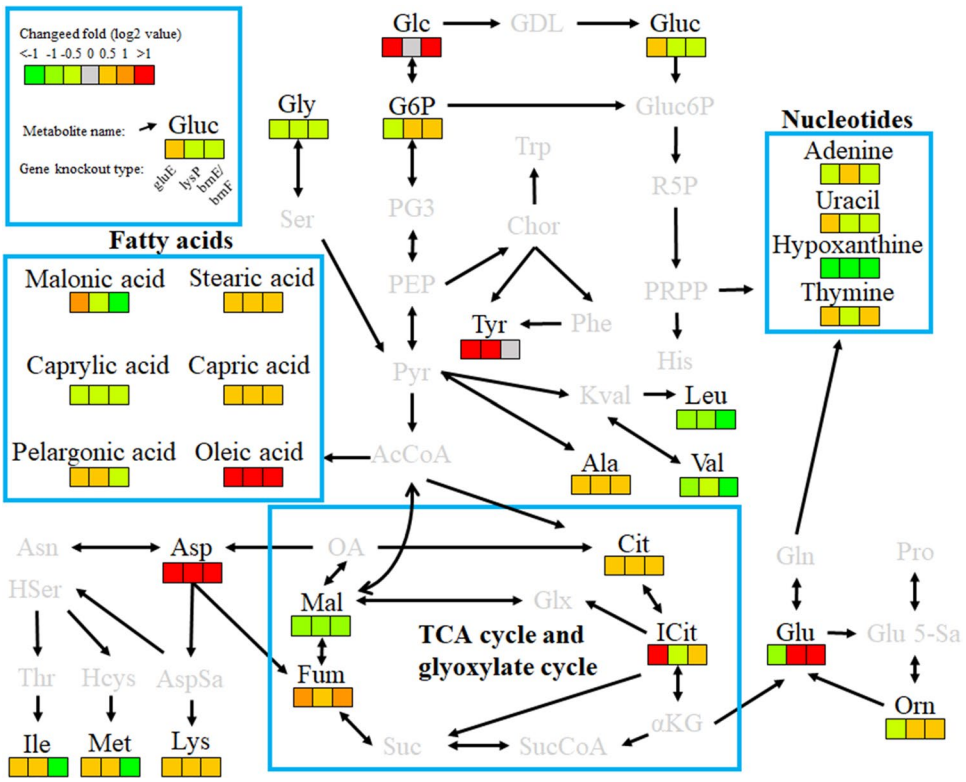

Fig. 8 Metabolic pathway analysis results in *C. glutamicum* after gene knocke out. Overview of the metabolic changes in *C. glutamicum* after *gluE*, *lysP* or *brnE/brnF* genes were knocked out. The left, middle and right boxes respectively indicate that the levels of metabolites after knocking out *gluE*, *lysP* or *brnE/brnF* genes have changed compared with the original strain

the fermentation medium increased by 9.0%, 12.3%, and 10.0% after the deletion of *gluE*, *brnE/brnF*, and *lysP*, respectively, in *C. glutamicum* 23604. However, after the inactivation of *gluE* and *brnE/brnF*, the strain retained the ability to transport L-glutamate and L-methionine, which means that other L-glutamate and L-methionine transporters are present in *C. glutamicum*.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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