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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Metabolic engineering of *Corynebacterium glutamicum* for increasing the production of L-ornithine by increasing NADPH availability

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Abstract The experiments presented here were based on the conclusions of our previous proteomic analysis. Increasing the availability of glutamate by overexpression of the genes encoding enzymes in the L-ornithine biosynthesis pathway upstream of glutamate and disruption of *speE*, which encodes spermidine synthase, improved L-ornithine production by *Corynebacterium glutamicum.* Production of L-ornithine requires 2 moles of NADPH per mole of L-ornithine. Thus, the effect of NADPH availability on L-ornithine production was also investigated. Expression of *Clostridium acetobutylicum gapC*, which encodes NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, and *Bacillus subtilis rocG*, which encodes NAD-dependent glutamate dehydrogenase, led to an increase of L-ornithine concentration caused by greater availability of NADPH. Quantitative real-time PCR analysis demonstrates that the increased levels of NADPH resulted from the expression of the *gapC* or *rocG* gene rather than that of genes (*gnd*, *icd*, and *ppnK*) involved in NADPH biosynthesis. The resulting strain, *C. glutamicum* ΔAPRE::*rocG*, produced 14.84 g l^{-1} of L-ornithine. This strategy of overexpression of *gapC* and *rocG* will be useful for improving production of target compounds using NADPH as reducing equivalent within their synthetic pathways.

Keywords *Corynebacterium glutamicum* · L-Ornithine · NADP-dependent glyceraldehyde-3-phosphate dehydrogenase gene · NAD-dependent glutamate dehydrogenase gene · NADPH availability

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Introduction

L-Ornithine, a non-essential amino acid and an important constituent of the urea cycle, is the precursor of other amino acids, such as citrulline and arginine. It is effective for the treatment and prophylaxis of liver diseases [24] and has been applied to promote wound healing [28]. Recently, it was demonstrated that L-ornithine supplementation increased serum levels of growth hormone and insulin-like growth factor-1 after strength-trained athletes engaged in heavy-resistance exercise [33]. Many studies have reported that high yields of L-ornithine can be produced from a citrulline- or arginine-requiring mutant of a coryneform bacterial strain generated by classical mutagenesis [3, 12, 15, 34]. Although this mutant produces a high yield of L-ornithine, cultures are unstable owing to reversion of the auxotrophic phenotype, which causes significant inhibition of the production of L-ornithine.

Several recent reports have described progress in the metabolic engineering of microorganisms used for L-ornithine production. Lee and Cho reported that an engineered *Escherichia coli* strain, W3110 (*ΔargFΔargIΔargRΔϖproBΔspeF*, P_{araB}*-arg214*), produced 13.2 mg per gram of dry cell weight (DCW) of L-ornithine and that addition of glutamate to the culture favored L-ornithine production [18]. Hwang et al. reported that overexpression of *argCJBD* by *C. glutamicum* strain ATCC 13032 (*ΔargFΔargRΔproB*) resulted in the production of 16.49 mg g^{-1} DCW of L-ornithine. The concentration of L-ornithine in the culture medium was 179.14 mg l^{-1} [8]. Proline can be converted into L-ornithine by ornithine cyclodeaminase,

which is a key enzyme responsible for enhancing L-ornithine production by *C. glutamicum* in proline-supplemented media [16]. Hwang and Cho reported that overexpression of the Ncgl1469 open reading frame, encoding *N*-acetylglutamate synthase activity, increased L-ornithine production in *C. glutamicum* by 39 % [6]. Recently, the same investigators deleted *gntK*, which encodes gluconate kinase, of *C. glutamicum* ATCC 13032 (*ΔargFΔargR*) to obtain *C. glutamicum* SJC8399, which produced 13.16 g l^{-1} of L-ornithine [7]. The L-ornithine-producing strain *C. glutamicum* ATCC 13032 (*ΔargF-ΔargR*) named ORN1 was constructed and shown to produce L-ornithine from arabinose when *araBAD* from *E. coli* was expressed [26]. Recently, this group also constructed the strain *C. glutamicum* ORN1 (pEKEx3-$xylA_{Xc}$-$xylB_{Cg}$) to effectively produce L-ornithine from xylose [23].

In a previous study [21], we reported the construction of a strain of *C. glutamicum* ATCC 13032 (*ΔargFΔϖ proBΔkgd*) that produced up to 4.78 g l^{-1} of L-ornithine. Comparative proteomic analysis revealed the mechanism of L-ornithine overproduction by the engineered strain. The expression levels of 202 proteins varied significantly in *C. glutamicum* ATCC 13032 (*ΔargFΔproBΔkgd*) compared with those in the wild-type strain. Of these proteins, 52 proteins were identified. L-Ornithine overproduction in the engineered strain was related to the upregulation of the expression levels of enzymes involved in the L-ornithine biosynthesis pathway and downregulation of the expression levels of proteins involved in the pentose phosphate pathway. The expression levels of enzymes in ornithine biosynthesis (ArgCJBD) downstream of glutamate are much higher than that in the upstream pathway of glutamate. The results suggested possible strategies for further enhancing L-ornithine production. The present study is based on this proteomic analysis and describes our efforts to genetically engineer *C. glutamicum* to produce even higher levels of L-ornithine. Production of L-ornithine required 2 moles of NADPH per mole of L-ornithine. Thus, the effect of NADPH availability on L-ornithine production was also investigated.

Materials and methods

Microorganism and media

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α was used for plasmid construction. *C. glutamicum* ΔAP was used as the parental strain for generating mutants. Luria–Bertani (LB) was used to propagate *E. coli* and *C. glutamicum* for generating recombinant DNA. For L-ornithine production by *C. glutamicum*, the seed medium consisted of (per liter) 25 g glucose, 10 g yeast extract, 10 g corn steep liquor, 15 g $(NH_4)_2SO_4$, 2.5 g $MgSO_4{\cdot}7H_2O$, 1 g of KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g Na_2HPO_4, and 10 g $CaCO_3$. The fermentation medium [20] consisted of (per liter) 80.0 g glucose, 14.0 g yeast extract, 37.9 g $(NH_4)_2SO_4$, 1.6 g $MgSO_4{\cdot}7H_2O$, 1.0 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g Na_2HPO_4, 20 mg $FeSO_4{\cdot}7H_2O$, 20 mg $MnSO_4{\cdot}4H_2O$, 2.0 g molasses, 5.0 g KAc, 5.0 g succinic acid, 10 g $CaCO_3$, and 1 ml Tween 80 (added after 8 h of fermentation). The initial pH of the above media was adjusted to 7.0.

Culture conditions

For L-ornithine fermentations, a 1.0-ml sample of the seed culture grown with shaking at 150 rpm at 30 °C for 12 h, was inoculated into 10 ml of the fermentation medium in a 100-ml flask, incubated at 30 °C and shaken at 150 rpm for 72 h. Kanamycin (50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *C. glutamicum*), chloramphenicol (20 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *C. glutamicum*), or ampicillin (50 μg ml^{-1} for *E. coli*) were added to the medium as required.

Primers, plasmid construction, and gene knockouts

Plasmids constructed for this study are listed in Table 1. The chromosomal DNA of *C. glutamicum* was isolated as described by Eikmanns et al. [4]. The preparation of competent cells and electroporation of *C. glutamicum* was performed as described by Van der Rest et al. [31]. The mutant genotypes of *C. glutamicum* were confirmed using colony PCR.

Amplification of *pgi, pfkA, gapA, pyk, pyc, gltA*, and *gdh* from the genomic DNA of *C. glutamicum* ATCC 13032 employed the primers listed in Table 2. The products were ligated to pEC-XK99E DNA [13]. *Clostridium acetobutylicum gapC* was amplified from pB3gapC [5] using the primers gapCF and gapCR (Table 2) and ligated to pEC-XK99E to obtain pEC-gapC. *B. subtilis rocG* was amplified from genomic DNA using the primers rocGF and rocGR (Table 2) and ligated to pEC-XK99E to obtain pEC-rocG.

The suicide vector pK18mobsacB [25] was modified to improve its efficiency. The lethality of the expression of *sacB* depends on its level of expression in corynebacteria [9]. Therefore, a 1.85-kb DNA fragment containing the *sacB* cluster was amplified from pK18mobsacB [25] DNA using PCR and the primers sacBF and sacBR (Table 2). This converted the native promoter of the *sacB* cluster to the *tac-M* promoter, which is a strong promoter in corynebacteria [32]. The entire backbone of pK18mobsacB, except for *sacB,* was amplified using the primers pk18msF and pk18msR (Table 2). The two fragments were digested with *Spe*I and *Eco*RV and ligated together to form the inducible suicide vector pK-JL (5,570 bp).

Table 1 *C. glutamicum* strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
C. glutamicum strains		
ATCC 13032	Wild-type	ATCC
ΔAP	ATCC 13032, Δ*argF*,Δ*proB*	21
ΔAPE	ATCC 13032, Δ*argF*,Δ*proB*,Δ*speE*	This study
ΔAPER	ATCC 13032, Δ*argF*,Δ*proB*,Δ*speE*,Δ*argR*	This study
ΔAPRE::*rocG*	ATCC 13032, Δ*argF*,Δ*proB*,Δ*argR*, Δ*speE*::$P_{tac\text{-}M}$-*rocG*	This study
ΔAPER::*gapC*	ATCC 13032, Δ*argF*,Δ*proB*,Δ*speE*, Δ*argR*::$P_{tac\text{-}M}$-*gapC*	
ΔAPE::*rocG*-R::*gapC*	ATCC 13032, Δ*argF*,Δ*proB*,Δ*speE*::$P_{tac\text{-}M}$-*rocG*, Δ*argR*::$P_{tac\text{-}M}$-*gapC*	
Clostridium acetobutylicum	Wild type, CICC8011	CICC
Bacillus subtilis	Wild type, CICC10033	CICC
Escherichia coli DH5α	supE44, hsdR17, recA1, thi-1, endA1, lacZ, gyrA96, relA1	Invitrogen
Plasmid		
PMD18-T vector	TA cloning vector, Amp^r	TaKaRa
pK18mobsacB	*sacB*, *lacZ*a, Kan^r, mcs mobilizable vector, allows for selection of double crossover *C. glutamicum*	25
pK-JL	K18mobsacB derivative, *sacB* under the control of the *tac-M* promoter, Kan^r	This study
pK18mobsacB-Δ*speE*	pK18mobsacB with 2162 bp *Bam*HI-*Sal*I fragment containing internal deletion of 199 bp fragment of *speE*	This study
pK18mobsacB-Δ*argR*	pK18mobsacB with 1574 bp *Bam*HI-*Sal*I fragment containing internal deletion of 506 bp fragment of *argR*	This study
pK-Δ*speE::rocG*	pK-JL with flanking fragment of *speE* internally inserted $P_{tac\text{-}M}$-*rocG*	This study
pK-Δ*argR::gapC*	pK-JL with flanking fragment of *argR* internally inserted $P_{tac\text{-}M}$-*gapC*	This study
pEC-XK99E	*C. glutamicum*/*E. coli* shuttle expression vector, Kan^r	13
pEC-pgi	pEC-XK99E derivative containing *C. glutamicum pgi*, Kan^r	This study
pEC-pfkA	pEC-XK99E derivative containing *C. glutamicum pfkA*, Kan^r	This study
pEC-gap	pEC-XK99E derivative containing *C. glutamicum gap*, Kan^r	This study
pEC-pyk	pEC-XK99E derivative containing *C. glutamicum pyk*, Kan^r	This study
pEC-pyc	pEC-XK99E derivative containing *C. glutamicum pyc*, Kan^r	This study
pEC-gltA	pEC-XK99E derivative containing *C. glutamicum gltA*, Kan^r	This study
pEC-gdh	pEC-XK99E derivative containing *C. glutamicum gdh* Kan^r	This study
pEC-gapC	pEC-XK99E derivative containing *C. acetobutylicum gapC*, Kan^r	This study
pEC-rocG	pEC-XK99E derivative containing *B. subtilis rocG*, Kan^r	This study

[a] *argF*, ornithine carbamoyltransferase gene; *proB*, γ-glutamyl kinase gene; *speE*, spermidine synthase gene; *argR*, arginine repressor gene; *rocG*, NADH-dependent glutamate dehydrogenase gene; *pgi*, glucose-6-phosphate isomerase gene; *pfkA*, 6-phosphofructokinase gene; *gap*, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase gene; *gapC*, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase gene; *pyk*, pyruvate kinase gene; *pyc*, pyruvate carboxylase gene; *gltA*, citrate synthase gene; *gdh*, NADPH-dependent glutamate dehydrogenase; *sacB*, levansucrase gene; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance

The disruption of genes was performed using the non-replicable integration vector pK18mobsacB or pK-JL, which allows for marker-free deletion of the target gene [25]. For the construction of pK18mobsacB-Δ*speE*, the flanking sequences of *speE* were amplified from *C. glutamicum* ATCC 13032 using the primers speEF5/speER5 and speEF3/speER3. The product and pK18mobsacB DNAs were digested with *Bam*HI/*Xba*I, *Xba*I/*Sal*I, and *Bam*HI/*Sal*I, respectively, and then ligated to generate pK18mobsacB-Δ*speE*. The construction of pK18mobsacB-Δ*argR* was similar, but the primers argRF5/argRR5 and argRF3/argRR3 were used. For the construction of pK-Δ*speE::rocG*, the primers speEF5 and speER3 were used with pK18mobsacB-Δ*speE* as template. The resulting fragment was ligated to the pMD18-T simple vector to generate pMD18-T-Δ*speE*. The *rocG* fragment was amplified using primers p_{tac}-rocGF/rocGR and pEG-rocG DNA as the template. The resulting fragment, $P_{tac\text{-}M}$-*rocG*, was digested with *Xba*I and inserted into the cognate site of pMD18-T-Δ*speE*. The Δ*speE*::$P_{tac\text{-}M}$-*rocG* fragment was cleaved from the resulting plasmid and was ligated to *Bam*HI/*Sal*I-digested pK-JL to obtain pK-Δ*speE::rocG*. For the construction of pK-Δ*argR::gapC*, primers argR5 and argR3 were used with pK18mobsacB-Δ*argR* DNA as template. The resulting fragment was ligated

Table 2 Primers used in this study

Primer	Sequence[a] and purpose (5′–3′)
sacBF	CGGCGACTAGTTGAGCTGTTGACAATTAATCATCGTGTGGTACCATGT GTGGAATTGTGAGCGGATAACAATTCCGCGGGTTCTTTAGGCCCGT AGTCT (*Spe*I, *Sac*II), PCR for *sacB*
sacBR	GCCGCGATATCTCTCGTGATGGCAGGTT (*Eco*RV), PCR for *sacB*
pk18msF	GCGCCGATATCGTTCGTCTGGAAGGCAGTA (*Eco*RV), PCR for the backbone of pK18mobsacB except for *sacB*
pk18msR	GCGCGACTAGTGCATGGGCATAAAGTTGC (*Spe*I), PCR for the backbone of pK18mobsacB except for *sacB*
speEF5	CGATGGATCCCGACCGCTACAAGGCATAA (*Bam*HI), deletion of *speE*
speER5	GCGTCTAGAGCGGAAATAATGGCGAAA (*Xba*I), deletion of *speE*
speEF3	CGCTCTAGACGATTCTGCCTCTGGATTA (*Xba*I), deletion of *speE*
speER3	CGAT*GTCGAC*CACCATCTGCCCAACG (*Sal*I), deletion of *speE*
argRF5	CGCT*GGATCC*TTTAAGCACGGCGTTATTT (*Bam*HI), deletion of *argR*
argRR5	CGG*TCTAGA*TGCGAGTCACGGGATTTA (*Xba*I), deletion of *argR*
argRF3	CGG*TCTAGA*GGTAAGGTATAACCCGAGTGT (*Xba*I), deletion of *argR*
argRR3	CGAT*GTCGAC*GACTTGATGCCCACGAGA (*Sal*I), deletion of *argR*
pgiF	GTAGGATCCAGGAGTTTTCATGGCGGAC (*Bam*HI), PCR for *pgi*
pgiR	GCGTCTAGAAGCGACTACCTATTTGCG (*Xba*I), PCR for *pgi*
pfkF	CGAGTCGACAAGGAGGAAGACATGCGAATTGC (*Sal*I), PCR for *pfkA*
pfkR	CCGCTGCA GACTATCCAAACATTGCCTG (*Pst*I), PCR for *pfkA*
gapF	GGCGGTACCAGGAGACACAACATGACCATT (*Kpn*I), PCR for *gap*
gapR	CGGGGATCCATTAGAGCTTGGAAGCTACGAG (B*am*HI), PCR for *gap*
pykF	CGAGAGCTCAAGGAGTAGGCTTATGGGCGT (*Sac*I), PCR for *pyk*
pykR	CCGGGTACCGATTAGAGCTTTGCAATCCT (*Kpn*I), PCR for *pyk*
pycF	CGCGAGCTCAAGGAGTACTCTAGTGTCGACTCACACAT (*Sac*I), PCR for *pyc*
pycR	CGCTCTAGATTAGGAAACGACGACGAT (*Xba*I), PCR for *pyc*
gltF	GCCGAATTCAAGGAGAACAAATATGTTTGAAAG (*Eco*RI), PCR for *gltA*
gltR	GTAGAGCTCTTTAGCGCTCCTCGCGAGGAACCAACT (*Sac*I), PCR for *gltA*
gdhF	CGCTCTAGAAAGGAGGAAATCATGACAGTTG (*Xba*I), PCR for *gdh*
gdhR	CGGGTCGACTCTTAGATGACGCCCTGT (*Sal*I), PCR for *gdh*
gapCF	GCGGGTACCGGAGGTAGTTAGAATGGCAAAGATAGC (*Kpn*I), PCR for *gapC*
gapCR	CGCGGATCCCTATTTTGCTATT (*Bam*HI), PCR for *gapC*
rocF	CCGTCTAGAGGAGGGAAAAAGATGTCAGCAA (*Xba*I), PCR for *rocG*
rocR	GGCGTCGACAAATTAGACCCATCCG (*Sal*I), PCR for *rocG*
p_{tac}-rocGF	GCTCTAGATGAGCTGTTGACAATTAATCATCGTGTGGTACCATGTGTGG AATTGTGAGCGGATAACAATTGGAGGGAAAAAGATGTCAGCAA (*Xba*I), PCR for the P_{tacM}-*rocG* fragment
rocGR	GCTGATCCTCTAGAAAATTAGACCCATCCG (*Xba*I), PCR for the P_{tacM}-*rocG* fragment
p_{tac}-gapCF	CGACTAGTTGAGCTGTTGACAATTAATCATCGTGTGGTACCATGTGTGGA ATTGTGAGCGGATAACAATTAAGGAGGAGTTAGAATGGCAAAGATAGC (*spe*I), PCR for the P_{tacM}-*gapC* fragment
gapCR2	CGCTCTAGACTATTTTGCTATT(*Xba*I), PCR for the P_{tacM}-*gapC* fragment
zwfF	ACCCGCAGGATAAACGA, qRT-PCR for *zwf*
zwfR	GCTAGATCATAAATGGC, qRT-PCR for *zwf*
ppnkF	GTTTACCGACCGACTTGTG, qRT-PCR for *ppnK*
ppnkR	GCTGACCTGGGATCTTTATT, qRT-PCR for *ppnK*
icdF	AGGACCAGGGCTACGACAT, PCR for *icd*
icdR	GCGGAACCCTTAACAGC, PCR for *icd*
gndF	AACCGCAGCACTGACAAA, PCR for *gnd*
gndR	CAGGGATGCTACGAACTCT, PCR for *gnd*
16s-F	TCGATGCAACGCGAAGAAC, PCR for *16 sRNA*
16s-R	GAACCGACCACAAGGGAAAAC, PCR for *16sRNA*

[a] Restriction enzyme cleavage sites are underlined

to the pMD18-T simple vector to generate pMD18-T-Δ*argR*. The *gapC* fragment was amplified using the primers p_{tac}-gapF and gapCR2 with pEG-gapC DNA as the template. The resulting fragment, $P_{tac\text{-}M}$-*gapC*, was digested with *Xba*I and inserted into the cognate site of pMD18-T-Δ*argR*. The Δ*argR*::$P_{tac\text{-}M}$-*gapC* fragment was cleaved from the resulting plasmid using *Bam*HI/*Sal*I and was ligated to *Bam*HI/*Sal*I-digested pK-JL DNA to obtain pK-Δ*argR::gapC*.

These nonreplicable integration vectors, including pK18mobsacB-Δ*speE,* pK18mobsacB-Δ*argR,* pK-Δ*speE::rocG*, and pK-Δ*argR::gapC* were used to transform *C. glutamicum* to disrupt the site-specific gene using the protocol described by Schäfer et al. [25].

Quantitative real-time PCR (qRT-PCR)

Total RNA from *C. glutamicum* grown for 54 h in shake flasks was isolated using an RNA extraction kit (Dongsheng Biotech, Guangzhou, China), following the manufacturer's instructions. The first-strand cDNA was synthesized using an All-in-One^{TM} First-Strand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China). qRT-PCR was performed using the All-in-One^{TM} qPCR Mix kit (GeneCopoeia, Guangzhou, China) on an iCycler iQ5 Real-Time PCR system (Bio-Rad Laboratories, Richmond, CA, USA). One hundred nanograms of cDNA was used as template. The PCR conditions were as follows: 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 15 s. The primers for qRT-PCR are listed in Table 2. The $2^{-\Delta\Delta Ct}$ quantification technique was used to analyze data [19]. The data were normalized to the level of expression of 16S rRNA.

NADPH assay

After aerobic cultivation of *C. glutamicum* on a rotary shaker (150 rpm) at 30 °C for 54 h, the cells were harvested by centrifugation and washed twice with water. Intracellular NADPH was extracted and quantified using the Enzychrom^{TM} $NADP^+$/NADPH Assay kit (BioAssay Systems, Hayward, CA), following the manufacturer's instructions.

Enzyme assay

Two-milliliter cell cultures at 50 h were harvested by centrifugation, washed twice with water, and subjected to sonication in 0.4 ml of 50 mM Tris–Cl (pH 7.5) containing 20 % glycerol, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Then the resulting broken-cells suspension was centrifuged for 15 min at 4 °C and 14,000 rpm to obtain cell-free extracts. Glutamate dehydrogenase activity was determined spectrophotometrically by measuring the oxidation of NADPH (or NADH) at a wavelength of 340 nm and 25 °C, as described previously [1]. The standard reaction mixture contained 2.9 ml of 55 mM Tris–Cl (pH 7.5) containing 2 % glycerol, 10 mM NaCl, 100 mM NH_4Cl, 10 mM 2-ketoglutarate, 0.2 mM NADPH (or NADH), and 100 μl crude extract. Reaction was initiated by the addition of the cell-free extracts. One unit of glutamate dehydrogenase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 mmol NADPH (or NADH) in 1 min under the above conditions. Glyceraldehyde-3-phosphate dehydrogenase activity was determined spectrophotometrically by measuring the reduction of NAD (or NADP) at a wavelength of 340 nm and 25 °C, as described previously [22]. The standard reaction mixture contained 2.9 ml of 50 mM Tris–Cl (pH 8.5) containing 3 mM β-mercaptoethanol, 1 mM NADP, 1 mM DL-glyceraldehyde 3-phosphate, 1 mM H_3PO_4, and 100 μl crude extract. Reaction was initiated by the addition of the cell-free extracts. One unit of glyceraldehyde-3-phosphate dehydrogenase activity was defined as the amount of enzyme that catalyzed the reduction of 1 mmol NAD (or NADP) in 1 min under the above conditions.

Analysis of cell growth and ornithine

Cell growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) using a spectrophotometer (UV2450, Shimadzu Corporation, Japan) after adding 0.2 mol l^{-1} HCl to dissolve $CaCO_3$. DCW was estimated according to the formula 1 OD_{600} = 0.28 g DCW l^{-1} [8]. L-Ornithine concentrations were determined by colorimetry using ninhydrin as described previously [2].

Statistical analysis

All experiments were conducted in triplicate, and data were averaged and presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significant differences using OriginPro (version 7.5) software. Statistical significance was defined as $p < 0.05$.

Results and discussion

Effect of expression of genes involved in glutamate biosynthesis on the production of L-ornithine

We previously conducted a comparative proteomic analysis between wild-type *C. glutamicum* and a triple knockout mutant of genes by 2-DE gel electrophoresis [21]. The results showed that the amount of enzymes involved in ornithine biosynthesis (ArgCJBD) downstream of

glutamate is much higher than that of enzymes acting upstream of glutamate. This indicated that overexpression of the enzymes upstream of glutamate would increase glutamate concentration, leading to increased production of L-ornithine in an appropriately engineered strain of *C. glutamicum*. Furthermore, the flux of Pgi, PfkA, GapA, Pyk, Pyc, GltA, and Gdh increases in parallel with increased glutamate production by *C. glutamicum* [29]. Thus, the genes *pgi, pfkA, gap, pyk, pyc, gltA*, and *gdh* were overexpressed in *C. glutamicum* ΔAP. As shown in Fig. 1, overexpression of *pgi, pfkA, gap, pyk,* and *gdh* slightly improved L-ornithine production compared with *C. glutamicum* ΔAP. These results support our assumptions based on our comparative proteomic analysis.

Of the aforementioned seven genes, overexpression of the genes *gap* or *gdh* showed a more positive effect on L-ornithine production. The reactions, catalyzed by the enzymes encoded by *gap* or *gdh,* require NAD or NADPH. Thus, we asked whether these cofactors affect the production of L-ornithine. Moreover, the three reactions utilizing NADPH in the L-ornithine biosynthetic pathway are catalyzed by NADP-dependent isocitrate dehydrogenase, NADPH-dependent glutamate dehydrogenase, or NADP-dependent *N*-acetyl-gamma-glutamyl-phosphate reductase. Therefore, we investigated the effect of the availability of endogenous NADPH on L-ornithine production by overexpressing these enzymes (Fig. 2). Overexpression of the genes *gap, gdh*, *gapC*, and *rocG* resulted in an increase of L-ornithine concentration compared with overexpression of pEC-XK99E by *C. glutamicum* ΔAP (10.14 ± 0.18 g l^{-1}, 10.05 ± 0.17 g l^{-1}, $10.59 \pm 0.0.30$ g l^{-1}, 10.62 ± 0.02 g l^{-1}, respectively, vs 8.66 ± 0.16 g l^{-1}, $P < 0.01$).

Glyceraldehyde-3-phosphate dehydrogenase, catalyzing oxidation of D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate, is a key enzyme of glycolysis and plays a crucial role in catabolic carbohydrate metabolism. Overexpression of NAD-dependent glyceraldehyde-3-phosphate dehydrogenase can drive more metabolic flux of carbon into the Embden–Meyerhof–Parnas (EMP) pathway, hence improving the internal glutamate pool. Overexpression of *gdh* in *C. glutamicum* resulted in an increased internal glutamate pool [11]. The increased internal glutamate pool resulted in enhanced L-ornithine production.

C. acetobutylicum gapC encodes NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. The GapC catalyzes glyceraldehyde-3-phosphate and NADP to form 3-phospho-D-glyceroyl phosphate and NADPH. Overexpression of the *gapC* gene results in an increase of the intracellular NADPH concentration. Overexpression of *C. acetobutylicum gapC* increases the production of CoQ_{10} [5]. The overexpression of *C. acetobutylicum gapC* together with the knockout of the endogenous *gapA* gene improves lycopene and ε-caprolactone production [22]. *B. subtilis rocG* encodes an NAD-dependent glutamate dehydrogenase that converts 2-oxoglutarate to glutamate in the presence of NADH. The expression of *rocG* provides more NADPH for L-ornithine production as an outcome of the consumption of 2-oxoglutarate by the NADPH-independent reaction.

Figure 2 also shows that overexpression of the *gdh* gene caused significant inhibition of growth. Kholy et al. [11] reported the same result. *C. glutamicum* overexpressing the *gdh* gene showed somewhat slower growth on glucose and acetate medium than that of the wild type. The overexpression

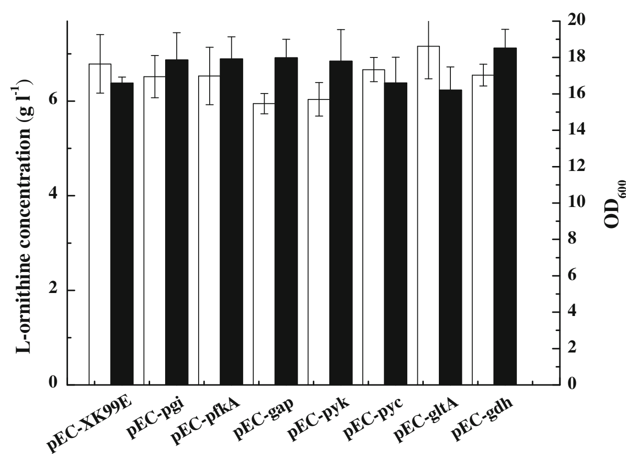

Fig. 1 Effect of overexpression of genes encoding enzymes in the upstream pathway of glutamate biosynthesis on L-ornithine production in *C. glutamicum* ΔAP. OD_{600} (*white bars*); L-ornithine concentration (*black bars*). Cells were cultured in the fermentation medium 25 μg ml^{-1} Kan and 0.5 mM IPTG at 30 °C for 72 h. Data are mean ± SD for three replicates

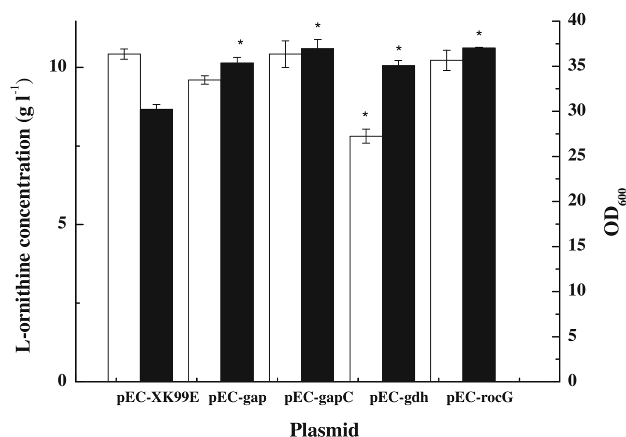

Fig. 2 Effect of the overexpression of genes encoding enzymes requiring NADPH on L-ornithine production by *C. glutamicum* ΔAP. OD_{600} (*white bars*); L-ornithine concentration (*black bars*). Cells were cultured in the fermentation medium 25 μg ml^{-1} Kan and 0.5 mM IPTG at 30 °C for 72 h. Data are mean ± SD for three replicates. *Significant different at $P < 0.01$ compared with corresponding controls

of the other three genes did not inhibit cell growth. Thus, the genes *gapC* and *rocG* were selected for further study.

Gene deletion strategy to increase L-ornithine production

Our previous proteome analysis [20] demonstrated that spermidine synthase encoded by *speE* was upregulated in the L-ornithine-producing strain *C. glutamicum* compared with wild type. We reasoned that this upregulation might result in the degradation of L-ornithine. Therefore, we deleted *speE* from *C. glutamicum* ΔAP to generate *C. glutamicum* ΔAPE. Deletion of *speE* enhanced the production of L-ornithine. Strain ΔAPE produced 10.87 ± 0.37 g l^{-1} of L-ornithine, which is 12.0 % higher than that of *C. glutamicum* ΔAP (9.71 ± 0.18 g l^{-1}; Table 3).

The expression of the *arg* operon that controls the L-ornithine biosynthetic pathway is regulated by the arginine repressor (ArgR). Further, the DNA-binding affinity of ArgR for the upstream of the *argB* gene plays an important role in the biosynthesis of L-ornithine biosynthesis by *C. glutamicum* [17]. Deletion of *argR* is another strategy for enhancing the level of expression of the *arg* operon. Thus, we deleted the *argR* gene of the *C. glutamicum* ΔAPE to generate *C. glutamicum* ΔAPER. *C. glutamicum* ΔAPER produced 12.12 ± 0.57 g l^{-1} of L-ornithine (Table 3), which was 11.5 % higher than that of *C. glutamicum* ΔAPE.

Effect of NADPH availability

To reduce the metabolic burden caused by plasmid replication, the genes *gapC* or *rocG* were integrated into the chromosome of *C. glutamicum* ΔAPER. The individual chromosomal integration of the *gapC* gene led to an increase in the concentration of L-ornithine from 12.12 ± 0.57 to 13.23 ± 0.54 g l^{-1} ($P < 0.01$; Table 3). The individual chromosomal integration of the *rocG* gene also led to an increase of L-ornithine concentration compared with *C. glutamicum* ΔAPER (14.84 ± 0.57 g l^{-1} vs 12.12 ± 0.57 g l^{-1}; $P < 0.01$; Table 3). Moreover, these modifications resulted in a significant increase in the concentration of intracellular NADPH. However, the simultaneous integration of *gapC* and *rocG* genes did not further increase the intracellular concentrations of L-ornithine and NADPH. In order to understand the mechanism of L-ornithine production, we determined the transcriptional level of *gapC* and *rocG*, and their corresponding enzyme activities. The data are shown in Table 4. The individual chromosomal integration of the *gapC* gene led to a reduction of Gap enzyme activity, and increase of total glyceraldehyde-3-phosphate dehydrogenase activities. The expression of GapC enzyme resulted in the evaluated NADPH concentration. The individual chromosomal integration of the *rocG* gene led to a reduction of Gdh enzyme activity, and increase of total glutamate dehydrogenase activities, which can provide more NADPH for L-ornithine biosynthesis, and thereby improving L-ornithine production. The data shown

Table 3 L-Ornithine production by the engineered strains

Strain	OD_{600}	L-Ornithine (g l^{-1})	NADPH (μmol g^{-1})
ΔAP	26.23 ± 0.51	9.71 ± 0.18	2.13 ± 0.16
ΔAPE	25.53 ± 0.65	10.87 ± 0.37	2.00 ± 0.42
ΔAPER	13.65 ± 0.28	12.12 ± 0.57	3.04 ± 0.21
ΔAPER::*gapC*	13.10 ± 0.21	13.23 ± 0.54	11.46 ± 0.63
ΔAPRE::*rocG*	11.65 ± 0.99	14.84 ± 0.57	11.37 ± 0.16
ΔAPE::*rocG*-R::*gapC*	12.50 ± 0.85	14.20 ± 0.71	11.46 ± 0.71

Table 4 Transcriptional levels of genes and specific activity of enzymes in the engineered strains

Strain	Relative mRNA expression[a]		Specific glyceraldehyde-3-phosphate dehydrogenase activity (U mg^{-1} protein)[a]			Specific glutamate dehydrogenase activity (U mg^{-1} protein)[a]		
	gapC	*rocG*	Gap	GapC	Total	Gdh	RocG	Total
ΔAPER			205.2		205.2	8,077.7		8,077.7
ΔAPER::*gapC*	1.4		122.9	468.1	591.0			
ΔAPRE::*rocG*		1.4				6,772.0	4,785.5	11,557.5
ΔAPE::*rocG*-R::*gapC*	1.6	1.4	106.8	516.2	623.0	6,774.9	4,800.7	11,575.6

[a] Values are averages based on the results obtained with at least three independent experiments and the standard deviations were consistently <10 %

in Table 4 also demonstrate that the level of transcription of the *gapC* gene and GapC activity in *C. glutamicum* ΔAPE::*rocG*-R::*gapC* was significantly different from that of *C. glutamicum* ΔAPER::*gapC*. However, the level of transcription of the *rocG* gene and RocG activity in *C. glutamicum* ΔAPE::*rocG*-R::*gapC* was not significantly different from that of *C. glutamicum* ΔAPRE::*rocG*.

To better understand the mechanism of increased NADPH concentration, we compared the levels of transcription of the genes (*zwf*, *gnd*, *icd*, and *ppnK*) involved in NADPH synthesis in *C. glutamicum* ΔAPER::*gapC*, ΔAPRE::*rocG*, and ΔAPE::*rocG*-R::*gapC* and compared these levels with those of *C. glutamicum* ΔAPER (Fig. 3). The levels of transcription of *gnd*, *icd*, and *ppnK* in *C. glutamicum* ΔAPER::*gapC*, ΔAPRE::*rocG*, and ΔAPE::*rocG*-R::*gapC* were lower than those of *C. glutamicum* ΔAPER, indicating that the increased level of NADPH in the three strains was not caused by the three genes. In *C. glutamicum* ΔAPRE::*rocG*, the levels of transcription of the four genes were lower than those of *C. glutamicum* ΔAPER, indicating that the increased level of NADPH in *C. glutamicum* ΔAPER::*rocG* was caused by chromosomal overexpression of the *rocG* gene and not by the three genes. The expression of the *rocG* gene can provide more NADPH for L-ornithine production, because the conversion of 2-oxoglutarate to glutamate is catalyzed by the NADPH-independent glutamate dehydrogenase. In *C. glutamicum* ΔAPER::*gapC* and ΔAPE::*rocG*-R::*gapC*, the level of transcription of the *zwf* gene was higher than that of *C. glutamicum* ΔAPER, indicating that the increased levels of NADPH in *C. glutamicum* ΔAPER::*gapC* and ΔAPE::*rocG*-R::*gapC* were caused by chromosomal overexpression of the *gapC/rocG* gene and increasing expression of *zwf* gene. However, the upregulation of the level of transcription of the *zwf* gene in the two strains can drive the metabolic flux of carbon from the EMP pathway to the pentose phosphate (PP) pathway. Moreover, given that the PP pathway is coupled to the production of CO_2, direct enhancement of the PP pathway may result in the release of CO_2, thereby decreasing the yield of L-ornithine. This reasoning would explain the lower concentration of L-ornithine by the two strains compared with that of *C. glutamicum* ΔAPRE::*rocG*.

The present study demonstrates that the availability of NADPH and the production of L-ornithine are tightly linked. Production of L-ornithine by *C. glutamicum* requires 2 moles of NADPH per mole of L-ornithine. Other strategies have been developed to improve the availability of NADPH. The inactivation of the gene encoding gluconate kinase (*gntK*) was shown to lead to a 51.8 % increase in intracellular NADPH concentration and a 49.9 % increase in L-ornithine production [7]. They also demonstrated that excess NADPH is not necessarily rate-limiting, but is required for increased

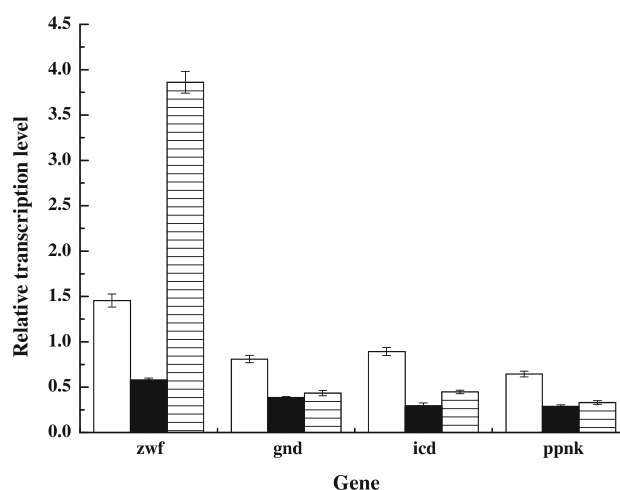

Fig. 3 Levels of transcription of genes involved in NADPH biosynthesis in *C. glutamicum* ΔAPER::*gapC*, ΔAPRE::*rocG*, and ΔAPE::*rocG*-R::*gapC* compared with those in the parental strain, *C. glutamicum* ΔAPER. ΔAPER::*gapC* (*white bars*), ΔAPRE::*rocG* (*black bars*), and ΔAPE::*rocG*-R::*gapC* (*hatched bars*)

L-ornithine production in *C. glutamicum*. Overexpression of *E. coli pntAB*, which encodes a membrane-bound transhydrogenase, enhances the availability of NADPH and thus increases the levels of L-lysine in *C. glutamicum* [10]. Replacement of the endogenous NAD-dependent glyceraldehyde-3-phosphate dehydrogenase with the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans* also increases the availability of NADPH and L-lysine in *C. glutamicum* [30]. Overexpression of *nadk*, which encodes NAD kinase, increases the NADPH/NADP ratio, which in turn enhances thymidine biosynthesis in *E. coli* [14]. Simultaneous chromosomal overexpression of transhydrogenase (*pntAB*) and NAD kinase (*yfjB*) genes had an effect on increasing NADPH supply and improving anaerobic isobutanol production [27]. These strategies, which are being explored in our laboratory, may be useful for further improving the production of L-ornithine by *C. glutamicum* ΔAPRE::*rocG*.

In conclusion, we implemented strategies to enhance L-ornithine production by *C. glutamicum* that were suggested by the results of our previous proteomic analysis [21]. Overexpression of *C. acetobutylicum gapC* and *B. subtilis rocG* resulted in increased production of L-ornithine, because these genetic manipulations provided increased levels of NADPH for L-ornithine biosynthesis. The increased levels of NADPH resulted from the expression of the *gapC* or *rocG* gene rather than that of the genes (*gnd*, *icd*, and *ppnK*) involved in NADPH biosynthesis. To the best of our knowledge, this is the first report on the expression of *gapC* and *rocG* to enhance the production of L-ornithine by *C. glutamicum*.

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References

1. Belitsky BR, Sonenshein AL (1998) Role and regulation of *Bacillus subtilis* glutamate dehydrogenase genes. J Bacteriol 180:6298–6305
2. Chinard FP (1952) Photometric estimation of proline and ornithine. J Biol Chem 199:91–95
3. Choi DK, Ryu WS, Choi CY, Park YH (1996) Production of L-ornithine by arginine auxotrophic mutants of *Brevibacterium ketoglutamicum* in dual substrate limited continuous culture. J Ferment Bioeng 81:216–219
4. Eikmanns BJ, Thum-Schmitz N, Eggeling L, Ludtke KU, Sahm H (1994) Nucleotide sequence, expression, and transcription analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology 140:1817–1828
5. Huang MT, Wang Y, Liu JZ, Mao ZW (2011) Multiple strategies for metabolic engineering of *Escherichia coli* for efficient production of coenzyme Q_{10}. Chin J Chem Eng 19:316–326
6. Hwang G-H, Cho J-Y (2010) Identification of a suppressor gene for the arginine-auxotrophic *argJ* mutation in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 37:1131–1136
7. Hwang GH, Cho JY (2012) Implication of gluconate kinase activity in L-ornithine biosynthesis in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 39:1869–1874
8. Hwang JH, Hwang GH, Cho JY (2008) Effect of increased glutamate availability on L-ornithine production in *Corynebacterium glutamicum*. J Microbiol Biotechnol 18:704–710
9. Inui M, Kawaguchi H, Murakami S, Vertès AA, Yukawa H (2004) Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. J Mol Microbiol Biotechnol 8:243–254
10. Kabus A, Georgi T, Wendisch VF, Michael B (2007) Expression of the *Escherichia coli pntAB* genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation. Appl Microbiol Biotechnol 75:47–53
11. Kholy ER, Eikmanns BJ, Gutmann M, Sahm H (1993) Glutamate dehydrogenase is not essential for glutamate formation by *Corynebacterium glutamicum*. Appl Environ Microbiol 59:2329–2331
12. Kinoshita S, Nakayama K, Udaka S (1957) The fermentative production of L-ornithine. J Gen Appl Microbiol 3:276–277
13. Kirchner O, Tauch A (2003) Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J Biotechnol 104:287–299
14. Lee HC, Kim JS, Jang W, Kim SY (2010) High NADPH/NADP ratio improves thymidine production by a metabolically engineered *Escherichia coli* strain. J Biotechnol 149:24–32
15. Lee HW, Yoon SJ, Jang HW, Kim CS, Kim TH, Ryu WS, Jung JK, Park YH (2000) Effects of mixing on fed-batch fermentation of L-ornithine. J Biosci Bioeng 89:539–544
16. Lee SY, Cho JY, Lee HJ, Kim YH, Min J (2010) Enhancement of ornithine production in proline-supplemented *Corynebacterium glutamicum* by ornithine cyclodeaminase. J Microbiol Biotechnol 20:127–131
17. Lee SY, Kim YH, Min J (2009) The effect of ArgR-DNA binding affinity on ornithine production in *Corynebacterium glutamicum*. Curr Microbiol 59:483–488
18. Lee YJ, Cho JY (2006) Genetic manipulation of a primary metabolic pathway for L-ornithine production in *Escherichia coli*. Biotechnol Lett 28:1849–1856
19. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408
20. Lu DM, Jiang L-Y, Chen L-A, Liu J-Z, Mao Z-W (2011) Optimization of fermentation conditions of the engineered *Corynebacterium glutamicum* to enhance L-ornithine production by response surface methodology. J Biotechnol Biomater 1:116. doi: 10.4172/2155-952X.1000116,15
21. Lu DM, Liu JZ, Mao ZW (2012) Engineering of *Corynebacterium glutamicum* to enhance L-ornithine production by gene knockout and comparative proteomic analysis. Chin J Chem Eng 20:731–739
22. Martínez A, Zhu J, Lin H, Bennett GN, San KY (2008) Replacing *Escherichia coli* NAD-dependent glyceraldehydes 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from *Clostridium acetobutylicum* facilitates NADPH dependent pathways. Metab Eng 10:352–359
23. Meiswinkel TM, Gopinath V, Lindner SN, Nampoothiri M, Wendisch VF (2012) Accelerated pentose utilization by *Corynebacterium glutamicum* for accelerated production lysine, glutamate, ornithine and putrescine. Microb Biotechnol 6:131–140
24. Salvatore F, Cimino F, Maria C, Cittadini D (1964) Mechanism of the protection by L-ornithine-L-aspartate mixture and by L-arginine in ammonia intoxication. Arch Biochem Biophys 107:499–503
25. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73
26. Schneider J, Niermann K, Wendisch VF (2011) Production of the amino acids L-glutamate, L-lysine, L-ornithine and L-arginine from arabinose by recombinant *Corynebacterium glutamicum*. J Biotechnol 154:191–198
27. Shi A, Zhu X, Lu J, Zhang X, Ma Y (2013) Activating transhydrogenase and NAD kinase in combination for improving isobutanol production. Metab Eng 16:1–10
28. Shi HP, Fishel RS, Efron DT, Williams JZ, Fishel MH, Barbul A (2002) Effect of supplemental ornithine on wound healing. J Surg Res 106:299–302
29. Shirai T, Fujimura K, Furusawa C, Nagahisa K, Shioya S, Shimizu H (2007) Study on roles of anaplerotic pathways in glutamate overproduction of *Corynebacterium glutamicum* by metabolic flux analysis. Microb Cell Fact 6:19
30. Takeno S, Murata R, Kobayashi R, Mitsuhashi S, Ikeda M (2010) Engineering of *Corynebacterium glutamicum* with an NADPH-generating glycolytic pathway for L-lysine production. Appl Environ Microbiol 76:7154–7160
31. Van der Rest ME, Lange C, Molenaar D (1999) A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol Biotechnol 52:541–545
32. Xu D, Tan Y, Shi F, Wang X (2010) An improved shuttle vector constructed for metabolic engineering research in *Corynebacterium glutamicum*. Plasmid 64:85–91
33. Zajac A, Poprzecki S, Zebrowska A, Chalimoniuk M, Langfort J (2010) Arginine and ornithine supplementation increases growth hormone and insulin-like growth factor-1 serum levels after heavy-resistance exercise in strength-trained athletes. J Strength Cond Res 24:1082–1090
34. Zhang JF, Wang JB, Huang JM, Zhang J (2009) Breeding of high-yield L-ornithine-producing strain by protoplast fusion. Amino acids Biotic Resour 31:53–57