Optimization of L-ornithine production in recombinant Corynebacterium glutamicum S9114 by cg3035 overexpression and manipulating the central metabolic pathway

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Abstract
Background: L-Ornithine is an important amino acid with broad applications in pharmaceutical and food industries. Despite lagging L-ornithine productivity and cost reduction, microbial fermentation is a promising route for sustainable L-ornithine production and thus development of robust microbial strains with high stability and productivity is essential.

Results: Previously, we systematically developed a new strain, SO1 originate from Corynebacterium glutamicum S9114, for L-ornithine production. In this work, overexpression of cg3035 encoding N-acetylglutamate synthase (NAGS) using a plasmid or by inserting a strong P_tac promoter into the chromosome was found to increase L-ornithine production in the engineered C. glutamicum SO1. The genome-based cg3035 modulated strain was further engineered by attenuating the expression of pta and cat, inserting a strong P_efe promoter in the upstream region of glycolytic enzymes such as pfka, gap, and pyk, and redirecting carbon flux to the pentose phosphate pathway. The final strain with all the exploratory metabolic engineering manipulations produced 32.3 g/L of L-ornithine, a yield of 0.395 g ornithine per g glucose, which was 35.7% higher than that produced by the original strain (23.8 g/L).

Conclusion: These results clearly demonstrated that enhancing the expression of NAGS promoted L-ornithine production and provide a promising alternative systematic blueprint for developing L-ornithine-producing C. glutamicum strains.

Keywords: Corynebacterium glutamicum, L-Ornithine, Metabolic engineering

Background
Chemicals produced by metabolically engineered strains have gained major importance in industrial biotechnology, which is developing as a stimulating field due to the advantages of providing environmentally friendly products to replace petrochemicals [1]. With the development of gene manipulation tools, there is an increasing number of strains such as Escherichia coli, Corynebacterium glutamicum, Bacillus subtilis and others that have been modified to produce economically valuable products. C. glutamicum is a favored industrial microorganism with promising applications in the industrial production of amino acids such as L-glutamate and L-lysine at a million-ton scale in the past decades [2, 3]. Recently, increasing studies have used C. glutamicum for producing several products including diamines [4, 5], terpene [6], diols [7], and amino acids such as L-isoleucine, L-arginine, and L-ornithine [3]. L-Ornithine is an...
intermediate metabolite in the urea cycle, a key precursor for the biosynthesis of l-citrulline, l-proline, and polyamines, and a non-essential amino acid that plays a critical role in post-traumatic treatment, liver protection [8] and treatment of liver disease, strengthening the heart, and maintaining the working of the immune system [9].

Due to the importance of l-ornithine in human health-promoting activities, economical and efficient production of ornithine has received much attention in the past years. The major strategy for producing l-ornithine is focused on the enzymatic action of arginase on arginine and microbial fermentation. Metabolic engineering of microorganisms to produce l-ornithine is an attractive alternative due to the high economic cost pressures of using l-arginine for the enzymatic reaction. Recently, several reports have focused on the development of metabolically engineered strains that rapidly convert high concentrations of simple sugars to l-ornithine. Jensen et al. constructed a metabolically engineered C. glutamicum strain that could produce 0.524 g/L l-ornithine per g glucose in CGXII medium through disruption of argFRG and overexpression of gdh and argCJBD [10]. Hwang and Cho modulated the NADPH supply to l-ornithine biosynthesis by inactivating three putative glucose dehydrogenases, which improved the yield of l-ornithine up to 14 g/L [11]. Kim et al. developed a high l-ornithine producing C. glutamicum strain by disrupting argF, argR, and proB, and overexpressing the operon argCJBD from C. glutamicum ATCC 21831. This strain produced 51.5 g/L l-ornithine from glucose in a fed-batch culture in a 6.6-L fermenter [12]. Jiang et al. engineered a C. glutamicum ATCC 13032-derived strain that produced 24.1 g/L l-ornithine in shake flask cultures by genetic modulation and adaptive evolution [13].

In previous study, we developed an engineered strain of C. glutamicum S9114 by deletion of argF, ncl1221, argR, and putP, attenuation of odhA, proB, and ncl2228, and overexpression of lysE, gdh, and argCJBD, which produced up to 25 g/L of l-ornithine in a shake flask culture [14, 15]. In the present study, further manipulation to improve l-ornithine production by the genetically engineered C. glutamicum strain was reported (see in Fig. 1). Overexpression of cg3035 using plasmid-based overexpression and promoter insertion experiments for improving l-ornithine production in C. glutamicum S01 was explored. In addition, the effects of overexpressing the key enzymes in glycolysis, along with attenuation of acetate biosynthesis were evaluated. Next, redirection of the metabolic flux to pentose phosphate pathway was also modulated.

Methods
Strains and plasmids
A high l-ornithine producing strain C. glutamicum S01 (S9114 with deletion of argF, ncl1221, argR, and putP, attenuation of odhA, proB, and ncl2228, and overexpression of lysE, gdh, and argCJBD) derived from C. glutamicum S9114 was used as the original strain for further strain development. The recombinant strains and the plasmid constructed in this study are listed in Table 1. For standard molecular manipulation, E. coli DH5α was used as the host for gene cloning. Luria–Bertani (LB) medium was used to propagate E. coli and C. glutamicum.

DNA manipulation and strain construction
To construct the recombinant strains, the genomic DNA of strain C. glutamicum S9114 was isolated using a genomic DNA extraction kit (Tiangen, Beijing, China) and employed for DNA fragment amplification. The PCR product and vectors were obtained using the PCR products Purification kit and the mini-plasmid isolation kit (Tiangen, Beijing, China), respectively. The primers used in this study are listed in Additional file 1: Table S1. For introducing modifications into the chromosome of C. glutamicum S9114, the suicide vector pK18mobacB containing the sucrose lethal gene sacB from Bacillus subtilis was employed by double crossover recombination, as described previously [16, 17].

For cg3035 overexpression in the high l-ornithine producing strain SO1, a widely used strong Pnod promoter and the open reading frame of cg3035 was amplified and spliced using PCR. The overlapped fragment was ligated

(See figure on next page.)

Fig. 1 l-Ornithine biosynthesis metabolic pathways in C. glutamicum and strategies to improve l-ornithine accumulation. The red x represented this pathways were inactivated. The blue font and arrows represented that pathways were attenuated. The green font and arrows indicated that pathways were overexpressed. The genes encoding enzymes involved in catalytically relevant reactions, pgk, encodes glucose-6-phosphate isomerase; zwf, encodes glucose-6-phosphate dehydrogenase; pgl, encodes 6-phosphogluconolactonase; tkt, encodes transketolase; tal, encodes transaldolase; opcA, encodes the putative glucose-6-phosphate dehydrogenase assembly protein; pka, encodes ATP-dependent phosphofructokinase; gap, encodes glyceraldehyde 3-phosphate dehydrogenase; pyk, encodes pyruvate kinase; pta, encodes phosphotransacetylase; ackA, encodes acetate kinase; cat, encodes succinyl-CoA:Acetate CoA-transferase; ncl2228, encodes a putative branched amino acid transporter protein; odhA, encodes a subunit of 2-ketoglutarate dehydrogenase; gdh, encodes glutamate dehydrogenase; ncl1221, encodes glutamate transporter; proB, encodes gamma-glutamate kinase; cg3035, encodes N-acetylglutamate synthase; argB, encodes N-acetylglutamate kinase; argC, encodes N-acetyl-gamma-glutamylphosphate reductase; argD, encodes acetylornithine aminotransferase; argF, encodes ornithine carbamoyltransferase; argJ, encodes ornithine acetyltransferase; argR, encodes arginine repressor; lysE, encodes lysing transporter.
Table 1 Strains and plasmids used in this study

| Strain/plasmid | Characteristic | Source |
|----------------|---------------|--------|
| **Strain** |  |  |
| E. coli DH5α | Clone host strain | Transgen |
| SO1 | C. glutamicum S9114 with deletion of argF, ncl2221, argR, putP, attenuation of adhA, proB, ncl2228, overexpression of lysE, gdh, argC/JBD | Lab stock |
| SO2 | SO1 carrying expression vector pEC-XK99E | This study |
| SO3 | SO1 carrying expression vector pEC-cg3035 | This study |
| SO4 | SO1 with P_{pta} promoter inserted in front of cg3035 | This study |
| SO5 | SO1 with terminator inserted in front of pta | This study |
| SO6 | SO1 with terminator inserted in front of cat | This study |
| SO7 | SO1 with P_{ehu} promoter inserted in front of pfkA | This study |
| SO8 | SO1 with P_{ehu} promoter inserted in front of gap | This study |
| SO9 | SO1 with P_{ehu} promoter inserted in front of pyk | This study |
| SO10 | SO1 with P_{ehu} promoter inserted in front of pfkA and P_{lac} promoter inserted in front of cg3035 | This study |
| SO11 | SO1 with P_{ehu} promoter inserted in front of pfkA, P_{lac} promoter inserted in front of cg3035 and a terminator inserted in front of pta | This study |
| SO12 | SO1 with P_{ehu} promoter inserted in front of pfkA, P_{lac} promoter inserted in front of cg3035 and a terminator inserted in front of cat | This study |
| SO13 | SO1 with P_{ehu} promoter inserted in front of pfkA, P_{lac} promoter inserted in front of cg3035 and terminator inserted in front of pta and cat | This study |
| SO14 | SO12 with an AAV degradation label inserted in the C terminal of pgi | This study |
| SO15 | SO14 with synthetic RBS and A1G replacement in front of zwf | This study |
| SO16 | SO15 with P_{lac} promoter inserted in front of tkt operon | This study |
| **Plasmid** |  |  |
| pK18mobsacB | Mobilizable vector, allows for selection of double crossover in C. glutamicum; Km^R, sacB | [16] |
| pEC-XK99E | A shuttle expression vector, Km^R | Lab stock |
| pEC-cg3035 | A derivative of pEC-XK99E, harboring cg3035 gene from C. glutamicum S9114 under its native promoter | This study |
| pK18-P_{lac}-cg3035 | A derivative of pK18mobsacB, harboring P_{lac}-cg3035 fragment | This study |
| pK18-T-pta | A derivative of pK18mobsacB, harboring T-pta fragment | This study |
| pK18-T-cat | A derivative of pK18mobsacB, harboring T-cat fragment | This study |
| pK18-P_{ehu}-pfkA | A derivative of pK18mobsacB, harboring P_{ehu}-pfkA fragment | This study |
| pK18-P_{ehu}-gap | A derivative of pK18mobsacB, harboring P_{ehu}-gap fragment | This study |
| pK18-P_{ehu}-pyk | A derivative of pK18mobsacB, harboring P_{ehu}-pyk fragment | This study |
| pK18- pgi-aav | A derivative of pK18mobsacB, harboring pgi-AAV fragment | This study |
| pK18-SB-zwf | A derivative of pK18mobsacB, harboring SB-zwf fragment | This study |
| pK18-P_{ehu}-tkt | A derivative of pK18mobsacB, harboring P_{ehu}-tkt fragment | This study |

Superscript “R” indicates resistance to the following antibiotics: Km kanamycin

into the expression vector pEC-XK99E by Gibson assembly to construct a constitutive expression cassette pEC-cg3035. After isolation from E. coli, this recombinant plasmid and empty vector pEC-XK99E were transferred into SO1 by electroporation. The positive transformants were pointed out and confirmed by colony PCR.

For P_{lac} promoter insertion in front of cg3035, the upstream region and the coding region of cg3035 was amplified. A strong P_{lac} promoter was introduced between the upstream region and coding region by primers. The overlapping fragment containing the upstream region, P_{lac} promoter, and the coding region was cloned into the Hind III/Xba I sites in the suicide vector pK18mobsacB by Gibson assembly. The engineered plasmid was then transferred into strain SO1 by electroporation. After double crossover recombination, the positive recombinant strain was detected by colony PCR. Similarly, the insertion of the P_{ehu} promoter in front of pfkA, gap and pyk or insertion of P_{lac} in front of tkt was also performed using these procedures.

To attenuate the expression of genes, strategy described in our previous study were carried out [18]. To attenuate the expression of pta and cat, a transcription terminator was introduced into the right upstream of the genes.
pta and cat which lines between the upstream fragment and downstream fragment by PCR. The recombinant fragment was then cloned into the Hind III/Xba I sites in the suicide vector pK18mobSacB by Gibson assembly. After extraction from E. coli, this engineered vector was transformed into SO1 by electroporation. After double crossover recombination, the mutant strains with a terminator inserted in the upstream region of pta and cat were determined by colony PCR. When constructing the mutant strains, an AAV taq was inserted in the C terminal of pgi with a synthetic RBS and G1A replacement in zwf. The RBS sequence with the predicted translation start strength of 50000 au was designed using the RBS Calculator (https://www.denovodna.com/software/doLogin) and is listed in Additional file 1: Table S2.

Fermentation in shake flasks
Fermentation with recombinant strains was performed in batch culture in shake flasks as described in our previous work [14, 15]. A single clone of the mutants was activated on LB agar plate for two cycles of 12 h. Subsequently, a ring of bacteria was inoculated into 10 mL of seed medium in a 100-mL normal shake flask. The seed medium consisted of (per liter) 25 g glucose, 10 g yeast extract, 10 g corn steep liquor, 2.5 g MgSO₄·7H₂O, 1 g KH₂PO₄, 0.5 g K₂HPO₄, 0.5 g Na₂HPO₄, and 10 g CaCO₃. After 11 h of cultivation at 32 °C and 220 rpm, the appropriate amount of culture was transferred to 24 mL of fermentation medium in a 32 °C and 220 rpm, the appropriate amount of culture, and then disrupted by sonication. After removing cell debris by centrifugation, the supernatant was collected as crude enzyme, and the protein concentration was determined by bicinchoninic acid (BCA) assay using bovine serum albumin as the standard. NAGS specific activity assay was performed as described previously [19].

RT-PCR
For RNA analysis, 500 µL of fermentation samples were collected at 12 h. RNA extraction and RT-PCR assays were performed as described in our early report [20].

Measurement of glucose consumption, optical density, and metabolite analysis
After dissolving CaCO₃ in 0.125 mol/L HCl, cell growth was monitored by measuring the OD₆₀₀ using a microplate reader (BioTek Instruments, Winooski, VT, USA). L-Ornithine concentrations were determined by colorimetry using ninhydrin, as described previously [21, 22]. L-Ornithine standard curve colorimetric assay was listed in Additional file 1: Figure S1. The fermentation supernatant was passed through a 0.22-µm filter and analyzed for glucose levels, using a SBA-40C biosensor (developed by Biology Institute of Shandong Academy of Sciences). Acetic acid were analyzed as described previously [23]. All experiments were conducted in triplicate; the data were averaged and presented as mean ± standard deviation (SD).

Results
Overexpression of cg3035 exerts a positive effect on L-ornithine production
Glutamate acetylation is the first step in the conversion of glutamate to L-ornithine and prevents glutamate from cyclisation and further conversion to L-proline. Petri et al. reported that glutamate acetylation is catalyzed by cg3035, which updated the previous knowledge that argF encodes the bifunctional enzyme for glutamate acetylation and N-acetylornithine deacetylation [19]. In a previous study, we constructed a high L-ornithine producing strain C. glutamicum strain SO1 (S9114 with deletion of argF, ngcl1221, argR, and putP, attenuation of odhA, proB, and ngcl2228, and overexpression of lyeE, gdh, and argCJBD) and found that argCJBD expression was not the rate-limiting step for L-ornithine accumulation. Therefore, glutamate acetylation prevented further improvement of L-ornithine accumulation. To overcome this barrier, cg3035 was overexpressed using an expression vector pEC-XK99E in the engineered strain SO1, thus generating strain SO3. Compared with strain SO1, NAGS enzyme activity in strain SO3 was improved by 5.63-fold, which suggested that cg3035 was successful overexpressed. The empty plasmid pEC-XK99E was also transformed into strain SO1 to produce strain SO2 as a control. To fully evaluate the performance of the
engineered strains SO2 and SO3, shake flask fermentation was performed. After 72 h of cultivation, the mutant strain SO3 produced 26.2 g/L of L-ornithine, which is 16.5% higher than that (22.5 g/L) obtained with strain SO2 (Fig. 2a). The cell growth of strain SO3 as well as its glucose consumption was comparable to that of the control strain SO2; indicating cg3035 overexpression is nontoxic to C. glutamicum (Fig. 2b). These results confirmed our speculation that glutamate acetylation plays an important role in L-ornithine biosynthesis. Inspired by the results of plasmid-based cg3035 overexpression, a strong mutant P_tac promoter developed by Jakob [24] was integrated into the chromosome of the parent strain SO1 to address the challenges associated with genetic instability using a vector, to generate strain SO4. This strategy was successfully applied for overexpressing lysE during our previous work. The relative mRNA level of cg3035 and NAGS enzyme activity in strain SO4 were 14.7-fold and 3.62-fold higher than that in the control strain, respectively, which illustrated that the expression of cg3035 was effectively strengthened through the insertion of P_tac promoter (Fig. 2c and Table 2). During 72 h of fermentation, the L-ornithine production titer of strain SO4 was measured to be 26.8 g/L, which was increased by 12.6% compared to that of the parent strain SO1 (23.8 g/L) (Fig. 2d). Similar to the result obtained by using a vector for cg3035 overexpression, the engineered strain SO4 exhibited coincident growth and glucose consumption as the control strain SO1 (Fig. 2e, f). In consequence, these results provide detailed knowledge of cg3035 expression to improve L-ornithine accumulation.

Improvement of L-ornithine production by attenuating the acetate synthesis pathway

Glutamate acetylation, a confirmed rate-limiting step for further improvement of L-ornithine production in engineered C. glutamicum SO1, is the first reaction in the L-ornithine pathway that uses acetyl-CoA as second sub-strate next to glutamate. We speculated that insufficient acetyl-CoA supplementation hindered the biosynthesis of L-ornithine in these strains. To spare acetyl-CoA for L-ornithine biosynthesis, we considered acetic acid biosynthesis that consumes acetyl-CoA and consists of two synthetic branches catalyzed by the products of pta and ack, or cat. Therefore, we attenuated these two acetate synthetic branches by insertion of a terminator, which was used to attenuate the expression of pfkA and pyk in previous work, in the upstream region of these genes, thus generating strains SO5 and SO6. Considering that pta and ack are listed as an operon, ack expression will also be attenuated by inserting a terminator in the upstream region of pta. The performance of these two strains was evaluated by shake flask fermentation. As shown in Fig. 3a, b, the expression of pta and ack in the mutant strain SO5 and the relative mRNA level of cat in the engineered strain SO6 was significantly reduced compared with that in the parental strain SO1. In addition, the yield of acetic acid produced by strain SO5 and SO6 were also reduced approximately 2.5-fold than strain SO1 (Table 2). This indicated that insertion of a terminator upstream of pta and cat was an efficient strategy to downregulate their expression. Strain SO5 and SO6 produced 25.1 and 26 g/L of L-ornithine, which was 5.5 and 9.1% higher than that of the control strain SO1 (23.8 g/L) (Fig. 3c). Results obtained per 12 h represent a significant improvement of L-ornithine synthesis compared with that of the control strain. Despite altered acetate biosynthesis, the engineered strains SO5 and SO6 grow robustly in the fermentation medium, maintaining a stable rate of glucose consumption (Fig. 3d). These results indicate that attenuating the expression of genes involved in acetate biosynthesis is a viable strategy for improving L-ornithine accumulation.

Effect of overexpressing enzymes of the glycolytic pathway on L-ornithine production

As previously demonstrated, plasmid-based overexpression of gap encoding NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, pfkA encoding 6-phosphofructokinase, and pyk encoding pyruvate kinase was able to improve L-ornithine production in the engineered C. glutamicum ATCC 13032 strain [22]. Therefore, to examine if enhancing the expression of these genes in strain SO1 could further improve L-ornithine production, we inserted a strong P_lys promoter into the upstream region of pfkA, gap, and pyk in strain SO1, to construct strains SO7, SO8, and SO9, respectively. After cultivation in the fermentation media for about 72 h, strains SO7, SO8, and SO9 produced 26.5, 22.8, and 21.5 g/L of L-ornithine (Fig. 4a). The yield of L-ornithine produced by strain SO7 with pfkA overexpression was 11.2% higher than that of the parental strain SO1. Strain SO8 with gap overexpression produced the same amount of L-ornithine as that of the parent strain SO1. The yield of L-ornithine produced by strain SO9 with P_lys inserted in front of pyk was decreased by 9.7% compared to strain SO1. The growth of strain SO8 and SO9 was slower than that of the engineered strains SO1 and SO7. Glucose consumption by strain SO7 was slightly faster than that by strains SO1, SO8, and SO9. Taken together, those results suggest that modulation of pfkA promotes L-ornithine accumulation in engineered C. glutamicum S9114.
Fig. 2 Improvement of L-ornithine production by cg3035 overexpression. a L-Ornithine production curves for strains SO2 (SO1 carrying pEC-XK99E) and SO3 (SO1 carrying pEC-cg3035). b Cell growth and glucose consumption of strains SO2 and SO3. c Relative transcription level of cg3035 in engineered strains SO1 and SO4 (SO1 with P_{tac} promoter inserted upstream of cg3035). d L-Ornithine production curves for the engineered strains SO1 and SO4. e Cell growth. f Glucose consumption. Samples were collected per 12 h for determining fermentation parameters. Results of standard deviations present in three individual experiments.
Combination of $P_{tac}$-cg3035, $P_{eftu}$-pfkA, T-pta, and T-cat targets and its influence on l-ornithine production

From the above results, several efforts including independently inserting a $P_{tac}$ in front of cg3035, inserting a $P_{eftu}$ in front of pfkA, and inserting a terminator in the upstream region of pta or cat were observed to enhance L-ornithine accumulation. Since our major goal is to construct a high L-ornithine producing strain, we combined these targets and constructed strain SO10 carrying $P_{tac}$-cg3035 and $P_{eftu}$-pfkA, SO11 with $P_{tac}$-cg3035, $P_{eftu}$-pfkA, and T-cat, SO12 with $P_{tac}$-cg3035, $P_{eftu}$-pfkA, and T-pta, and SO13 with $P_{tac}$-cg3035, $P_{eftu}$-pfkA, T-pta, and T-cat. Next, the effects of these modifications on L-ornithine accumulation were tested in the context of shake flask fermentation. As shown in Fig. 5a, mutant strains incorporating the modulation of these targets produced more L-ornithine compared to the parent strain SO1. The highest L-ornithine production titer in 72 h cultivation was produced by strain SO12 and reached 29 g/L, which was 21.8% higher than that obtained with strain SO1 (23.8 g/L). Unexpectedly,

### Table 2 Acetic acid concentration in 72 h fermentation broth

| Strains | Relative NAGS specific activity | Acetic acid concentration (g/L) |
|---------|--------------------------------|-------------------------------|
| SO1     | 1 ± 0.13                        | 3.85 ± 0.14                   |
| SO3     | 6.63 ± 0.07                     | –                             |
| SO4     | 4.62 ± 0.21                     | –                             |
| SO5     | –                               | 1.05 ± 0.05                   |
| SO6     | –                               | 1.34 ± 0.06                   |

![Fig. 3](image)

**Fig. 3** Blocking acetate biosynthesis by inserting a terminator into the upstream region of pta and cat, and its effect on L-ornithine production.

- **a** Relative pta and ack mRNA levels in SO1 and SO5 (SO1 carrying modulation in pta). Samples at the 12 h time point were subjected to transcript analysis during fermentation cultivation.
- **b** Relative cat mRNA levels in SO1 and SO6 (SO1 carrying modulation in cat).
- **c** L-Ornithine production by the engineered strains SO1, SO5, and SO6.
- **d** Cell growth and glucose consumption. Samples were collected per 12 h for determining the fermentation parameters. Results of standard deviations present in three individual experiments.
strain SO13, which possessed all of these modifications, exhibited a lower L-ornithine production performance and relatively slow glucose utilization efficiency compared to strain SO12 (Fig. 5b). In conclusion, these results illustrate that these targets exhibit a positive synergistic effect on improving L-ornithine production.

**Enhancement of L-ornithine production by redirecting the metabolic flux into pentose phosphate pathway**

L-Ornithine biosynthesis in *C. glutamicum* requires 2 mol of NADPH. Improving NADPH supplementation is an important factor for L-ornithine accumulation. Several strategies such as redirecting carbon flux to the pentose phosphate pathway, overexpression of
Clostridium acetobutylicum gapC, deletion of putative oxidoreductases, and so on for enhancing NADPH supplementation were confirmed to increase the production titer of L-ornithine. Nevertheless, as NADPH supply is frequently described as a limiting factor for L-ornithine biosynthesis and the engineered strain SO13 does not carry any genetic modification to enhance NADPH supply, the question of whether NADPH supply is limiting or not remains open. Therefore, we redirected the carbon flux to the pentose phosphate pathway through attenuation of pgI, overexpression of the tkt operon, and changing the RBS and translation start codon of zwf in the engineered strain SO12, to generate strains SO14 (SO12 with an AAV taq inserted in the C-steam of pgI), SO15 (SO14 with a strong P_tac promoter inserted in front of the tkt operon), and SO16 (SO15 with G1A and RBS replacement in zwf). The relative tkt and zwf mRNA levels in strain SO16 were approximately 1.5-fold higher than those in strain SO15, indicating that insertion of the P_tac promoter is able to improve the transcription level of the tkt operon (Fig. 6a). Fermentation in a shake flask revealed that strains SO14, SO15, and SO16 produced 30.38, 32.30, and 32.38 g/L L-ornithine with productivities of 0.359, 0.389, and 0.395 g/g glucose, respectively, which is 4.5, 11.4, and 11.4% higher than that of strain SO12 (29 g/L). Compared with strain SO12, SO14, and SO15, strain SO16 exhibited a slightly lower growth (Fig. 6c). Strain SO14 demonstrated relatively lower
glucose consumption compared to other strains (Fig. 6d). These results illustrate that improving the availability of NADPH is an inevitable modulation for L-ornithine accumulation in C. glutamicum.

Discussion
In this study, we demonstrate system-wide engineering and optimization of cellular metabolism in C. glutamicum SO1 to further improve strain performance with respect to L-ornithine biosynthesis. Depending on the current knowledge about L-ornithine biosynthesis pathway, cg3035 was overexpressed using a plasmid that significantly improved L-ornithine production. This confirmed our speculation that acetyl CoA acetylation is a rate-limiting step for L-ornithine accumulation after removing the feedback inhibition of ArgR on the argCBDF operon. In the incipient fermentation period, the improvement of L-ornithine production was not obvious probably due to slow glucose utilization rate. In addition, we induced genome-based cg3035 overexpression to ensure strain stability, which also promote L-ornithine accumulation. The improved percentage in ornithine production by genome-based cg3035 overexpression was lower than plasmid based cg3035 overexpression probably due to the not superior NAGS enzyme activity. We believe that this intriguing target could be extended to L-arginine and L-citrulline production.

Notably, N-acetylglutamate biosynthesis also requires acetyl-CoA as a precursor in addition to N-acetylglutamate synthase. Therefore, we attenuated the acetate biosynthesis pathway to reduce acetyl-CoA consumption and improved L-ornithine production titer, thus indicated that acetyl-CoA supplementation is an important factor for L-ornithine production, which further confirmed previous reports that deletion of dtstR1 encoding a subunit of acetyl-CoA carboxylase is beneficial for L-glutamate [25] and L-arginine accumulation [26]. This result is also consistent with previous work reported that block the acetate biosynthesis pathway promote the production of several compounds [27–30]. Attenuating acetate biosynthesis also conserves carbon flux into the glycolytic pathway, which may also account for the high L-ornithine production performance. This encouraged us to explore the glycolytic pathways. Overexpression of pkA by the insertion of a strong Peftu promoter tremendously promoted L-ornithine production, whereas insertion of a strong Peftu promoter in front of gap or pyk did not contribute to L-ornithine accumulation, which is in contrast with previous results [22]. The differences in genetic background between C. glutamicum ATCC 13032 and C. glutamicum S9114 may account for these diverse outcomes. There is a one percent gap in the genomes of those two strains. In addition, C. glutamicum S9114, a mutant strain derived from Brevibacterium tianjinense T6–13, processed a faster cell growth than C. glutamicum ATCC 13032. Modulations in pyk and gap slightly affected cell growth and glucose consumption in C. glutamicum S9114 probably acting as the main reason for the reduced L-ornithine yield. Based on the aforementioned results, Plac-cg3035, Peftu-pfka, T-pta, and T-cat were confirmed to independently increase L-ornithine accumulation. To further improve the yield of L-ornithine, these targets were assembled. The highest performance was obtained in strain SO12 with Plac-cg3035, Peftu-pfka, and T-pta, but not in strain SO13 with Plac-cg3035, Peftu-pfka, T-pta, and T-cat, which illustrated that attenuating pta and cat simultaneously can affect L-ornithine production by interfering with normal physiological metabolism in engineered strains.

Several previous works have demonstrated that NADPH availability is strongly correlated with L-ornithine production. In this work, L-ornithine production was improved by suppositional redirecting the carbon flux to the pentose phosphate pathway according to previous study [12], which further demonstrated the importance of NADPH supplementation in developing L-ornithine producing strains. This is consistent with previous work by Kim et al., where the pentose phosphate pathway was reinforced by replacing the native promoter of the tkt operon with a strong sod promoter and changing the start codons of zwf and pgI [12].

In summary, compared with previous studies shown in Table 3, this study provide more systematic and comprehensive steps in the construction of an engineered high L-ornithine producing C. glutamicum S9114 strain. Currently, the highest L-ornithine production titer (51.5 g/L) was reported by Kim et al. [12], which employ a bioreactor for fed-batch fermentation (Table 3). However, L-ornithine production yield per gram glucose via fed-batch fermentation (0.24 g/g) was lower than that of shake flask batch fermentation (0.524 g/g) developed by Jensen et al. [10]. In the following work, we will try more strategies such as releasing feedback inhibition of argB, which is probably the main reason why L-ornithine yield in this study was lower than 0.524 g/g glucose, and continue to modulate the metabolic pathway to further improve the production titer of L-ornithine. In addition, inspired by previous work [12], employ a fermentation bioreactor and change the fermentation mode to fed-batch cultivation was supposed to promote L-ornithine accumulation.

Conclusion
Redesigning and engineering strains for use in the industrial production of L-ornithine has significant potential application for reducing the economic cost pressures...
of using L-arginine for enzymatic reaction and extending the fermentation organism. C. glutamicum has been extensively studied for its ability to produce L-ornithine, though its yield and productivity are still low compared to other strategies. Very recently, we reported a C. glutamicum S9114 derived recombinant strain with high L-ornithine production titer. Here, we have further enhanced the L-ornithine yield through genome-based cg3035 overexpression and systematic manipulation of central metabolic pathways including glycolysis, acetate metabolism, and pentose phosphate pathway, consequently suggesting that improved L-ornithine production can be obtained by these modulations. C. glutamicum is an excellent producer for producing L-glutamate and L-glutamate-derived products including L-ornithine, L-citrulline, and L-arginine. We supposed that the metabolic engineering strategies reported in this work can be applied to constructing strains producing such products.

### Additional file

**Additional file 1: Table S1.** Primers and their sequences in this study. **Table S2.** Promoter and terminator sequence used in this study. **Figure S1.** L-Ornithine standard curve of colorimetric assay using ninhydrin.

### Authors’ contributions

BZ planned and conducted the experiments, analyzed and interpreted data, and wrote the manuscript; MY and W-PW participated in the research; B-CY supervised the research and finalized the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

### Availability of data and materials

Gene sequences used in this project are from Genbank (http://www.ncbi.nlm.nih.gov/) and the material and data supporting their findings can be found in the main paper and in additional file.

### Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

### Informed consent

Informed consent was obtained from all individual participants included in the study.

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### Table 3 Comparison of other C. glutamicum strains engineered for L-ornithine production

| Strains (C. glutamicum) | L-Ornithine titer (g/L) | L-Ornithine yield (g/g glucose) | Cultivation | Modulations | References |
|------------------------|-------------------------|-------------------------------|-------------|-------------|------------|
| SO16                   | 32.3                    | 0.395                         | Shake flask; batch | Deletion of argF, ncgl1221, argR, and putP; attenuation of odhA, proB, pta, cat and ncgl2228; overexpression of lysE, gdh, cg3035, pknA, pyk, tkt, and argCJD | This study |
| YW06 (pSY223)          | 51.5                    | 0.240                         | Bioreactor; fed-batch | Deletion of argF, argR, and proB; Reinforcement of the PPP pathway flux; The use of a feedback-resistant enzyme | [12] |
| ORN6                   | 20.96a                  | 0.524                         | Shake flask; batch | Deletion of argF, argR, and argG; overexpression of argB; attenuation of pgi. | [10] |
| SO16Δncgl2281Δncgl2582 | 14a                     | ND                            | Shake flask; batch | Deletion of argF, argR, and proB; Blocking gluconate biosynthesis | [11] |
| ΔAPE6937R42            | 24.1                    | 0.298                         | Bioreactor; batch | Deletion of argF, argR, and proB; Adaptive evolution in presence of L-ornithine | [13] |
| 1006ΔargR-argJ         | 31.6                    | 0.396                         | Shake flask; batch | Deletion of argR, overexpression of argJ. | [31] |

* These values were not described in the main text of the original reference and thus estimated from the figure or graph.
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