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REVIEW ARTICLE

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L-arginine production in *Corynebacterium glutamicum*: manipulation and optimization of the metabolic process

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ABSTRACT

As an important semi-essential amino acid, L-arginine is extensively used in the food and pharmaceutical fields. At present, L-arginine production depends on cost-effective, green, and sustainable microbial fermentation by using a renewable carbon source. To enhance its fermentative production, various metabolic engineering strategies have been employed, which provide valid paths for reducing the cost of L-arginine production. This review summarizes recent advances in molecular biology strategies for the optimization of L-arginine-producing strains, including manipulating the principal metabolic pathway, modulating the carbon metabolic pathway, improving the intracellular biosynthesis of cofactors and energy usage, manipulating the assimilation of ammonia, improving the transportation and membrane permeability, and performing biosensor-assisted high throughput screening, providing useful insight into the current state of L-arginine production.

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L-arginine; *Corynebacterium glutamicum*; molecular breeding; metabolic engineering; fermentation

Introduction

Amino acids are valuable organic acids, which constitute a multi-billion dollar market [1,2]. This number is expected to increase because of the high value of human health. L-arginine is a semi-essential amino acid that plays an important role in food, pharmaceutical, and industrial fields [3]. Owing to its beneficial function in the enhancement of wound healing, stimulation of hormone secretion, and nitric oxide formation, L-arginine can also be applied for the relaxation and dilation of blood vessels or the relief of hyperammonemia [4]. In industry, L-arginine can be used as a raw material for the enzymatic conversion of L-ornithine and L-citrulline by the exploitation of arginine deaminase [5–7]. Hence, it is necessary to develop appropriate methods for the large-scale production of L-arginine.

Microbial fermentation is a powerful method that is widely employed for the production of numerous bulk chemicals. Like with most other amino acids [8–10], commercial L-arginine production is dependent on microbial fermentation by using *Escherichia coli* or *Corynebacterium glutamicum* as the host strain. Owing to the advantages in cell growth and conversant genetic background, *E. coli* is widely used to produce valuable bulk chemicals, including amino acids [11], alcohols [12], pigments [13], amines [14], organic acids [15], vitamins [16], grease [17], and others [18,19]. Currently, it is feasible to engineer *E. coli* for the production of L-arginine by inactivation of arginine repressor and ornithine decarboxylases and overexpression of the feedback-resistant *argA214* or *argA215* [20]. However, the low L-arginine production titer (only 11.64 g/L) [20] and synthesized endotoxin [21] limit the application of *E. coli* as an industrial strain for the production of L-arginine.

C. glutamicum is a promising microbial host that can be intensively engineered to utilize multitudinous substrates to produce valuable bulk chemicals [22–24]. Recent progress in the development of gene editing tools, such as CRISPRi [25], RNAi [26], and CRISPR-Cpf1 [27,28], has enabled the fast development of *C. glutamicum* through metabolic engineering [29]. As listed in Table 1, numerous *C. glutamicum* strains are capable of secreting L-arginine. In particular, *Corynebacterium crenatum*, a subspecies of *C. glutamicum*, shows 99% genomic homology to model *C. glutamicum* ATCC 13032

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Table 1. Microorganisms for L-arginine production developed by metabolic engineering.

Strains	L-ornithine titer (g/L)	yield (g/g substrate)	Cultivation	Modulations	Reference
E. coli					
SJB009	11.64	0.17	Batch; Shake flask	Deletion of *adiA*, *speC*, *speF*, *argR*, *argA*, introducing high gene copy number of *argA214* and *argO*	[20]
C. glutamicum					
AR6	92.5	0.40	Fed-batch; Bioreactor	Random mutagenesis; deletion of *argR*, *farR*, and *ncgl1221*; attenuation of *pgi*; overexpression of *tkt* operon, *argGH*, and *carAB*	[30]
ARG2Δ*cgmR*	7.14*	ND	Batch; Shake flask	Deletion of *cgmR*	[31]
JML07	67.01	0.35	Fed-batch; Bioreactor	Deletion of *argR*, *farR*, and *ldh*; overexpression of *pntAB* and *ppnk*	[32]
C. crenatum					
Cc6	87.30	0.43	Fed-batch; Bioreactor	Deletion of *proB*; attenuation of *odhA*, *lysC*, *pgi*; overexpression of *iolT1*, *ptsG*, *ppgk*, *pyc*, *gltA*, *gdh*, *argCJBDF*, *argGH*	[33]
ARG (*glnA–aspA–gdh*)	53.20	0.32	Fed-batch; Bioreactor	Overexpression of *glnA*, *aspA*, *gdh*	[34]
5-5Δ*frd12* Δ*nox*Δ*amn*(*pyk-pgk*)	57.30	0.33	Fed-batch; Bioreactor	Deletion of *frd12*, *nox*, and *amn*; overexpression of *pyk* and *pgk*	[35]
SYPA/pJCE	35.91	ND	Batch; Bioreactor	Overexpression of *lysE*	[36]
Cc–amtB2	60.90	0.36	Fed-batch; Bioreactor	Deletion of *amtR*; overexpression of *amtB2*	[37]
SYPA-EH3	61.20	0.43*	Batch; Bioreactor	E19Y/I74V/F91H/K234T mutation in CcNAGK	[38]
CCM07	24.85	0.33	Batch; Shake flask	Deletion of *putP*, *pta*, *cg12310* and *ncgl1221*; overexpression of *lysE*	[39]
Cg/pXMJ19-*glnK*	49.98	0.34*	Batch; Bioreactor	Overexpression of *glnK*	[40]
MT-M4 Δ*proB*	16.50	0.39	Batch; Shake flask	Mutation of CcNAGK; deletion of *proB*	[41]
P2	41.11	0.24	Batch; Bioreactor	Overexpression of *phbCAB* operon and *ppnK*	[42]
H-7-GH	67.92	0.34	Fed-batch; Bioreactor	Mutation of CcNAGK; overexpression of *argGH*	[43]
ARG 3-15	45.36	0.32	Batch; Bioreactor	Atmospheric and room temperature plasma (ARTP)	[44]
CMB03	29.97	0.34	Batch; Shake flask	Deletion of *dtsR1* and *ncgl1221*; addition of tween 40	[45]
SYPA/pJCE	35.91	ND	Batch; Bioreactor	Overexpression of *lysE*	[36]
SYPA-CCB_{M3} (pJC*tac-argJ*)	45.60 42.40	0.30 0.29	Batch; Bioreactor Batch; Bioreactor	Overexpression of Mutated CcNAGK Overexpression of *argJ*	[46] [47]

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

and has been isolated from soil. Thus, *C. glutamicum* has been extensively engineered for the production of L-arginine. In this review, we have summarized the recent advances in the development of high L-arginine-producing *C. glutamicum* and provide systematic modifications in metabolic pathways that have been demonstrated for the improvement of L-arginine production.

Main biosynthesis pathways of L-arginine

Three main biosynthesis pathways in microorganisms are employed for the biosynthesis of L-arginine, which requires L-glutamate as a substrate that is catalyzed by different enzymes (Figure 1) [48]. According to the difference in acetyl groups involved, the biosynthesis pathways of L-arginine can be divided as follows: firstly, the linear pathway (pathway I) (Figure 1(A)). In microorganisms, such as *E. coli*, L-glutamate is converted into L-ornithine, L-citrulline, and L-arginine by the catalysis *via* enzymes encoded by *argA*, *argB*, *argC*, *argD*, *argE*, *argF*, *argG*, and *argH*. Among them, *N*-acetylglutamate synthetase (NAGS), encoded by *argA*, is the key rate-limiting enzyme that is feedback inhibited by the final product L-arginine. Second is the economic circulation pathway (pathway II) (Figure 1(B)). This pathway mainly exists in microorganisms including *C. glutamicum*, *Corynebacterium crenatum*, *Bacillus thermophilus*, and *Saccharomyces cerevisiae*, in which the biosynthesis of L-arginine occurs through the urea cycle. The genes encoding the enzymes involved in pathway II are listed as two operons, *argCJBDFR* and *argGH*, which are feedback repressed by ArgR and FarR [49,50]. In addition, the generation of 1 mole of L-arginine requires 2 moles of NADPH through two enzyme reactions including *N*-acetyl-gamma-glutamyl-phosphate reductase and L-glutamate dehydrogenase, which converts *N*-acetyl-γ-glutamyl-phosphate to *N*-acetylglutamate semialdehyde and α-oxoglutarate to glutamate, respectively.

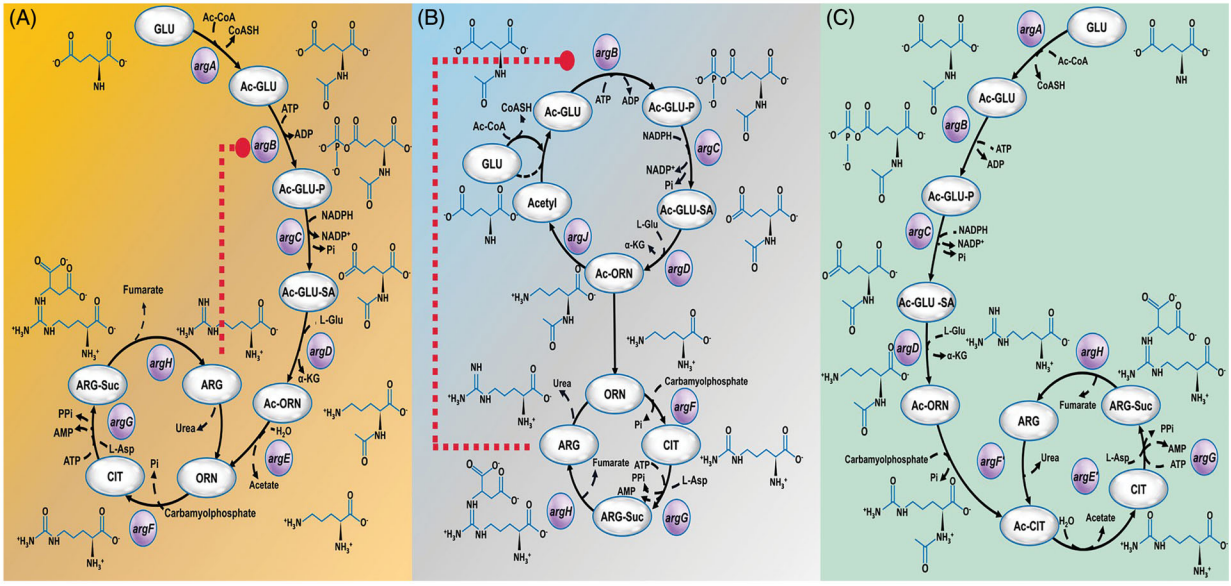

Figure 1. The biosynthesis pathways of L-arginine in microorganisms. (A) Linear pathway (pathway I); (B) Economic circulation pathway (pathway II); (C) Newly discovered special pathway (pathway III). *argA*: encoding N-acetylglutamate synthase; *argB*: encoding N-acetylglutamate kinase; *argC*: encoding N-acetylglutamate semialdehyde dehydrogenase; *argD*: encoding N-acetylornithine transaminase; *argE*: encoding N-acetylornithine deacetylase; *argJ*: encoding ornithine acetyltransferase (a bifunctional enzyme); *argF*: encoding ornithine transcarbamylase; *argG*: encoding argininosuccinate synthase; *argH*: encoding arginosuccinase; *argF'*: encoding N-acetylornithine carbamoyltransferase; *argE'*: encoding N-acetylcitrulline deacetylase; Glu: glutamate; Ac-Glu: N-acetylglutamate; Ac-Glu-P: N-acetylglutamal-phosphate; Ac-Glu-SA: N-acetylglutamal-semialdehyde; Ac-ORN: N-acetylornithine; Ac-CIT: N-acetylcitrulline; ORN: ornithine; CIT: citrulline; ARG-Suc: argininosuccinate; ARG: arginine.

Moreover, compared with pathway I, pathway II has introduced ornithine acetyltransferase (Ornithine Acetyltransferase, OAT), which can not only catalyze the conversion of *N*-acetylornithine to L-ornithine but is also able to convert L-glutamate to *N*-acetylglutamate. OAT functions as an isoenzyme that exerts NAGS and *N*-acetylornithinase activity though NAGS (encoded by *cg3035*) in *C. glutamicum*. *N*-acetylornithine provides an acetyl group for the acetylation of L-glutamate. In this pathway, *N*-acetylglutamate kinase (NAGK), encoded by *argB*, is the key rate-limiting enzyme inhibited by the final product L-arginine. Third is the newly discovered special pathway (pathway III) (Figure 1(C)). This special metabolic pathway for the biosynthesis of L-arginine was found in *Xanthomonas* sp. In pathway III, *N*-acetylornithine is converted into *N*-acetylcitrulline through the enzymatic activity of *N*-acetylornithine carbamyltransferase (encoded by *argF*), and *N*-acetylcitrulline is then directly converted to L-citrulline *via* biocatalysis by *N*-acetylcitrulline deacetylase (encoded by *argE*) [51].

Manipulating the principal metabolic pathway of L-arginine biosynthesis

For metabolic engineering of microorganisms, manipulation of the principal metabolic pathway is necessary and is the most important strategy for the overproduction of valuable target compounds [52,53]. To overproduce L-arginine, deletion of ArgR and FarR was initially employed in *C. glutamicum* or *C. crenatum* to remove the feedback repression of those genes on the *argCJBDFR* and *argGH* operon, involved in the principal pathway of L-arginine biosynthesis (Figure 2). In general, inactivation of *argR* and *farR*, which stimulates the expression of the *argCJBDFR* and *argGH* operon, is a powerful strategy for enhancing the L-arginine production titer. For instance, deletion of *argR* and *farR* in the *C. glutamicum* JML00 strain improved the yield of L-arginine (from 41.5 to 51.96 g/L) and the yield of glucose (from 0.19 to 0.30 g/g glucose) [32]. In addition, fed-batch cultivation of *C. glutamicum* AR2 with double deletion of *argR* and *farR* produced 61.9 g/L of L-arginine, which entails a 28 g/L improvement when compared to the parent strain *C. glutamicum* AR1 [37]. Furthermore, the genes involved in the biosynthesis are tightly controlled by ArgR in *C. crenatum*, whereas inactivation of ArgR can significantly enhance the production titer of L-arginine [54,55]. Therefore, it can be concluded that the expression of the *argCJBDFR* and *argGH* operon is a major limitation for the production of L-arginine, and this notion was further confirmed by plasmid-based homologous overexpression of the *argC*$\sim$*H* cluster in *C. crenatum*, which improved the L-arginine production titer by 24.9% [56].

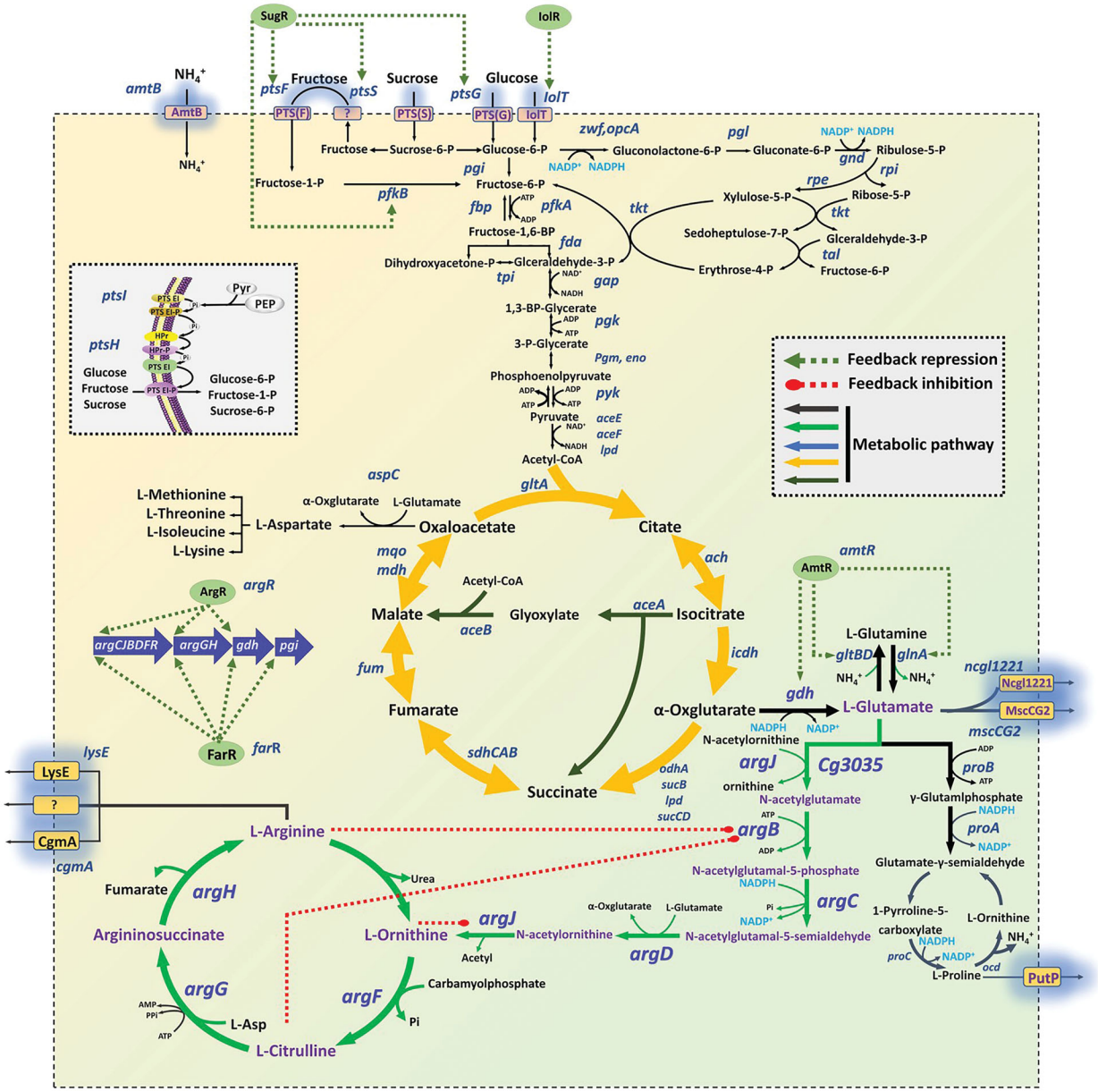

Figure 2. Biosynthesis pathways and regulation mechanism of L-arginine in *Corynebacterium glutamicum*. These gene annotations are listed in the support materials.

As described above, double deletion of transcription factors *argR* and *farR* stimulates the expression of arginine operons and improves the L-arginine production titer. However, due to the existence of another tightly controlled mechanism, the enzymatic activity of *N*-acetylglutamate kinase is inhibited by less than 1 mM of L-arginine in *Corynebacterium* strains, extremely limiting progress in the development of high L-arginine-producing strains (Figure 2). To overcome this obstacle, various site-directed mutagenesis approaches have been extensively evaluated in *N*-acetylglutamate kinase in an attempt to relieve its sensitivity to L-arginine [57]. Firstly, heterologous expression of *argB* from *E. coli*, natively insensitive to L-arginine, results in a threefold increased production of L-arginine. Introducing mutations of *argB26* and *argB31* causes a dramatic increase in L-arginine production in the mutant strain *C. glutamicum* RB [58]. In addition, desensitization of NAGK was further investigated by the site-directed mutagenesis in *argB*, and thus a wide range of *argB* mutations promoting the development of high L-arginine-producing strains, including $argB^{E19R,\ H26E,\ H268N}$ [46,59], $argB^{A49V,\ M54V,\ H268N}$ [60], $argB^{K47H,\ V65E}$ [61], $argB^{E19R,\ H26E,\ D311R,\ D312R}$ [41], $argB^{E19Y,\ I74V,\ F91H,\ K234T}$ [38], and $argB^{W23R,\ R209W}$ [62], were discovered. Among them, $argB^{E19Y,\ I74V,\ F91H,\ K234T}$ exhibits favorable specific activity and

thermostability that improved the yield of L-arginine from 43.1 to 61.2 g/L in engineered *C. crenatum*. Moreover, this feedback inhibition process is also regulated by CgGlnK, a PII signal transduction protein, which interacts with NAGK. Overexpression of CgGlnK in engineered *C. crenatum* resulted in a 1.4-fold increase in the L-arginine semi-inhibition constant of NAGK and a 22.61% increase in the yield of L-arginine [40].

Improving the supplementation of precursor by optimizing metabolic pathways and blocking the precursor secretion system

As shown in Figure 2, L-glutamate has been identified as the direct precursor in the biosynthesis of L-arginine. However, L-glutamate is also converted to L-proline *via* a multistep enzymatic reaction [63] (Figure 2). The biosynthesis of L-proline is in competition with L-arginine production by occupation of carbon sources and redox NADPH cofactor [64]. Therefore, blocking the L-proline biosynthesis pathway *via* disruption of the *proB* (encodes γ-glutamyl kinase) gene is commonly utilized to develop high L-arginine-producing strains. In practice, deletion of *proB* exerts a positive effect on L-arginine production in several strains including *C. crenatum* MT-M4 [41] and *C. crenatum* Cc5*lysC*-30 [33]. However, cell growth is significantly affected by disruption of the L-proline biosynthetic pathway, which requires the addition of L-proline in the fermentation medium that increases the complexity and operational cost. Instead, a recent study suggested that deletion of L-proline transport protein PutP in *C. crenatum* MT resulted in secretion of negligible amounts of L-proline and a 14.8% increase in L-arginine production without any influence on cell growth [39]. Attenuating the L-proline biosynthetic pathway by disrupting the L-proline transporter seems to be more suitable for the development of high L-arginine producing strains that cannot only avoid the negative effect on growth caused by L-proline deficiency but also saves precursor for L-arginine production. Inspired by the progress on inactivation of the L-proline transporter, the biosynthesis pathways of other amino acids, such as valine, isoleucine, and leucine, were also attenuated by removal of the branched amino acid transporter Cgl2310/Ncgl2228, which resulted in a 27% (from 12.2 to 15.6 g/L) increase in L-arginine production in *C. crenatum* MT [39]. These results were consistent with L-ornithine production in *C. glutamicum* orn8 [65]. It is obvious that deletion of the corresponding transporter could be identified as the first choice for reducing the secretion of byproducts in fermentation broth, which provides more carbon sources and cofactors for the biosynthesis of valuable target compounds.

In addition to the disruption of byproducts metabolic pathways, optimizing the biosynthesis pathway of the precursor L-glutamate is another powerful strategy for enhancing L-arginine production. Overexpression of the endogenous glutamate dehydrogenase, blocking the glutamate secretion system, and attenuation of α-ketoglutarate dehydrogenase have been performed in engineered *C. glutamicum* strains and have significantly improved the L-arginine production titer. For instance, overexpression of *icd* and *gdh*, as well as attenuation of *odhA*, in the *C. crenatum* Cc4 strain increased L-arginine production from 68.6 g/L to 76.8 g/L [33]. In addition, extracellular secretion of glutamate is unfavorable for the biosynthesis of downstream products. Thus, deletion of *ncgl1221* (encodes a glutamate transporter) has been explored in L-arginine-producing *C. glutamicum*, which remarkably improved the L-arginine production titer [66]. Therefore, improving glutamate availability is a powerful strategy for L-arginine overproduction in *C. glutamicum*.

Modulation in carbon uptake system

Currently, L-arginine production is mainly dependent on microbial fermentation by using glucose as substrate. It can, thus, be reasoned that enhancing the glucose uptake system is critical for developing robust L-arginine-producing strains (Figure 2). In *C. glutamicum*, deletion of *iolR*, a regulator involved in myo-inositol uptake and degradation, removed the repression of *iolT1* and has been widely applied to improve the glucose uptake for the production of various products including L-serine [67], xylonic acid [68], cis, and cis-muconic acid [69]. Unfortunately, previous studies attempted to improve glucose uptake through the replacement of the native promoters of the *iolT1* and *ppgk* gene with the *sod* promoter that slightly reduced the L-arginine production titer in the *C. crenatum* Cc2 strain [33]. This unexpected result suggests that obstruction of metabolic pathways other than the glucose uptake system may be more critical for L-arginine production. It is obvious that the central metabolic pathways, including the glycolytic pathway and Krebs cycle pathway, play an important role in providing the carbon skeleton for the biosynthesis of L-arginine. Thus, appropriate modulation in central metabolic pathways is necessary for redirecting metabolic flux to L-arginine metabolism. To channel carbon metabolic flux into the Krebs cycle pathway, overexpression of pyruvate carboxylase and citrate synthase, through substitution of the *pyc* native

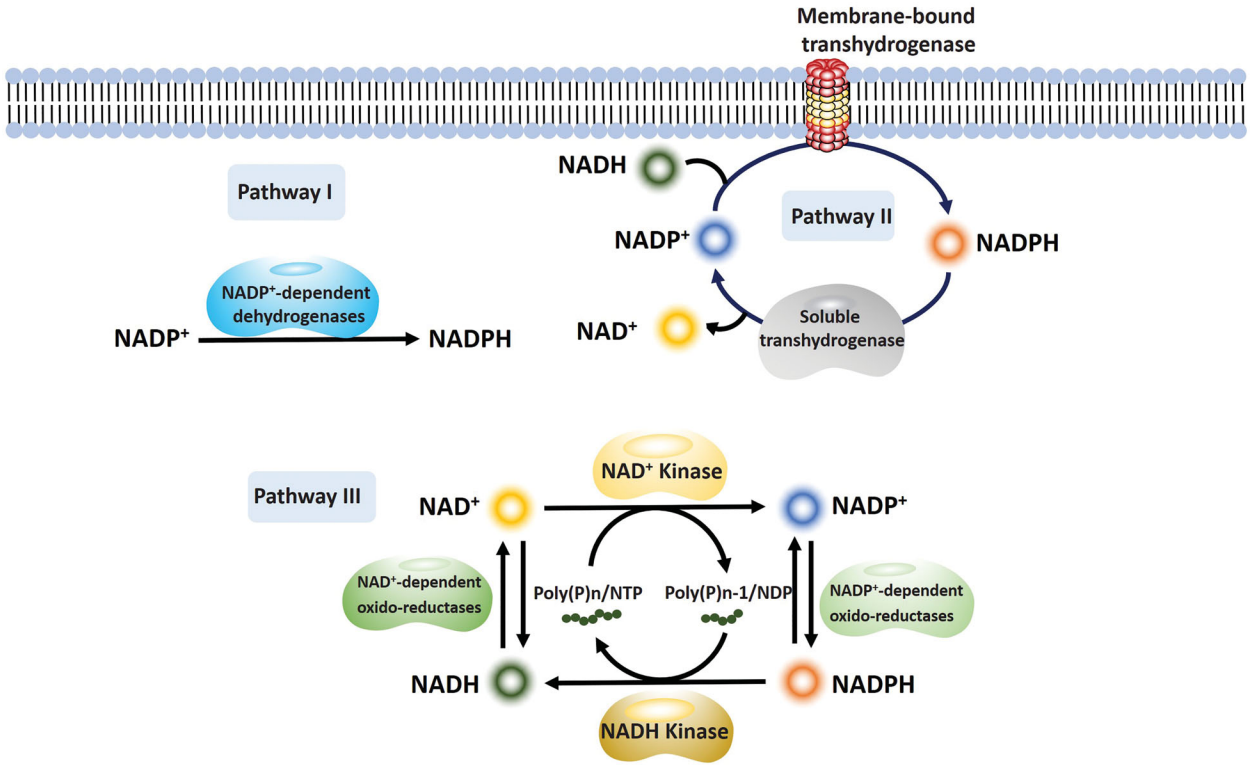

Figure 3. Three pathways to regenerate cofactor NADPH in *Corynebacterium glutamicum*. Pathway I. NAD kinase-based circulation pathway of NADH and NADPH. Pathway II. Membrane-bound transhydrogenase-based phosphorylation pathway. Pathway III. Dehydrogenases coupled biosynthesis pathway.

translation start codon GTG with ATG and the implementation of an additional copy of *gltA* on the chromosome, was achieved in the engineered *C. crenatum* Cc3 strain. This approach improved the L-arginine production titer by 12.5% [33]. This result was further confirmed in a recent study suggesting that overexpression of *glt* improved L-ornithine production in the *C. glutamicum* SO16 strain [70].

Enhancement of the co-factor regenerating pathway and energy status

The biosynthesis of valuable chemicals is an anabolic process that consumes cofactor to provide reducing power [71]. Efficient supplementation of NADPH, a widely used cofactor, significantly stimulates the biosynthesis of numerous amino acids, including L-lysine [72], L-ornithine [73], L-methionine [74], L-valine [75], L-arginine [45], and L-isoleucine [76]. Consequently, NADPH is frequently identified as a rate-limiting factor for the production of valuable compounds in *C. glutamicum*. To overcome this limitation, a variety of strategies, including improving the metabolic flux of the pentose phosphate pathway [77], heterologous expression of a NADP-dependent glyceraldehyde 3-phosphate dehydrogenase [78–80], and overexpression of NAD kinase [42,81], have been explored to improve the intracellular supplementation of NADPH. For the production of L-arginine using *C. glutamicum*, two enzymatic reactions require NADPH for providing reductive power. Redirection of the carbon metabolic flux to the pentose phosphate pathway by substitution of a translation start codon and native promoter in the *C. glutamicum* AR3 strain increased the yield of L-arginine from 61.9 to 80.2 g/L [30]. This result was further confirmed by later research suggesting that downregulation of the expression level of *pgi* by ribosome binding site (RBS) substitution in *C. crenatum* Cc2-6500 improved the yield of L-arginine from 53.2 to 66.4 g/L [33]. Additionally, co-expression of *pntAB* (membrane-bound transhydrogenase) and *ppnK* (NAD^+ kinase) in the *C. glutamicum* JML03 strain increased the intracellular NADPH concentration by 53.8% (from 182 ± 17 to 280 ± 41 μmol/g DCW) and increased the L-arginine production titer from 41.5 to 61 g/L [32]. Therefore, it is feasible to develop high L-arginine-producing *C. glutamicum* strains by increasing the intracellular NADPH supply.

In addition to the pentose phosphate pathway, there are two other metabolic pathways related to NADPH regeneration (Figure 3). Among them, NAD^+ kinase (PpnK) catalyzes the transformation of NAD^+ to $NADP^+$, consuming ATP and poly(P), indicating that the

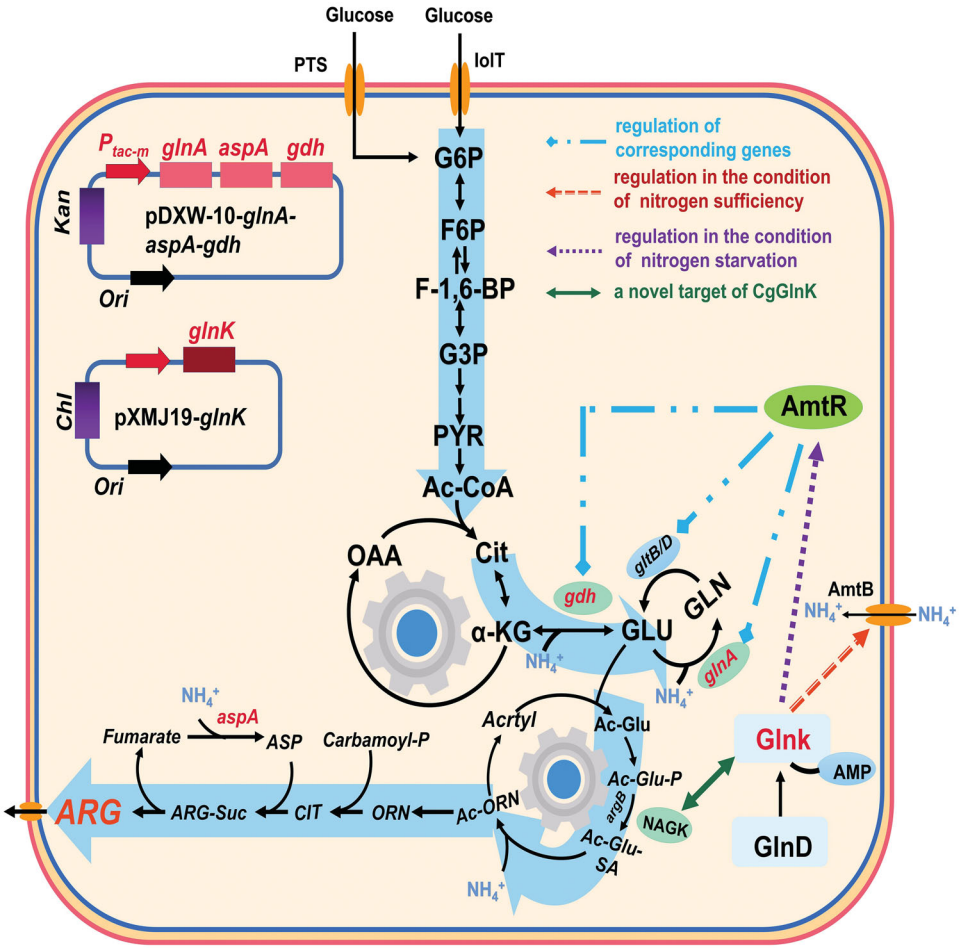

Figure 4. Schematic representation of ammonia metabolic pathway in *Corynebacterium glutamicum* and modulation strategies preformed to improve L-arginine production by enhancing the assimilation of ammonia. G6P: glucose-6- phosphate; F6P: Fructose-6-phosphate; F1,6BP: Fructose-1,6-biphosphate; G3P: glyceraldehyde-3-phosphate; PYR: pyruvate; Ac-CoA: acetyl-CoA; OAA: oxaloacetate; Cit: citrate; α-KG: α- oxoglutarate; GLU: glutamate; GLN: glutamine; Ac-Glu: N- Acetyl-glutamate; Ac-Glu-P: N-Acetyl-glutamate phosphate; Ac-Glu-SA: N-acetylglutamal-5-semialdehyde; Ac-ORN: N-acetylornithine; ORN: ornithine; CIT: citrulline; ARG-Suc: Argininosuccinate; ARG: arginine. ASP: aspartate. These gene annotations are listed in the support materials.

intracellular ATP concentration is a crucial factor for the biosynthesis of L-arginine. However, intracellular ATP concentration is highly related to the active oxygen species (ROS) level, which is coupled to the electron transport chain. Previous studies have tested the feasibility of improving oxygen supply for the biosynthesis of the L-arginine by plasmid-based overexpression of *Vitreoscilla* hemoglobin in *C. crenatum* SYPA 5-5 strain. As compared to the wild-type strain, this recombinant *C. crenatum* exhibited a 38.8% improvement in oxygen uptake rates, which resulted in 17.3% and 10.5% increases in L-arginine concentration and biomass, respectively [82]. Subsequently, inactivation of two flavin reductases, Frd181 and Frd188, which reduce the formation of H_2O_2 and the intracellular reactive oxygen species (ROS) level, was introduced in *C. crenatum* to increase intracellular NADH and ATP levels. In combination with the deletion of *noxA* and *amn*, and the overexpression of *pgk* and *pyk* resulted in the *C. crenatum* 5-5Δ*frd12*Δ*nox*Δ*amn* (*pyk-pgk*) strain producing 57.3 g/L of L-arginine, which is 49.2% higher than the amounts obtained from the parent strain [35]. These published data illustrated that intracellular energy status also has an important role in the biosynthesis of L-arginine by using *C. glutamicum*.

Modulation in the ammonia uptake system

In addition to the carbon skeleton, one L-arginine molecule also contains four nitrogen atoms, which is assembled through metabolic pathways that convert 2-oxoglutarate to L-glutamate, acetylglutamate semialdehyde to acetylornithine, L-ornithine to L-citrulline, and L-citrulline to argininosuccinate (Figure 4). Among them, the last two reactions, catalyzed by ornithine carbamoyltransferase (encoded by *argF*) and

arginosuccinate synthetase (encoded by *argG*), as well as the biosynthesis of carbamyl phosphate, frequently hampered the biosynthesis of L-arginine. In practice, this limitation has been solved by replacing the native promoter of the *carAB* and *argGH* genes with strong promoters P_{sod} and P_{eftu}, respectively, improving the yield of L-arginine by 29% [30]. Further, to enhance the intracellular ammonium assimilation, addition of L-glutamine and L-aspartate to the fermentation medium were employed and promoted the biosynthesis of L-arginine in the *C. crenatum* SDNN403 (ARG) strain. Inspired by the result of nitrogen atom donor addition, rational combination of numerous modulation strategies including reinforcing the intracellular supplementation of L-glutamine and L-aspartate synthesis by heterologous overexpression of glutamine synthetase gene *glnA* and aspartase gene *aspA* from *E. coli* and co-overexpression of the endogenous glutamate dehydrogenase gene *gdh* have improved the L-arginine production titer by 41.5% (from 37.6 to 53.2 g/L) [34] (Figure 4). Furthermore, a recent study focused on upregulating intracellular nitrogen metabolism to provide more nitrogen atoms for L-arginine production in *C. crenatum*. Deletion of *amtR*, coding for a nitrogen metabolism global transcriptional regulator, improved the assimilation of NH_4^+ by upregulation of the enzyme activity of glutamate dehydrogenase and glutamine synthetase, which resulted in a 16.67% increase in L-arginine production. Comparative transcriptional level analysis between *C. crenatum* Cc–Δ*amtR* and *C. crenatum* Cc5-5 suggested that the expression of *amtB* was increased 31.2-fold, while expression of *glnK* and *glnD* increased two-fold, respectively. Next, overexpression of *amtB*, an ammonium transporter, by insertion of a new copy of P_{tacM}-*amtB* into the *amtR* locus not only accelerated NH_4^+ uptake but also resulted in a 37.7% increase in L-arginine production (from 26.5 to 36.5 g/L) [37]. Therefore, rebalancing of carbon and nitrogen fluxes is a promising and useful strategy for the development of high L-arginine producing strains.

Improving the export of L-arginine by modulating membrane permeability and the specific transportation system

Modulation of membrane permeability is a critical factor for the overproduction of valuable compounds. There are two common strategies, namely overexpression of specific transporters and changes in the structure of the cell membrane or cell wall, which can be performed to improve membrane permeability for developing robust microbial cell factories. For the secretion of amino acids in *C. glutamicum*, numerous specific transport proteins, including glutamate transporters Ncgl1221 [83,84] and MscCG2 [85], phenylalanine transporter (AroP) [86], lysine transporter (LysE) [87,88], and branched chain amino acid transporter (BrnFE) [89,90], were discovered and targeted for increasing production titers. Results from dipeptide feeding experiments have indicated that although LysE has been identified as a lysine transporter, it can also transport other basic amino acids such as L-arginine, L-citrulline, and L-ornithine [31,65,91]. Transcriptional cross-regulation analysis demonstrated that *C. glutamicum* LysE is orthologous to the *E. coli* L-arginine transporter ArgO [92]. Tracking the intracellular and extracellular L-arginine concentration in the minimum culture CGXII suggested that LysE can also serve as an L-arginine transporter [91]. Hence, plasmid-based overexpression of LysE in the L-arginine producing *C. crenatum* SYPA strain resulted in a 13.6% increase in the yield of L-arginine [36]. Besides LysE, the permease CgmA was shown to compensate for the lack of LysE with regard to L-arginine transportation. Deletion of *cgmR*, which activated the expression of *cgmA*, resulted in a 5% improvement in L-arginine production titers, indicating that CgmA may function as another export system for L-arginine [31]. Moreover, optimization of the structure of the cell membrane or cell wall has been applied to achieve improved production of L-arginine through the addition of surfactant. In one example, the effect of adding Tween 40, a fermentation trigger, on L-arginine production was investigated in an engineered *C. crenatum* CCM01 strain. By optimizing the addition time and concentration of Tween 40, the yield of L-arginine increased by 16.5% when compared to the controls. Transcriptional level analysis indicated that addition of Tween 40 can also improve the precursor glutamate availability by downregulating the expression of *dtsR1*, *odhA*, *pknG*, and *sucB* for the biosynthesis of L-arginine. Furthermore, remarkable improvement in the yield of L-arginine was achieved by the deletion of DtsR1, which is probably related to upregulated biosynthesis of NADPH, channeled metabolic flux to glutamate, and changes in membrane permeability [45]. Therefore, it can be concluded that rational modulation of the transportation and membrane permeability is an effective approach for promoting L-arginine production.

Biosensor-assisted irrational optimization of the L-arginine metabolic process

The previously discussed breeding strategies were based on rational genetic modulations. However, direct

manipulation of the genome is limited and is likely to break the original balance of cell metabolism, which would negatively affect cell growth. Traditionally, despite the enormous amount of work involved in screening, irrational strain breeding methods, such as induced mutation and adaptive laboratory evolution [93], were proven to be feasible methods that have provided extensive genetic manipulation targets for improving the performance of industrial microorganisms. Numerous high-throughput screening (HTS) approaches have been established to overcome the drawbacks in screening effort driven by random mutagenesis methods [94]. For the construction of high-throughput screening methods, a wide range of biosensors have been extensively studied and characterized. These biosensors recognize a variety of target compounds, including 3-hydroxypropionic acid [95], glutarate [96], itaconic acid [97], flavonoids [98,99], lactam [100], amino acids [101,102], acrylic acid [103], salicylate [104], isoprene [105], and shikimate [106]. Thus, an L-arginine biosensor has been established based on the theory that translation of rare codons cannot be effectively processed in amino acid starvation conditions. By inserting several L-arginine rare codons into the kanamycin resistance gene *kan*, the growth of *C. glutamicum* harboring modified *kan* gene was coupled with the yield of L-arginine, while the mutant strain with high yield of L-arginine could be selected from random mutant libraries by increasing kanamycin dosage and screening vigorous strains. Consequently, a mutated *C. glutamicum* CGL-4 strain was obtained and indicated a 3.7-fold increase in the yield of L-arginine (2.742 mg/g (biomass)) compared with the parent strain *C. glutamicum* ATCC13032 [107]. Although there is still space for advancement in the dynamic range, this biosensor assistant strategy has stimulated the development of high-throughput screening of L-arginine-producing strains. Recently, a more practical L-arginine biosensor named "ARG-Select" has been constructed based on the regulation mechanism of ArgR, in which the expression of the reporter gene in the downstream region of *argC* promoter was repressed by ArgR in response to high L-arginine concentration. As a result, mutant strain *C. crenatum* HArg1 and DArg36 were obtained by integrated usage of atmospheric room temperature plasma (ARTP) mutagenesis and *SacB*-based "ARG-Select" that produced 56.7 and 95.5 g/L of L-arginine, respectively, during fed-batch fermentation [108].

Conclusions and perspectives

In this review, we discussed several useful strategies that promote metabolic engineering of L-arginine-producing strains. These strategies encompass manipulation of the principal metabolic pathway, modulation of the carbon metabolic pathway, improving the intracellular biosynthesis of cofactor and energy status, manipulating the assimilation of ammonia, improving the transportation and membrane permeability, and biosensor-assisted high-throughput screening. Developing an engineered L-arginine-producing strain will require the combination of the aforementioned strategies to systematically manipulate L-arginine metabolism. For instance, Park et al. [30] reported the development of metabolically engineered *C. glutamicum* ATCC 21831 for overproduction of L-arginine by random mutagenesis, deletion of L-arginine regulatory repressors, optimization of NADPH level, overexpression of *carAB* and *argGH* operons, and inactivation of L-glutamate exporter. Fed-batch fermentation of the final strain in 5 L and 1500 L bioreactors resulted in L-arginine production titers of 92.5 and 81.2 g/L (with a yield of 0.40 and 0.35 g/g (glucose)), respectively. In addition, Man *et al.* [33] systematically manipulated L-arginine metabolism in *C. crenatum*, involving removal of feedback inhibition, overexpression of the arginine operon, strengthening the metabolic flux of the pentose phosphate pathway, strengthening of the glucose uptake system, channeling more metabolic flux into the L-arginine biosynthetic pathway, and attenuation of byproducts formation, all of which together generated a highly assembled recombinant strain that produced 87.3 g/L of L-arginine with yield up to 0.431 g/g (glucose) in fed-batch fermentation. Nonetheless, we anticipate that the adequate adoption of the strategies described here will allow for the development of engineered strains capable of efficiently producing L-arginine on a large scale with reduced effort, time, and cost.

Currently, although extensive investigation has been carried out for systems metabolic engineering of L-arginine-producing strains, some challenges remain. First, most developed strains are capable of producing L-arginine from glucose, which is in competition with human food. During the past several years, *C. glutamicum* has been extensively engineered to utilize newly emerging and more sustainable non-food or waste substrates for the production of valuable chemicals [109,110]. Motivated by sustainable development of the global economy, further research efforts are needed for the development and introduction of optimized L-arginine-producing recombinant *C. glutamicum* strains, which will consume alternative carbon substrates including hemicellulose hydrolysate, seaweed hydrolysate, energy gas, etc. Second, independent modulation of genes frequently causes cell damage, which breaks

the intrinsic metabolic balance and affects the physiological status of recombinant strains. Owing to the complexity of cell metabolism, it is critical to systematically handle the balance of biomass formation, L-arginine production, and redox systems by using genome-scale metabolic modeling and simulation. Third, a rational combination of random mutagenesis and high-throughput screening strategies provides an attractive prospect for developing L-arginine-producing strains. Nonetheless, the dynamic range of existing L-arginine biosensors is not capable of screening mutant strains with high L-arginine production titers. Application of carefully designed synthetic biological elements, including the insertion of disparate promoters, ribosome binding sites, and a disparate number of rare codons, for optimization of the L-arginine biosensor needs to be further investigated. With the rapid development of systems metabolic engineering, we anticipate the above challenges will be rapidly addressed in the coming decades. Together with the development of research techniques, such as genomics [111], transcriptomics [112], proteomics [113,114], and CRISPR/Cas9-assisted gene-editing technology [27,28,115,116], adequate adoption of these strategies will also become imperative for accelerating the development of high L-arginine-producing strains.

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