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Production of L-glutamate family amino acids in *Corynebacterium glutamicum*: Physiological mechanism, genetic modulation, and prospects

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ABSTRACT

L-glutamate family amino acids (GFAAs), consisting of L-glutamate, L-arginine, L-citrulline, L-ornithine, L-proline, L-hydroxyproline, γ-aminobutyric acid, and 5-aminolevulinic acid, are widely applied in the food, pharmaceutical, cosmetic, and animal feed industries, accounting for billions of dollars of market activity. These GFAAs have many functions, including being protein constituents, maintaining the urea cycle, and providing precursors for the biosynthesis of pharmaceuticals. Currently, the production of GFAAs mainly depends on microbial fermentation using *Corynebacterium glutamicum* (including its related subspecies *Corynebacterium crenatum*), which is substantially engineered through multistep metabolic engineering strategies. This review systematically summarizes recent advances in the metabolic pathways, regulatory mechanisms, and metabolic engineering strategies for GFAA accumulation in *C. glutamicum* and *C. crenatum*, which provides insights into the recent progress in L-glutamate-derived chemical production.

1. Introduction

Amino acids, the major categories of nutrients in human and animal diets, have been predominantly utilized in the pharmaceutical, cosmetic, and feed industries [51,87,97,184]. After ethanol and antibiotics, amino acids are the third most important fermentation products, which have reached a million-ton production titer, owing to the development of biotechnology (Fig. 1A) [95]. In particular, large-scale production of L-glutamate family amino acids (GFAAs), consisting of L-glutamate, L-arginine, L-citrulline, L-ornithine, L-proline, L-hydroxyproline (HYP), γ-aminobutyric acid (GABA), and 5-aminolevulinic acid (5-ALA), has undergone rapid development owing to the rapidly increasing demands in the world [51,97,184] (Fig. 1B). For instance, L-glutamate, the first commercialized amino acid [4], is found at high levels in cheese, green tea, tomato, and human breast milk, occupying more than four million tons of the global market per year [185]. In addition, L-arginine is a semiessential amino acid that is widely used in the medicinal and industrial fields and has a demanding capacity of 1200 tons per year [60,159]. The enormous demand puts forward higher requirements for the production of these compounds. From the perspective of sustainable development, fermentation approach, which is an eco-friendly biotechnology, has been widely applied for the fermentative production of GFAAs [9,83,188].

The economic importance of GFAAs promotes the constant improvement in production techniques, which require the continuous development of robust engineering strains. Currently, three model strains have been developed and applied for the biosynthesis of GFAAs. *Escherichia coli* is a "superstar" in microbial fields and has the advantages of rapid cell division and a shorter fermentation cycle. Therefore, it is a model strain capable of producing various compounds at a high yield [43,189,198]. *E. coli* has been extensively genetically engineered to synthesize L-glutamate, L-arginine [40], L-citrulline [149], L-ornithine [94], L-proline, HYP [18], GABA [86] and 5-ALA [115,220]. However, lipid A, also known as endotoxin, serves as the main component of membranes in *E. coli* and is banned in the pharmaceutical and food

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Abbreviations

GFAAs	L-glutamate family amino acids
HYP	L-hydroxyproline;
GABA	γ-aminobutyric acid
5-ALA	5-aminolevulinic acid
GRAS	generally recognized as safe
EMP	glycolysis pathway
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
PTS	phosphotransferase systems
GlcN	glucosamine;
GlcNAc	ODHC: 2-oxoglutarate dehydrogenase complex
GDH	glutamate dehydrogenase
NAGK	N-acetylglutamate kinase
ROS	reactive oxygen species
NADK	NAD kinase
Ac-ORN	acetylornithine;
NAOD	N-acetylornithine deacetylase
NAGS	N-acetylglutamate synthase
OATase	L-ornithine acetyltransferase
GAD	L-glutamate decarboxylase
RBS	ribosomal binding site;
G6DH	glucose-6-phosphate dehydrogenase
6-PGD	6-phosphogluconate dehydrogenase
BCAA	branched-chain amino acid

application [128]. This disadvantage restricts the utilization of *E. coli* as a suitable GFAA-producing strain. *Saccharomyces cerevisiae* is regarded as a generally recognized as safe (GRAS) microbial cell factory that produces various chemical compounds owing to its robustness in harsh growing conditions [42,106,124]. As a GRAS strain [98,188], *S. cerevisiae* has been successfully engineered to produce L-ornithine [133]. However, owing to complex and rigorous regulation, producing GFAAs using *S. cerevisiae* faces great limitations in the improvement in the yiled of L-ornithine. *Corynebacterium glutamicum*, a GRAS strain accumulated considerable amounts of L-glutamate, was discovered by Japanese scientists in 1956, providing a feasible process for the fermentative production of amino acids [116] and other high-value chemicals [17,21,183]. For GFAAs, in addition to L-glutamate, *C. glutamicum* has been successfully engineered to produce L-arginine [129], L-proline [67,212], L-citrulline [29,46], L-ornithine [32], HYP, GABA, 5-ALA [37]. *C. crenatum*, a subspecies of *C. glutamicum* isolated from soil by Chinese scientists, is safe, robust, and possesses genetic tractability. *C. crenatum* has been extensively applied for the biosynthesis of L-arginine [30,190], L-ornithine [161], succinate [20], and biofuel [167]. Although various strains can be identified and used to produce GFAAs, further engineering optimization will help scale up production.

Efficient strain breeding strategies are crucial for the development of mutant strains with high productivity and stability, which are the core elements in the industrial fermentation of GFAAs (Fig. 1B). Traditionally, the random mutagenesis approach has been used to generate robust strains, which limits multiple rounds of mutagenesis and represents a major expenditure of time and effort, as well as causing undesirable alterations in the nontarget genes [11]. With the development of gene editing technology and in-depth knowledge of the biosynthetic pathways involved, we may explore more strains with potential in industrial applications via metabolic engineering approaches [10,11]. Numerous studies focusing on modulating local amino acid biosynthesis pathways have been continuously conducted. However, limited information and available research experience means that few genes can be manipulated, and engineering of the entire pathway network is still difficult [8,91]. Hence, being able to modify a complex pathway network on a global scale would be indispensable for GFAA production to meet productivity, yield, and titer requirements. This review systematically summarizes the metabolic pathways and regulatory mechanisms of GFAAs accumulation in *C. glutamicum*. Next, comprehensive information and breeding strategies for developing GFAA-producing *C. glutamicum* and *C. crenatum* strains are discussed.

2. Central metabolism and its genetic modifications

GFAA accumulation can be promoted by rational manipulation of central metabolic pathways, including uptake of carbon sources, glycolysis pathway (EMP), pentose phosphate pathway (PPP), anaplerotic pathway, acetyl-CoA metabolism, tricarboxylic acid (TCA) cycle, and energy balance.

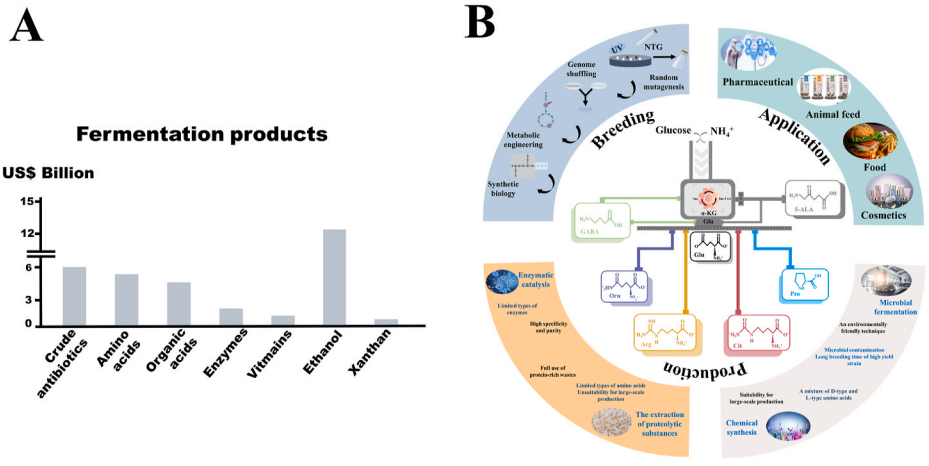

Fig. 1. GFAAs and global market for fermented products. A. The strain breeding strategies, applications, and production pattern of GFAAs and its derivatives; B. Global market for fermented products, including crude antibiotics, amino acids, organic acids, enzymes, ethanol, and xanthan.

2.1. Carbon source assimilation

2.1.1. Improving GFAA accumulation by reinforcing the sugar transport systems

In *C. glutamicum*, there are four sugar transport systems on the cell membrane that are capable of uptake of glucose, fructose, and sucrose (Fig. 2A). Three phosphotransferase systems (PTSs) were originally found in *Brevibacterium flavum* (*C. glutamicum*), which consist of three components: PTS EI, PTS EII, and HPr (Fig. 2B) [117,118]. Among them, PTS EI (encoded by *ptsI*) and HPr (encoded by *ptsH*) are common components that are identified as cytoplasmic proteins. PTS EII possesses diverse types, including PTS^{Glu} (encoded by *ptsG*), PTS^{Fru} (encoded by *ptsF*), and PTS^{Suc} (encoded by *ptsS*), corresponding to the transport of glucose, fructose, and sucrose, respectively [25]. In addition to the PTS, the inositol transporters IolT1 and IolT2 are alternative sugar uptake pathways involved in glucose uptake and phosphorylation [99].

There are three repressors, namely SugR, IolR, and FruR, which regulate the sugar uptake systems in *C. glutamicum* (Fig. 2A). Among them, SugR represses the expression of *ptsG*, *ptsF*, *ptsS*, *pfkA*, *pfkB*, *fda*, *gapA*, *eno*, *pyk*, and *ldhA* [34,169,173]. Simultaneously, SugR functions as a transcriptional repressor of *ramA* in response to the perturbation of the extracellular and intracellular environments [172]. FruR (encoded by *fruR* or *cg2118*), a DeoR-type regulator, reduces the expression of PTS-relevant genes (*ptsI*, *ptsH*, and *ptsF*) in the presence of fructose [39]. The primary function of IolR is negative regulation of the *iol* genes involved in myo-inositol catabolism and activating the expression of *pck* (encoding phosphoenolpyruvate carboxykinase) (Fig. 2A) [82].

Currently, genetic engineering of the sugar transport systems has been applied to the production of L-arginine and L-ornithine in *C. crenatum* and *C. glutamicum* (Fig. 3). By replacing the native promoters of *ptsG*, *iolT1*, and *ppgk* with a strong *sod* promoter, the transcription of these genes was significantly improved, which accelerated the biosynthesis of L-arginine in *C. crenatum*. The resulting engineered strain produced 61 g/L L-arginine at a yield of 0.294 g/g glucose. In addition, to improve the yield of L-ornithine, studies on the enhancement of glucose consumption rate were conducted in *C. glutamicum*, which generated the mutant strain SO26. The results indicated that the yield of L-ornithine reached 38.5 g/L, which increased by approximately 10% compared with the control strain SO24 [204]. Therefore, it can be concluded that accelerating glucose utilization is a proper strategy for improving GFAA production.

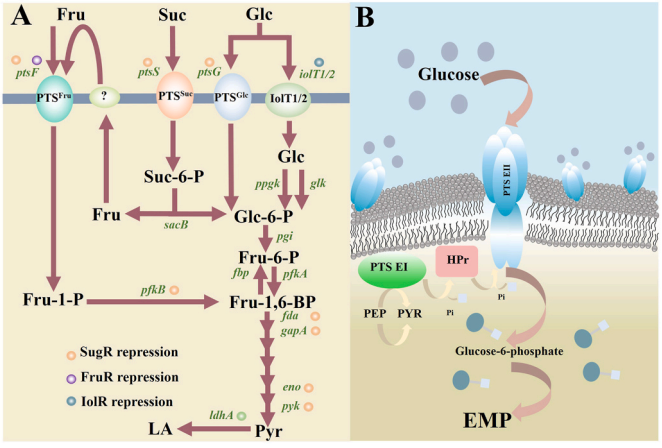

Fig. 2. Sugar uptake systems in *C. glutamicum*. A. Thumbnail of sugar assimilation and absorption pathway. B. Transport mechanism of phosphotransferase systems (PTSs): PTS EI, Enzyme I; PTS EII, Enzyme I; HPr, heat-stable carrier protein; PEP, Phosphoenolpyruvate; PYR, pyruvate. *ptsF*, encoding fructose-specific IIABC component; *ptsG*, glucose-specific IIABC component; *ptsS*, sucrose-specific IIABC component; *iolT1/2*, encoding two myo-inositol transporters; *glk*, encoding glucokinase; *ppgk*, encoding glucokinase; *scrB*, encoding sucrose-6-phosphate hydrolase; *pgi*, encoding glucose-6-phosphate isomerase; *pfkA*, encoding 6-phosphofructokinase; *pfkB*, encoding 1-phosphofructokinase; *fbp*, encoding fructose-1, 6-bisphosphatase; *fda*, encoding fructose bisphosphate aldolase; *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase; *eno*, encoding enolase; *pyk*, encoding pyruvate kinase; *ldhA*, encoding lactate dehydrogenase; Fru, fructose; Suc, sucrose; Glc, glucose; Suc-6-P, sucrose-6-phosphate; Glc-6-P, glucose-6- phosphate; Fru-6-P, fructose-6- phosphate; Fru-1,6-BP, fructose-1,6-bisphosphate; Pyr, pyruvate; LA, lactate; SugR, a DeoR-Type regulator; FruR, a DeoR-Type regulator; IolR, a GntR-Type regulator; PTS^{Fru}, the related components of fructose utilization; PTS^{Glc}, the related components of glucose; PTS^{Suc}, the related components of sucrose; Iol1/2, two myo-inositol transporters.

2.1.2. Exploring alternative carbon resource for GFAA production

In addition to glucose, other alternative carbon feedstocks, including xylose, arabinose, glucosamine (GlcN), N-acetyl-glucosamine (GlcNAc), sucrose, molasses, and glycerol, have been intensively applied to produce GFAAs (Fig. 4) [3]. Xylose, which is a cheap and attractive feedstock, has been applied to produce L-ornithine [113,204] and GABA [7,187]. Overexpression of the *xylAB* operon (encoding xylose isomerase and xylulokinase originating from *Xanthomonas campestris*) by employing a recombinant plasmid pXMJ19-*xylAB* generates the recombinant strain *C. glutamicum* SO29, which produces L-ornithine at a titer of 18.9 g/L [204]. Heterologous expression of *E. coli gadB* (encoding glutamate decarboxylase, GAD) and *xylAB* resulted in engineered *C. glutamicum* H36GD1852 that produced 35.47 g/L GABA during batch culturvation [7]. Similarly, the GABA-producing strain was engineered to utilize alternative carbon sources, such as GlcN and GlcNAc. The uptake of GlcN was identified to be transported by the PTS^{Glc} system and repressed by NanR (encoded by *nanR*) [179]. Seventeen *Corynebacterium* strains were tested for possibility to grow on GlcNAc, which revealed that only *Corynebacterium glycinophilum* DSM45794 harboring an EII permease for the assimilation of GlcNAc [110]. Deletion of NanR and overexpression of *nagE* in *C. glutamicum* generated the mutant strain GABA6C, which produced 3.9 g/L of GABA [72]. Arabinose is a pentose that comprises much of rice straw hydrolyzate. By expressing *araBAD* from *E. coli* MG1655, *C. glutamicum* strains were engineered to produce L-glutamate, L-ornithine, and L-arginine at a titer of 37 ± 10 mM, 124 ± 35 mM, and 25 ± 2 mM, respectively, from arabinose [148]. Sucrose and molasses, as industrial raw materials, are highly attractive for the fermentative production of bio-products because they are inexpensive and readily available [84,140]. Sucrose-6-phosphate hydrolase (encoded by *sacB*) in the genome of *C. glutamicum* can hydrolyze sucrose to form fructose and glucose-6-phosphate [33]. β-Fructofuranosidase (encoded by *sacC*), an extracellular enzyme from *Mannheimia succiniciproducens* is favorable for engineering microorganisms, has been successfully applied to the *E. coli* K-12 strain to produce L-threonine [88]. Recombinant *C. glutamicum* harboring the *sacC* gene from *M. succiniciproducens* was constructed and produced 22.0 g/L and 27.0 g/L of L-ornithine from sucrose and molasses, respectively [216]. Recently, with the rapid development of the biodiesel industry, more than 1.6 million tons of waste glycerol has been generated per year [114]. To utilize glycerol in *C. glutamicum*, coexpression of the *glpFKD* operon from *E. coli* were performed in *C. glutamicum* WT (pVWEx1-$glpFKD^{Eco}$), *C. glutamicum* ORN1(pVWEx1-$glpFKD^{Eco}$) and *C. glutamicum* ARG1(pVWEx1-$glpFKD^{Eco}$), which produced L-glutamate, L-ornithine, and L-arginine at yields of 7.2 ± 0.2 mM, 17.9 ± 0.4 mM, and 23.6 ± 0.9 mM, respectively [114].

In summary, slow glucose uptake rate frequently leads to a decrease in GFAA productivity [107]. Manipulating the glucose uptake system is a feasible strategy to improve GFAA production. In addition, it is emphasized that using glucose in fermentation industry has the problem of competing with the food industry and human nutrition. To avoid this competition, xylose, arabinose, sucrose, GlcNAc, and glycerol may serve as environmentally friendly and renewable natural carbon resources for the biotechnological production of GFAAs.

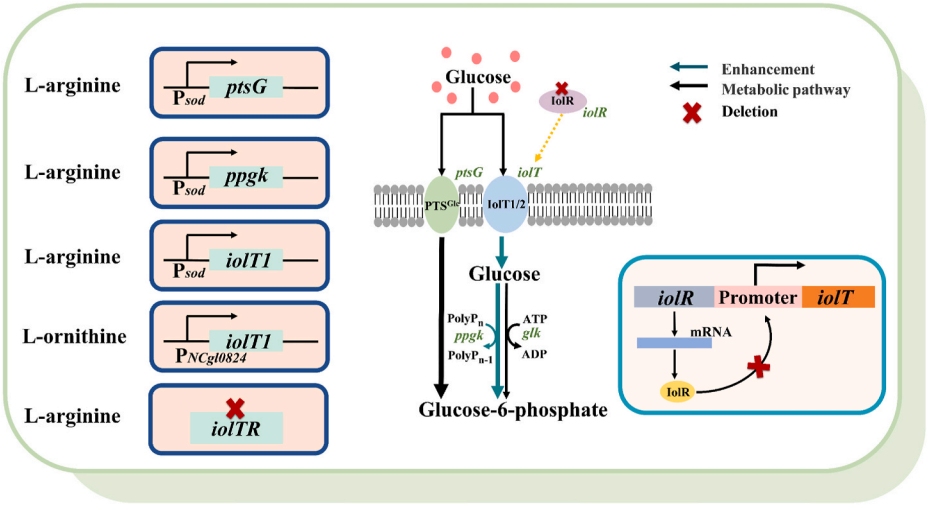

Fig. 3. Genetic engineering of the sugar transport systems for GFAA production. Red fork represents gene deletion; atrovirens arrow represents gene overexpression.

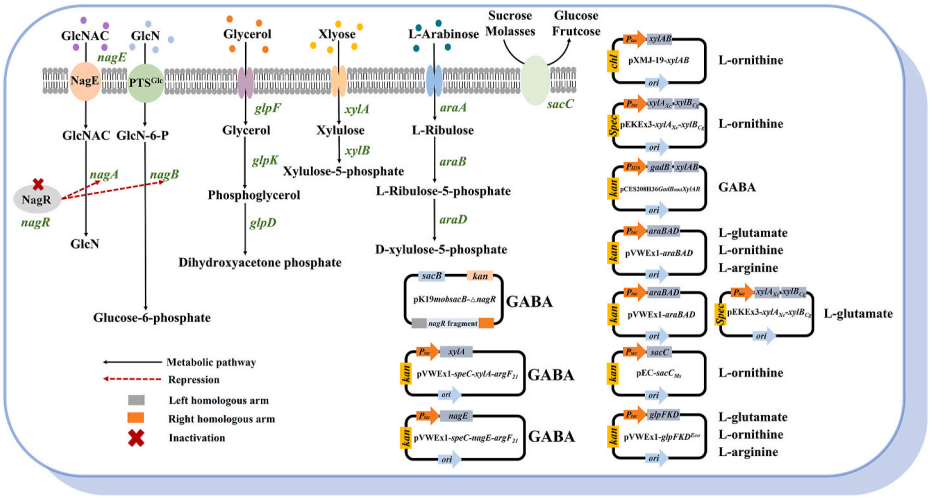

Fig. 4. The utilization of alternative carbon sources to produce GFAAs. *nagB*, encoding glucosamine 6-phosphate deaminase; *nagA*, encoding *N*-acetylglucosamine 6-phosphate deacetylase; *nanR*, encoding transcriptional regulator; *nanE,* encoding *N*-acetylglucosamine-specific transporter; *glpF*, encoding glycerol facilitator; *glpK*, encoding glycerol kinase; *glpD* encoding glycerol-3-phosphate dehydrogenase; *xylA*, encoding xylose isomerase; *xylB*, encoding xylulokinase; *araA*, encoding arabinose isomerase; *araB*, encoding ribulokinase; araD, encoding ribulose-5-phosphate-4-epimerase; *sacC*, encoding β-fructofuranosidase.

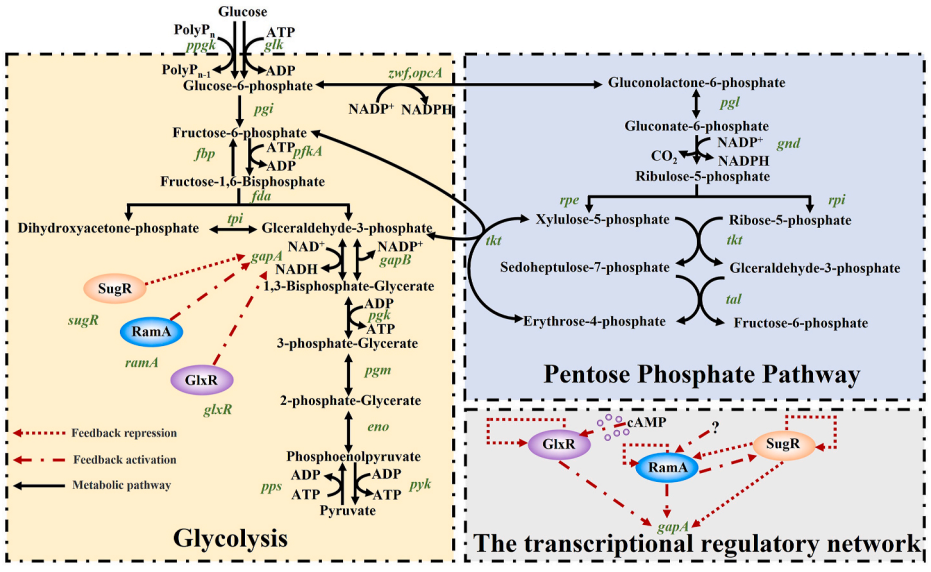

Fig. 5. Glycolysis and the pentose phosphate pathway in *C. glutamicum*. *gapB,* encoding NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; *pgk,* encoding 3-phosphoglycerate kinase; *pgm*, encoding phosphoglycerate mutase; *eno*, encoding enolase; *pps*, encoding phosphoenolpyruvate synthetase; *pyk*, encoding pyruvate kinase; *zwf* and *opcA*, encoding glucose-6-phosphate dehydrogenase; *pgl*, encoding 6-phosphogluconolactonase; *gnd*, encoding 6-phosphogluconate dehydrogenase; *rpe*, encoding ribulose-5-phosphate epimerase; *rpi*, encoding ribose-5-phosphate isomerase; *tkt*, encoding transketolase; *tal*, encoding transaldolase; *sugR*, encoding a DeoR-Type transcriptional regulator SugR; *ramA*, encoding a LuxR-type transcriptional regulator RamA; *glxR*, encoding a CRP-type transcriptional regulator GlxR; SugR, a DeoR-Type regulator responsible for global regulation; RamA, a CRP-type transcriptional regulator; GlxR, a CRP-type transcriptional regulator; cAMP, cyclic adenosine monophosphate.

2.2. EMP and PPP

2.2.1. Natural metabolic regulation mechanism of EMP and PPP in C. glutamicum

After phosphorylation sugar transport from the extracellular to the intracellular environment, further metabolism occurs via both the EMP and the PPP (Fig. 5). EMP involves ten steps, and 11 enzymes catalyze one molecule of glucose to 2 × pyruvate, 2 × ATP, and 2 × NADH. In this pathway, the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate catalyzed by glyceraldehyde-3-phosphate dehydrogenase is a rate-limiting step, which responds to changes in the intracellular NADH/NAD^+ ratio and is regulated by the global regulators SugR, RamA, and GlxR [26]. SugR, a DeoR-type regulator, is involved in the negative transcriptional regulation of *gapA* in the absence of sugar, whereas fructose-1-phosphate (F-1-P) and fructose-1,6-bisphosphate (F-1,6-BP) negatively affect the binding of SugR and the *gapA* promoter [173,175]. In addition, RamA (encoded by *ramA* or *cg2831*) is a LuxR-type regulator that is directly controlled the upregulation of *sugR* expression [174]. PPP is a bypass of EMP that provides reducing power and intermediates for the biosynthetic pathway of various metabolites. This pathway contains seven enzymes that can be divided into two components: an irreversible oxidative route, consisting of glucose-6-phosphate dehydrogenase (G6DH), 6-phosphogluconolactonase, and 6-phosphogluconate dehydrogenase (6-PGD), which catalyzes the conversion of glucose-6-phosphate into ribulose-5-phosphate with the formation of two molecules of NADPH. Second, the reversible nonoxidative route, which catalyzes the conversion of ribulose-5-phosphate into glyceraldehyde-3-phosphate, and involves precursor metabolites including triose phosphates, tetrose phosphates, pentose phosphates, and heptose phosphates.

2.2.2. Metabolic engineering of PPP and EMP for improving GFAA production

It is widely acknowledged that flux distribution in the EMP and the PPP plays a crucial role in maintaining the balance of cellular metabolism. Under L-glutamate-producing conditions, the carbon metabolic flux distribution in the EMP and PPP was estimated at 80:20, whereas it was 30:70 and 40:60 during L-arginine and L-lysine fermentation, respectively [78]. Channeling the metabolic flux to PPP can directly provide more NADPH for the biosynthesis of GFAAs. For instance, coexpression of *zwf* and *gnd* in the strain CgΔ*argG* resulted in 287% and 363% improvement in intracellular NADPH content, which stimulated L-citrulline accumulation [102]. In addition, optimization of intracellular NADPH supplementation by downregulating the expression of *pgi* and upregulating the expression of the *tkt* operon is a feasible strategy to improve L-arginine production (as shown in Fig. 6) [129]. Similar strategies have been applied for the development of L-ornithine and L-proline-producing strains. Replacing native start codons of *pgi* and native promoter of *tkt* operon resulted in an engineered strain that produced L-ornithine at a yield of 51.5 g/L, with an overall productivity of 1.29 g/L/h [77]. Simultaneously, modulation of *zwf* and *gnd* to improve the supplementation of NADPH is the preferred genetic engineering strategy for L-proline production [191].

Overexpression of enzymes in glycolysis for optimization of carbon metabolic velocity and ATP supply has been applied in the construction of engineered strains (Fig. 6). For instance, insertion of a strong P_{efu} promoter into the upstream region of *pfkA* and *gap*A resulted in engineered strains SO7 and SO9 that produced 26.5 and 22.8 g/L of L-ornithine, which was 11.2% and 9.7%, respectively, higher than the parent strain. In addition, to conjugate the glycolytic pathway with the generation of NADPH, *gapC* from *Clostridium saccharobutylicum* was introduced into *C. glutamicum* strainΔAPER, which showed a 277% increase in intracellular NADPH content and a 10% improvement in L-ornithine

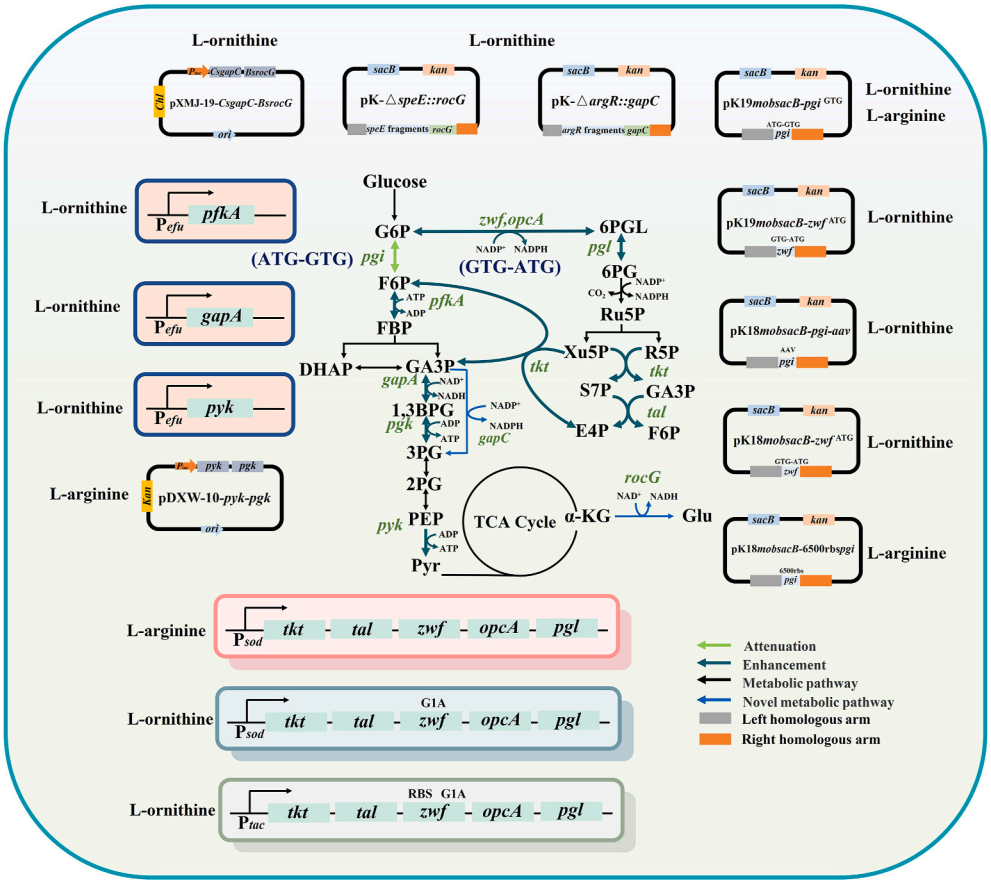

Fig. 6. Genetic modification of EMP and PPP to produce GFAA in *C. glutamicum*. *gapC*, encoding NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; *rocG*, encoding NADH-dependent glutamate dehydrogenase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, Dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate; 1,3BPG, 1,3-bisphosphate-glycerate; 3 PG, 3-phosphate-glycerate; 2 PG, 2-phosphate-glycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; 6PGL, gluconolactone-6-phosphate; 6 PG, gluconate-6-phosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; α-KG, 2-oxoglutarate; Glu, L-glutamate.

production titer [70]. This useful strategy was further confirmed by its application in *C. glutamicum* strain KBJ11, which produced 88.26 g/L of L-ornithine during fed-batch culturvation [27].

In addition to EMP and PPP, the gluconate bypass pathway frequently consumes the mid-metabolite, which reduces the generation of NADPH. Therefore, approaches aimed at the gluconate bypass pathway have been used to improve the NADPH pools for GFAA production. Deletion of *gntK* enables the absence of gluconate kinase activity in strain SJC8039, which showed a 51.8% increase in intracellular NADPH content and a 49.9% increase in the yield of L-ornithine [58]. Inactivation of glucose dehydrogenase by chromosomal in-frame deletion of *NCgl0281*, *NCgl2582*, and *NCgl2053* led to the improvement in specific G6DH and 6-PGD activities, which promoted the supplement of intracellular NADPH and L-ornithine accumulation [59].

In summary, metabolic engineering of EMP and PPP to increase NADPH availability and metabolic velocity, has been applied to four categories to improve GFAA production: (1) Redistribution of the carbon flux between the EMP and PPP, (2) enhancing the expression of key enzymes involved in energy metabolism, (3) blocking the gluconate-bypass pathway, and (4) the heterologous expression of NADP -or NAD-dependent enzymes. These strategies increase intracellular NADPH supplementation, which is universally applicable to anabolism.

2.3. TCA cycle and related pathways

The TCA cycle, as an amphibolic pathway, serves as a central catabolic and anabolic metabolism that catalyzes the total oxidation of acetyl units, generating energy and providing precursors for the biosynthetic pathways of valuable products [181] (Fig. 7). This cycle pathway is tightly regulated and provides the necessary trunk metabolism for the biosynthesis of GFAAs, which includes three critical metabolic nodes: acetyl-CoA metabolism node, pyruvate-oxaloacetate metabolic node, and α-ketoglutarate metabolic node.

2.3.1. The regulation mechanism of the TCA cycle pathway

The TCA cycle is subject to strict regulation not only at the transcriptional level [13,16], but also at the posttranscriptional level [80,135,200]. In the case of the α-oxoglutarate dehydrogenase complex (ODHC), posttranscriptional regulation is controlled by the DstR1 protein (acetyl-CoA carboxylase, encoded by *dstR1*) (Fig. 7) and OdhI protein (a signal transduction protein encoded by the *odhI* gene) [19]. First, DtsR is a subunit of acetyl-CoA carboxylase that participates in the anabolism pathway of fatty acids, which also activates the activity of ODHC. Deletion of *dstR1* can reduce the activity of ODHC by 80%. In addition, the presence of Tween 40, sublethal concentrations of penicillin, and limited amounts of biotin also inhibited the activity of ODHC [79,200]. In addition, the phosphorylated and unphosphorylated forms of OdhI can regulate ODHC activity. The OdhI protein consists of a forkhead-associated domain at the C-terminus and an extension of 44 amino acids at the N-terminus. The reduction in ODHC activity is caused by the combination of the forkhead-associated domain of unphosphorylated OdhI protein and the OdhA subunit. The OdhI protein phosphorylation status at Thr15 is controlled by four serine/threonine protein kinases PknG (encoded by the *pknG* or *cg3046* gene), PknA (encoded by the *pknA* or *cg0059* gene), PknB (encoded by the *pknB* or *cg0057* gene), and PknL (encoded by the *pknL* or *cg2388* gene) [38,152]. The activity of ODHC is recovered during this phosphorylation process of the OdhI protein, whereas the OdhI protein dephosphorylation status is catalyzed by the phospho-serine/threonine-protein phosphatase (Ppp, encoded by the *ppp* or *cg0062* gene) [151,152]. Based on the above mechanism, experimental results suggest that overexpression of *odhI*, deletion of *dtsR1*, and Tween 40 addition are effective strategies for improving GFAA accumulation in *C. glutamicum* (Fig. 8) [76].

2.3.2. Modulation of the acetyl-CoA metabolism node for improving GFAA production

When *C. glutamicum* is cultivated on C6 or C5 substrates, acetyl-CoA, derived from the catalysis of pyruvate by pyruvate dehydrogenase complex, is the fueling substrate for the TCA cycle involved in the acetate biosynthesis pathway, glutamate acetylation, and fatty acid biosynthesis pathway (Fig. 8). Ensuring continuous and abundant supplementation of acetyl-CoA is crucial for the biosynthesis of GFAAs. To

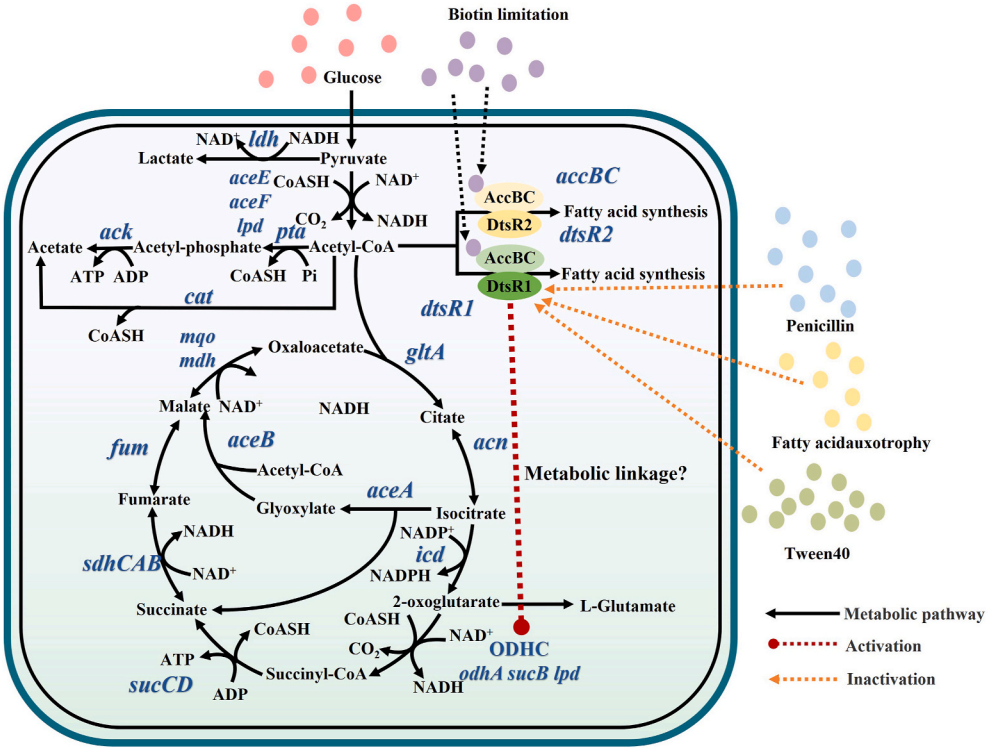

Fig. 7. Thumbnail of TCA cycle in *C. glutamicum*. *ldh*, encoding lactate dehydrogenase; *ack*, encoding acetate kinase; *pta*, encoding phosphotransacetylase; *cat*, encoding acetyl-CoA: CoA transferase; *aceE*, encoding pyruvate dehydrogenase complex (PDHC), E1p subunit; *aceF*, encoding PDHC, E2p subunit; *lpd*, encoding PDHC and OGDHC, LPD subunit *was*; *gltA*, encoding citrate synthase; *acn*, encoding aconitase; *icd*, encoding isocitrate dehydrogenase; *odhA*, encoding oxoglutarate dehydrogenase complex (ODHC), Elo subunit; *sucB*, encoding ODHC E2o subunit; *sucCD*, encoding succinyl-CoA synthetase; *sdhCAB*, encoding succinate: menaquinone oxidoreductase; *fum*, encoding fumarase; *mqo*, encoding malate: quinone oxidoreductase; *mdh*, encoding malate dehydrogenase; *aceA*, encoding isocitrate lyase; *aceB*, encoding malate synthase; *accBC*, encoding a protein with a biotin-binding motif, AccBC; *dtsR1* and *dtsR2*, encoding acetyl-CoA carboxylase; AccBC, a protein with a biotin-binding motif; DtsR1 and DtsR2, acetyl-CoA carboxylase.

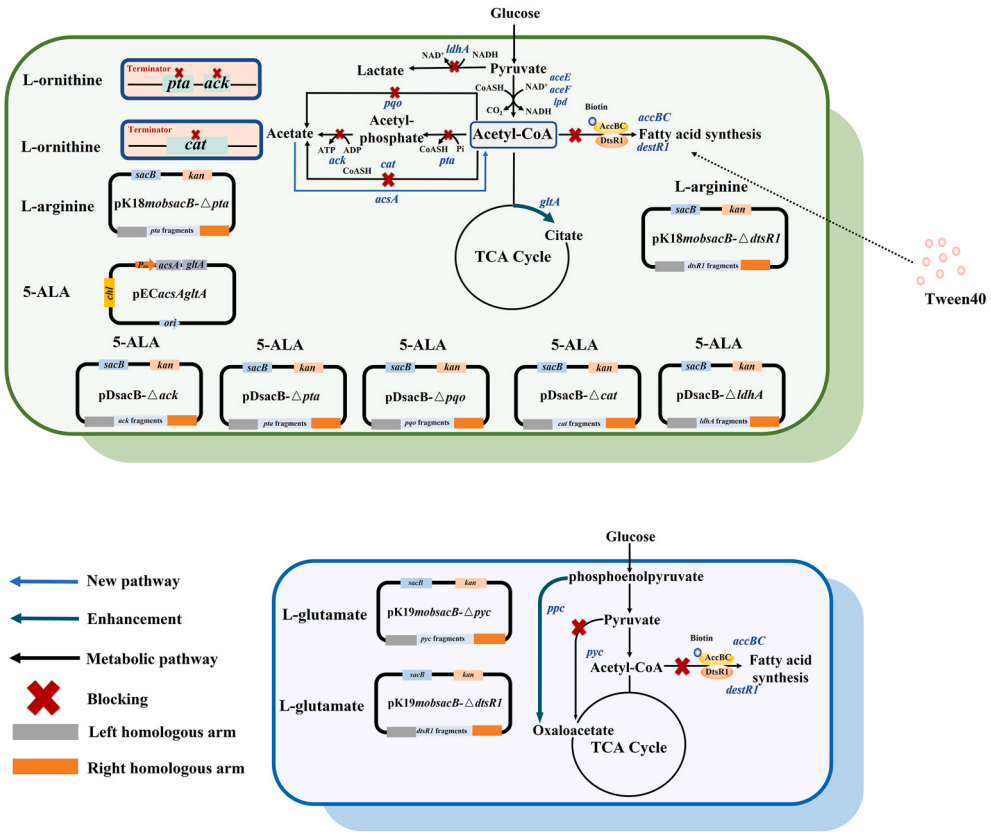

Fig. 8. Genetic modification of acethyl-CoA metabolism and pyruvate-oxaloacetate metabolic node to produce GFAA. *ppc*, encoding PEP carboxylase; *pyc*, encoding pyruvate carboxylase; *pqo*, encoding pyruvate: menaquinone oxidoreductase; *acsA*, encoding acetyl-CoA synthetase. Red fork represents gene deletion; atrovirens arrow represents gene overexpression.

reduce the consumption of acetyl-CoA in the biosynthetic pathway of by-products, attenuation of the acetate metabolic pathway resulted in the engineered strain SO6, which produced 26 g/L of L-ornithine, exhibiting approximately 9.15% increase as compared with the parent strain [208]. In addition, on the basis of genome-scale metabolic network model prediction, deletion of *pta* for improving L-arginine production titer has also been investigated in *C. crenatum*, which indicated that the mutant strain CCM03 could produce 15.27 g/L of L-arginine, which is 27.0% higher than that of the control strain CCM01 (12.02 g/L) [54]. Moreover, reducing acetate formation by inactivation of *pqo, pta*, *ack*, and *cat* promotes 5-ALA production in the engineered strain ALA1 [37]. Therefore, continuous sufficient acetyl-CoA

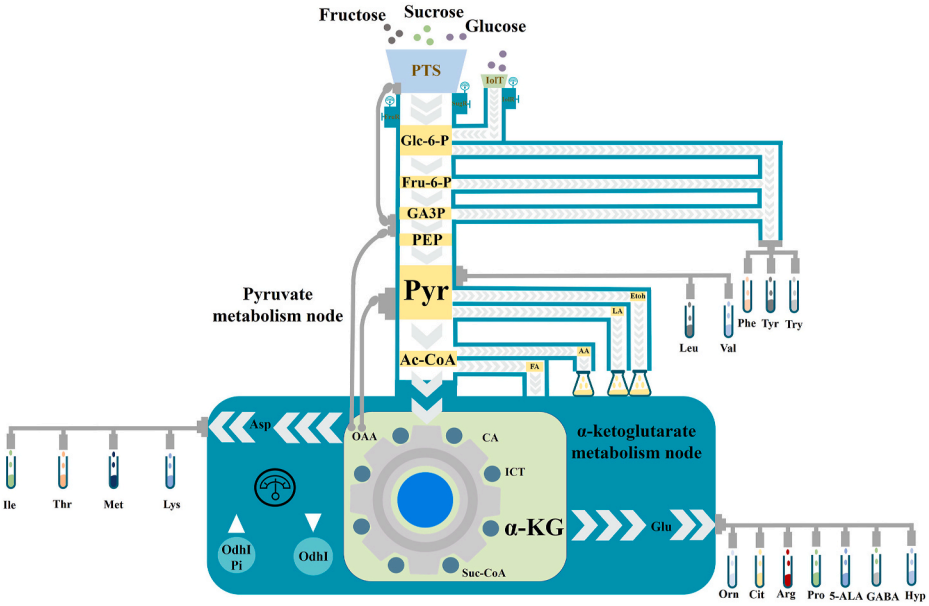

Fig. 9. Schematic diagram of pyruvate-oxaloacetate metabolic node and α-ketoglutarate metabolic node in *C. glutamicum*.

supplementation contributes to the biosynthesis of GFAAs, which is crucial for the accumulation of L-arginine, L-ornithine, and L-citrulline, which requires acetyl-CoA as a cofactor to generate N-acetylglutamate. Eliminating the biosynthetic pathway of the by-products improved the supplementation of acetyl-CoA, which was identified as an effective strategy to promote GFAA accumulation.

2.3.3. Modulation of the pyruvate-oxaloacetate metabolic node for improving GFAA production

To maintain the high-speed stability of the rotational TCA cycle, regulation of the activity of various enzymes at the pyruvate-oxaloacetate node is a nonnegligible strategy, which exerts a crucial effect on GFAA production [48] (Fig. 9). Redirecting carbon flux from pyruvate to OAA by anaplerotic pathways is beneficial for the biosynthesis of GFAAs [52]. *C. glutamicum* carries two anaplerotic pathways (Table 1), which are catalyzed by phosphoenolpyruvate carboxylase [126] and pyruvate carboxylase [131]. Previously, the effect of modification of the analperotic pathway on 5-ALA biosynthesis was investigated by changing the native promoter of *ppc* with P_{sod} promoter in *C. glutamicum* ALA4. In response to this genetic modification, the production of 5-ALA by the mutant strain ALA4 (2.06 ± 0.05 g/L) increased by 7.29% and biomass increased by 8.98% as compared with the parent strain ALA2 [6,101]. Corresponding modification approaches have been applied to the biosynthesis of L-arginine. To redirect more carbon flux into the TCA cycle, the strain Cc4 was constructed based on strain Cc3 by replacing the start codon GTG with ATG in the *pyc* gene and implementing an additional copy of the *gltA* gene on the chromosome. The fed-batch fermentation results showed that the strain Cc4 produced L-arginine up to 68.6 g/L, at a yield of 0.336 g/g glucose [107]. In addition, attenuating the expression of *lysC* (encoding aspartokinase), which catalyzes the phosphorylation of L-aspartate to form L-aspartyl--phosphate, is also a useful method to control the carbon flux and ensure the supply of oxaloacetate, which increased the yield of L-arginine by 10% in the engineered strain Cc5-800, as well as reducing the biosynthesis of L-lysine and L-isoleucine [107]. For L-ornithine, overexpression of the *gltA* (encoding citrate synthase) gene via insertion of a strong promoter resulted in a 10.7% increase in the yield of L-ornithine [204].

In addition to the anaplerotic pathways, NADH-dependent lactate dehydrogenase can convert pyruvate into L-lactic acid, which is disadvantageous for GFAA production [162]. Deletion of the *ldh* gene not only reduces by-product formation, but also saves pyruvate and NADH for the biosynthesis of GFAAs. For instance, to investigate the effect of disrupting *ldhA* on L-glutamate accumulation, *C. glutamicum* strain GDK-9Δ*ldhA* was constructed to produce 70.4 ± 1.33 g/L L-glutamate, which was improved by 9.15% as compared with the control group. Simultaneously, the deletion of the *ldhA* gene led to a 97.6% and 44.6% decrease in L-lactate and L-alanine, respectively, compared to the strain GDK-9 [211]. Similarly, this strategy was performed in *C. glutamicum* JML05, which promoted L-arginine accumulation [203]. The above reports demonstrate that inactivation of *ldhA* markedly improved L-glutamate and L-arginine production and reduced by-product formation.

2.3.4. Modulation of the α-ketoglutarate metabolic node for improving GFAA production

α-Ketoglutarate, as a crucial metabolite listed in the TCA cycle, is catalyzed by the ODHC to form succinyl-CoA but is also catalyzed by glutamate dehydrogenase (GDH) to form L-glutamate (a precursor of GFAAs). Hence, the α-ketoglutarate metabolic node is regarded as the pivotal metabolic node for GFAA production (Fig. 9). Redirecting more metabolic flux from the TCA cycle to the biosynthesis of L-glutamate by attenuation of the *odhA* gene improved the biosynthesis of L-ornithine [209], L-arginine, L-proline [212], putrescine [122], and GABA production [72]. In addition, increasing the supply of precursor L-glutamate through the overexpression of *gdh* is an efficient strategy for the accumulation of GFAAs [185]. For instance, overexpression of GDH (encoded by the *gdh* gene or *CgS9114_12202*) and GDH2 (encoded by the *gdh2* gene or *CgS9114_07576*) resulted in obvious improvement in L-ornithine production [204]. In addition, replacing the natural promoter of *gdh* with a strong promoter in *C. glutamicum* ZQJY-3 is a feasible method for improving L-proline accumulation [212]. Furthermore, it has been proven that L-glutamate supply is essential for L-arginine production in the ARG strain (*glnA–aspA*). Hence, the synthesis capacity of L-glutamate should be improved to further enhance L-arginine biosynthesis. To improve L-arginine production by enhancing GDH activity, the strain ARG (*glnA–aspA–gdh*) was engineered by coexpression of *glnA*, *aspA*, and *gdh*. The resulting strain produced L-arginine at a titer of 53.2 ± 1.27 g/L during fed-batch cultivation. Compared to the control strain, L-arginine production, productivity, and the yield of strain ARG (*glnA–aspA–gdh*) increased by 41.5, 41.3, and 27.0%, respectively [41]. These examples demonstrate that redistributing the metabolic flux to L-glutamate is a feasible strategy for improving GFAA production.

In conclusion, modulation of the key metabolic nodes in the TCA cycle is a useful genetic modification strategy for improving GFAA production. The phosphoenolpyruvate-pyruvate-oxaloacetate metabolic node and α-ketoglutarate metabolic node are two important nodes in the TCA cycle, which represent the bonding point between EMP, GFAAs biosynthesis, and the TCA cycle. The purpose of modulating these nodes is to achieve the rational distribution of carbon flux between the TCA cycle and the branch of the GDH pathway, to provide more L-glutamate precursors for the anabolism of GFAAs. The phosphoenolpyruvate-pyruvate-oxaloacetate metabolic node contains acetyl-CoA and anaplerotic pathways. Thus, modulating the two metabolic pathways in this key node is to increase the availability of acetyl-CoA and oxaloacetate for the TCA cycle and L-glutamate precursor for the biosynthesis of GFAAs. Acetyl-CoA metabolism is also involved in the biosynthesis of

Table 1
Genetic modification of pyruvate-oxaloacetate metabolic node for GFAAs production.

Strains	Substrate	Titer (g/L)	Product	Cultivation	Modulations	Reference
C. glutamicum ALA6	Glucose	2.07	5-ALA	Shake flask; batch	the insertion of a strong P_{sod} promoter in the upstream region of *ppc* gene; the deletion of *pyk* gene	[37]
C. crenatum Cc4	Glucose	68.6	Arginine	Bioreactor; fed-batch	replacing the start codon GTG of *pyc* gene with ATG; implementing an additional copy of *gltA* gene on chromosome	[107]
C. crenatum Cc5 *lysC*30	Glucose	25.8	Arginine	Shake flask; batch	the attenuation of *lysC* gene by replacing the natural RBS with the sequence of synthetic RBS with strengths of 30 au	[107]
C. glutamicum SO21	Glucose	34.1	Ornithine	Shake flask; batch	the overexpression of *gltA* gene via inserting strong promoter	[204]
C. glutamicum GDK-9△*ldhA*	Glucose	70.4	Glutamate	Shake flask; batch	the deletion of *ldhA* gene	[211]
C.glutamicum JML06	Glucose	52.7	Arginine	Bioreactor; fed-batch		[203]
C.glutamicum ZQJY-6	Glucose	8.01	Proline	Shake flask; batch	the deletion of *avtA* gene	[212]

fatty acids. DtsR1 does not only form a complex with AccBC to participate in fatty acid synthesis, but also activates ODHC activity. Thus, the inactivation of the *dtsR1* and the addition of Tween 40 can directly reduce the consumption of acetyl-CoA and indirectly reduce the activity of ODHC to strengthen the biosynthesis pathway of GFAAs. Regulation of the anaplerotic pathways involves the redistribution of carbon flux into the TCA cycle. Two different approaches consisting of the simultaneous overexpression of *pyc* and *gltA* and double deletion of *pyc* and *dstR1,* significantly improved GFAA production. The α-ketoglutarate metabolic node is involved in energy, reducing power, and precursor supply for the biosynthesis of GFAAs. Therefore, genetic modification of the flux controlling genes in this metabolic node is crucial for improving GFAA accumulation (Table 2). From the current reports, the modulations of these genes, including *gltA*, *icd*, *odhA*, *gdh*, and *gdh2*, can be divided into two aspects. On the one hand, attenuation of *odhA* is a key factor in redistributing more metabolic flux from α-ketoglutarate to L-glutamate. To attenuate the expression of *odhA*, various specific modification approaches, including changing the natural start codon or the ribosomal binding site (RBS) of *odhA*, deletion of *dtsR1*, and removing the phosphorylation motif site of OdhI, were employed, which showed a great promoting effect on GFAA accumulation. On the other hand, overexpression of the *gltA*, *icd*, *gdh*, and *gdh2* genes is a useful approach to redistribute more carbon flux into the GDH pathway to provide L-glutamate precursors for GFAAs production on the basis of balancing cell growth.

3. Nitrogen metabolism and its genetic modifications

Microorganisms have developed rigorous regulatory mechanisms for nitrogen metabolism to meet the requirements of varying environmental conditions, which is important for the bio-based production of GFAAs.

3.1. Uptake of nitrogen sources and assimilation of ammonium

Ammonium, urea, L-glutamine, and other nitrogenous compounds are available nitrogen sources for *C. glutamicum*. Among them, ammonia and urea can accross the cell membrane using diffusion, whereas the assimilation of ammonium [164], urea [163], and L-glutamine [165] require specific uptake systems. The ammonium uptake system is mainly dependent on two special carriers, Amt (encoded by *amt*) and AmtB (encoded by *amtB*, originally named *amtP*) [65] (Fig. 10). In contrast to Amt permease, the AmtB permease accepts ammonium and transports ammonia into the cell [66,164]. Uptake of urea mainly depends on passive diffusion and urea transporters in the presence of abundant and insufficient urea feeding conditions, respectively [163]. In addition to the common nitrogen sources, other nitrogen-containing compounds can also be utilized as nitrogen sources. For instance, although the transport efficiency of L-glutamate is lower than that of L-glutamine, L-glutamate can still regard as a nitrogen source for *C. glutamicum* [158, 171]. Two L-glutamate uptake channel composed of proteins encoded by *gluABCD* [85] and a secondary, sodium-coupled carrier (encoded by the *gltS* gene) [176] have been investigated. The expression of the *gluABCD* cluster is not only under nitrogen control but also under the control of glucose catabolite repression. Under a high nitrogen supply, the *gluABCD* cluster is repressed and the growth of *C. glutamicum* is inhibited [14]. When glucose or sucrose was used as the carbon source, this gene cluster was also downregulated [15].

3.2. Regulating ammonium assimilation using AmtR and PII signal transduction systems

The transcriptional regulator AmtR (encoded by *amtR*) regulates the expression of at least 35 genes, which are related to changes of nitrogen concentration in *C. glutamicum*. These genes encode transporters and enzymes involved in the uptake of nitrogen sources, assimilation of ammonium, and signal transduction [12,121] (Fig. 10). AmtR expression is controlled by a signal cascade of uridylyltransferase (UTase, encoded by *glnD*) and GlnK (encoded by *glnK*) in *C. glutamicum* [125]. When ammonium is performed at high concentrations, AmtR represses the transcription of related genes, whereas in response to nitrogen starvation, the GlnK signal transduction protein is acetylated to form the GlnK-Amp complex and directly interacts with AmtR to release the repression [112].

PII signal transduction protein is a highly conserved regulatory protein that is widely distributed in microorganisms. The regulatory mechanism of this signal transduction protein is posttranscriptional regulation, which can coordinate the activities of multiple target proteins and regulate nitrogen metabolism through protein-protein interactions in different nitrogen states [2,55,137]. When a nitrogen source is present at low concentrations, adenylated GlnK interacts with the transcriptional regulator AmtR, thereby facilitating the uptake of ammonium. In nitrogen-sufficient conditions, deadenylated GlnK binds to the AmtB protein to reduce the uptake of ammonium and maintain the intracellular nitrogen balance [23,136]. A previous study showed that GlnK can alleviate the inhibition of N-acetylglutamate kinase (NAGK) and regulate the expression of numerous L-arginine biosynthesis-related genes, which causes excessive biosynthesis of L-arginine. Further analysis indicated that the interaction between the C and N domains of NAGK and the B-and T-loops of GlnK exerts a crucial effect on the release of feedback inhibition [196]. The regulatory mechanisms mentioned above provide insights into nitrogen metabolism in *C. glutamicum*, which provides the foundation for the

Table 2
Genetic modification of α-ketoglutarate metabolic node for GFAAs production.

Strains	Substrate	Titer (g/L)	Product	Cultivation	Modulations	Reference
C. glutamicum Sorn7	Glucose	16	Ornithine	Shake flask; batch	replacing the start codon ATG of *odhA* gene; replacing the natural RBS of *odhA* with the sequence of synthetic RBS with strengths of 837 au	[209]
C. glutamicum ZQJY-7	Glucose	12.8	Proline	Bioreactor; fed-batch	the attenuation of *odhA* gene by mutation of RBS	[212]
C. glutamicum GABA6B	Glucose	9.8	GABA	Shake flask; batch	replacing the start codon GTG *odhA* gene with the TTG; removing phosphorylation motif site of OdhI protein	[72]
C. glutamicum ORN11	Glucose	34.1	Ornithine	Shake flask; batch	the insertion of a strong P_{eftu} promoter in the upstream region of *gdh* gene;	[205]
C.glutamicum ZQJY-7	Glucose	6.59	Proline	Shake flask; batch		[212]
C. glutamicum SO18	Glucose	33.7	Ornithine	Shake flask; batch	the insertion of a strong P_{eftu} promoter in the upstream region of *gdh2* gene;	[204]
C.glutamicum ARG (*glnA–aspA–gdh*)	Glucose	53.2	Arginine	Bioreactor; fed-batch	the co-expression of *glnA*, *aspA* and *gdh* genes	[41]
C. crenatum Cc5-800	Glucose	76.8	Arginine	Bioreactor; fed-batch	the overexpression of *icd* and *gdh* genes; the attenuation of *odhA* by replacing the natural RBS of *odhA* gene with the sequence of synthetic RBS with strengths of 800 au	[107]

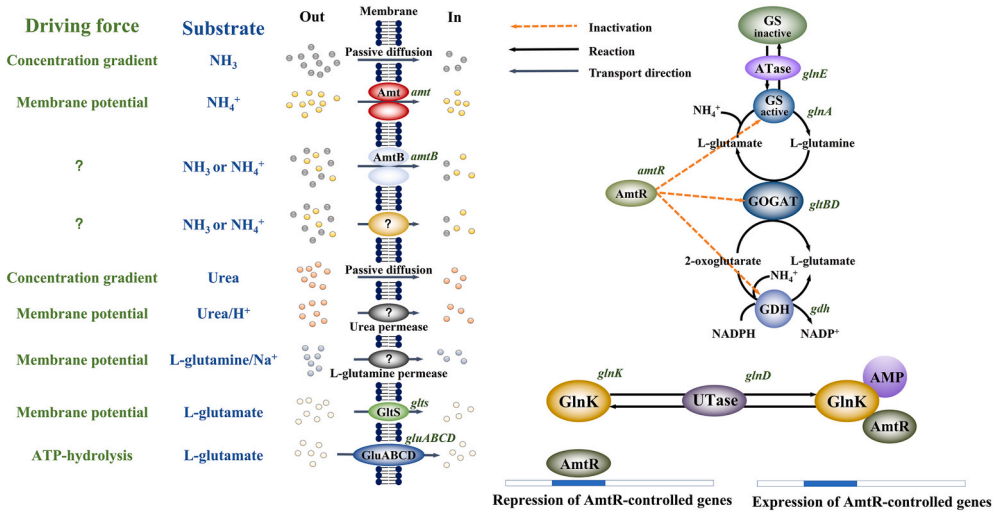

Fig. 10. Schematic diagram of nitrogen metabolism in *C. glutamicum*. *amt*, encoding an ammonium transporter; *amtB*, encoding a permease; *glts*, encoding a sodium-coupled carrier; *gluABCD*, encoding a binding protein-dependent ABC transporter; *amtR*, encoding a TetR-type repressor; *glnA*, encoding glutamine synthetase; *glnD,* encoding uridylyltransferase; *glnE*, encoding adenylyltransferase; *glnK*, encoding a nitrogen signal transduction protein; *gltBD*, encoding glutamate synthase.

development of GFAA-producing strains.

3.3. Improvement in GFAA accumulation by modulating nitrogen metabolism

The uptake and assimilation of nitrogen sources by microorganisms are extremely important for amino acid production. In particular, nitrogen source metabolism in *C. crenatum* was modified for L-arginine production. First, the AmtR was deleted to release feedback repression of genes listed in the biosynthesis of L-arginine. The mutant strain suggested a 16.67% increase in the yield of L-arginine compared to that of the control strain, reaching 30 ± 1.5 g/L during 72 h of cultivation. Next, the transporter AmtB was overexpressed to enhance ammonium uptake, which resulted in engineered strain *Cc–amtB2* that produced 60.9 ± 1.31 g/L of L-arginine, representing a 54.3% increase compared to the parent strain [192]. Furthermore, the PII signal transduction protein, GlnK, was overexpressed to regulate nitrogen source metabolism in *C. crenatum* SYPA5-5, which generated strain Cg/pXMJ19-glnK. The results of the 5-L bioreactor test showed that the production of this recombinant strain by L-arginine reached 49.978 g/L, which was 22.61% higher than that of strain Cg/5-5 (40.978 g/L) [196]. Moreover, the addition of L-glutamate, L-glutamine, and L-aspartate as nitrogen atom donors has a significant effect on L-arginine biosynthesis [41]. Thus, overexpression of three ammonium assimilation genes, namely *glnA* (encoding glutamine synthetase), *gdh*, and *aspA* (encoding aspartase from *E. coli*), in *C. crenatum* SDNN403 significantly improved L-arginine production [41].

In summary, accelerating nitrogen metabolism by deletion of *amtR*, overexpression of *amtB*, and overexpression of *glnK* were identified as effective modification strategies for improving L-arginine accumulation. Based on the knowledge of the nitrogen assimilation pathway, coexpression of *glnA*, *aspA*, and *gdh* improved nitrogen fixation metabolic reactions, which provided more ammonium for the biosynthesis of L-arginine. Therefore, the supply of a nitrogen source is also an important factor affecting the yield increase of GFAAs, especially alkaline amino acids such as L-arginine. Along with the improved cognition of the assimilation pathway and transcriptional and posttranscriptional regulation mechanisms of nitrogen metabolism, the above approaches do not only provide feasible modification strategies for modifying L-arginine biosynthesis, but also have great potential for further improving other GFAA accumulation in *C. glutamicum*.

4. Transport engineering of GFAAs

L-glutamate is a direct precursor for the biosynthesis of GFAAs. Thus, providing excess precursor L-glutamate is essential for promoting the GFAAs accumulation. To convert L-glutamate into other GFAAs rather than excreting it into the fermentation medium, specific transport systems have been modified, which are regarded as efficient strategies to improve GFAA production (Table 3). To reduce L-glutamate secretion during the biosynthesis of L-arginine, *ncgl1221* was deleted, resulting in *C. crenatum* strain CCM02 on the basis of strain CCM01. The results indicated that extracellular L-glutamate decreased to an undetectable level, whereas the production titer of L-arginine increased from 17.73 g/L to 19.56 g/L [19]. Similarly, the inactivation of glutamate transport protein in the construction of high-yielding L-ornithine strain can accomplish this goal in the same way, providing more precursors for L-ornithine biosynthesis by reducing L-glutamate secretion [209]. To meet the requirement for the biosynthesis of 5-ALA, *ncgl1221* was deleted in strain SA7, resulting in a mutant strain SA9, which indicated that the yield of ALA was improved by 9.8% [207]. In addition to Ncgl1221, MscCG2 was identified as a novel glutamate transporter, which is speculated to be a potential target for improving GFAA production. Since MscCG2 exists in *C. glutamicum* S9114, double deletion of *mscCG* and *ncgl1221* can completely block L-glutamate transport, which significantly promotes L-ornithine production [204].

LysE is a transmembrane protein that exerts a major role in the transport of L-lysine, L-arginine, and L-citrulline. For GFAAs, this transport protein ensures the fluent transport of L-arginine, which can maintain a lower intracellular L-arginine concentration, thereby releasing its feedback inhibition to the key enzyme ArgB. For this purpose, *lysE* was overexpressed in *C. crenatum* SYPA 5-5, which improved the production titer of L-arginine by 13.6% [194]. Similarly, *lysE* was selected as a gene enhancer site for L-arginine production in *C. crenatum* by prediction of the genome-scale metabolic network model (GSMM). Sequentially, inserting *lysE* with P*tac* promoter in the flanking regions of *ncgl1221* resulted in the mutant strain CCM05, which produced 14.9 g/L L-arginine during 108 h of shake flask fermentation, representing a 12.1% increase as compared with the parent strain [54]. For L-ornithine production, a recombinant strain Sorn11 was constructed based on plasmid-based overexpression of *lysE* that produced L-ornithine up to 18.4 ± 0.49 g/L, represents a 21.8% increase than the control group [209]. Additionally, to overcome the problem of genetic instability due to the introduction of a plasmid or antibiotic addition, the upstream region of this gene was inserted into a *tac* promoter [141], showing the

Table 3
Modification strategies of transport systems for GFAAs production.

Strains	Substrate	Titer (g/L)	Product	Cultivation	Modulations	Reference
C. crenatum CCM02	Glucose	19.56	Arginine	Shake flask; batch	the deletion of *ncgl1221* gene	[19]
C. crenatum CCM05	Glucose	13.29	Arginine	Shake flask; batch		[54]
C. glutamicum Sorn2	Glucose	9.8	Ornithine	Shake flask; batch		[209]
C. glutamicum SA9	Glucose	0.616	5-ALA	Shake flask; batch		[207]
C. glutamicum SO17	Glucose	32.7	Ornithine	Shake flask; batch	the deletion of *mscCG2* gene	[204]
C. crenatum SYPA-*lysE*	Glucose	35.9	Arginine	Bioreactor; fed-batch	the overexpression of *lysE* gene	[194]
C. glutamicum Sorn11	Glucose	18.4	Ornithine	Shake flask; batch		[209]
C. crenatum CCM06	Glucose	14.9	Arginine	Shake flask; batch	the insertion of a strong P_{sod} promoter in the upstream region of *lysE* gene;	[54]
C. glutamicum orn8	Glucose	19	Ornithine	Shake flask; batch	the insertion of a strong mutant P_{tac} promoter in the upstream region of *lysE* gene	[205]
C. glutamicum SA13	Glucose	0.734	5-ALA	Shake flask; batch	the deletion of *lysE* and *putP* genes	[207]
C. glutamicum GABA6C	Glucose	9.8	GABA	Shake flask; batch	the deletion of *yggB* and *cgmA* genes	[72]
C. crenatum CCM02	Glucose	14.2	Arginine	Shake flask; batch	the deletion of *putP* gene	[54]
C. glutamicum Sorn4	Glucose	13.2	Ornithine	Shake flask; batch		[209]
C. glutamicum orn9	Glucose	20.6	Ornithine	Shake flask; batch	the insertion of a transcription terminator upstream of *ncgl2228* gene	[205]
C. crenatum CCM04	Glucose	15.34	Arginine	Shake flask; batch	the deletion of *cgl2310* gene	[54]
C. glutamicum ALA7	Glucose	3.14	5-ALA	Shake flask; batch	heterologous expression of *rhtA* gene from *E. coli*	[37]
C. glutamicum CgS1/pEC-SB-*rhtA*	Glucose	14.7	5-ALA	Shake flask; batch	the co-expression of *hemA* and *RhtA* genes	[199]
C. glutamicum SA15	Glucose	0.872	5-ALA	Shake flask; batch	heterologous expression of *rhtA* gene from *E. coli*	[207]

same amount of improvement in L-ornithine production as that of plasmid-based *lysE* overexpression [205]. L-proline and L-arginine are regarded as competing for metabolic by-products of 5-ALA that share a common precursor, L-glutamate. Thus, the accumulation of by-products can accelerate the consumption of L-glutamate, which is a disadvantage for 5-ALA production. Therefore, LysE and PutP, two membrane proteins involved in the transportation of L-arginine and L-proline, were inactivated in strain SA9, resulting in a 19.1% increase in the yield of 5-ALA as compared with the control group [207].

CgmA is a permease responsible for the transport of L-arginine, L-citrulline, diamine putrescine, and cadaverine, which is regulated by the transcript repressor CgmR. Thus, deletion of *cgmR* to upregulate cgmA expression has been processed to increase cadaverine [81] and putrescine production [123]. Similarly, inactivation of the repressor CgmR improves L-arginine production by 5% [105]. The biosynthesis pathway of L-proline is regarded as a competing metabolic pathway for other GFAAs. To save the carbon skeleton for production of other GFAAs, PutP, identified as a sodium/proline symporter, was inactivated to block the secretion of L-proline, which led to intracellular accumulation of L-proline, inhibiting gamma-glutamyl kinase (encoded by the *proB* gene) and reducing the biosynthesis of L-proline. Instructed by the modification site prediction of GSMM, *putP* was disrupted in *C. crenatum* strain CCM01, resulting in an 18.1% increase in the yield of L-arginine [54]. Similarly, the deletion of *putP* has been used as a modification method to improve L-ornithine production, which can reduce L-proline accumulation as much as possible without affecting growth [209].

Ncgl2228 (encoded by *ncgl2228*) is a BCAA transporter protein [204,205]. During L-ornithine fermentation, BCAAs, such as L-leucine, L-valine, and L-isoleucine, are treated as by-products, owing to the consumption of carbon sources and negative effects on downstream separation. To reduce the accumulation of BCAAs, attenuation of *ncgl2228* was adopted, which resulted in an 8.4% increase in L-ornithine production [205]. Commonly, this BCAA transporter was identified as one of the deletion sites for developing an L-arginine-producing strain by GSMM [134]. This prediction was based on the theory that if intracellular metabolites cannot be secreted into the extracellular environment, the related anabolic pathway is prevented by feedback inhibition to protect cells from excessive accumulation of metabolites, resulting in toxic effects [160]. Therefore, the *cgl2310/ncgl2228* gene was deleted in *C. crenatum* MT strain CCM01, which increased the yield of L-arginine by 27.5% [54].

RhtA is a transmembrane protein derived from *E. coli* that capable of transport threonine and homoserine [103], but also exports 5-ALA [74]. For efficient production of 5-ALA, *rhtA* was introduced into *C. glutamicum* via a low-copy plasmid pEP2 that promotes 5-ALA production [37]. In addition, a heterologous HemA (5-aminolevulinate synthase, encoded by *hemA*) from *Rhodobacter capsulatus* together with RhtA from *E. coli* were coexpressed in *C. glutamicum* that increased the 5-ALA titer from 12.46 to 14.7 g/L [199]. For GABA, deletion of *gabP* blocks the GABA reuptake system, which has a positive effect on GABA production. For example, GabP was inactivated in the *C. glutamicum* strain RES167/pGXKZ9, which represents a 12.5% increase in GABA production [219].

In summary, inactivation of Ncgl1221 and MscCG2 completely blocks the secretion channels of L-glutamate, which promotes L-ornithine accumulation and provides a reference for the genetic engineering of L-arginine, L-proline, and L-citrulline-producing strains. In addition, excessive accumulation of by-products such as L-proline, L-isoleucine, L-leucine, and L-valine during the L-arginine fermentation process exerts a negative effect on the separation and purification processes. Reducing the biosynthesis of by-products is beneficial for improving the production of GFAAs. Traditionally, blocking by-product biosynthesis by inactivation of key enzymes of the anabolic pathways, such as LysC and ProB, has been adopted, resulting in nutritional deficiencies, which have a negative effect on cell growth. Therefore, deletion of export transport proteins has been proven to be a suitable method to prevent the secretion of by-products. The basic principle of this method is that the accumulation of by-products by blocking export channels can result in feedback inhibition, thereby regulating by-product biosynthesis while ensuring cell growth. Furthermore, the overexpression of export proteins related to the desired products is enhanced to ensure the supply of the L-glutamate precursor. LysE, CgmA, and RhtA (from *E. coli*) are various amino acid carriers that are related to the transportation of L-arginine, L-ornithine, L-citrulline, or 5-ALA. Overexpression of *lysE* or *cgmA* does not only reduce the accumulation of intracellular amino acids, but also alleviates feedback inhibition of the end products. Introduction of the *rhtA* from *E. coli* in *C. glutamicum* does not only ensure the transport of 5-ALA to the extracellular space, but also improves 5-ALA production. Moreover, blocking the product reuptake system is also a widely used method to develop high-yielding strains. In *C. glutamicum*, a reuptake system encoded by *gabP* is responsible for GABA transport, and its inactivation precluded the utilization of GABA,

thereby improving GABA production. At present, consolidated strategies related to genetic modification for export systems can be summarized into four strategies: a) Blocking the secretion channels to reduce the secretion of precursors. b) Blocking the secretion channels to reduce the biosynthesis of by-products. c) Enhancing the expression of specific transporters to promptly export GFAAs. d) Blocking the reuptake system to reduce the reabsorption process for GFAAs.

5. Anabolic pathways of GFAAs and their derivatives

The main anabolic pathways of GFAAs, their derivatives, and related regulations involved in *C. glutamicum* are described below. In addition, the genetic modulation for the production of GFAAs is also included in this section.

5.1. Metabolic engineering of the anabolic pathways for L-ornithine, L-citrulline, and L-arginine production

L-ornithine, L-citrulline, and L-arginine are important amino acids for food, medicinal, pharmaceutical, and industrial applications. Sustained efforts have been made to uncover the molecular mechanisms of these anabolic pathways and to explore efficient metabolic engineering strategies in *C. glutamicum* (Table 4).

5.1.1. Anabolic pathway of L-ornithine, L-citrulline, and L-arginine

In prokaryotes, three metabolic pathways, namely the "linear," "recycling," and "special" pathways, for the biosynthesis of L-ornithine, L-citrulline, and L-arginine have been found. These pathways require L-glutamate as a common substrate that is catalyzed by a series of different enzymes [30,104] (Fig. 11). These three biosynthetic pathways consist of eight steps of the enzymatic reaction, but their main difference is that the transfer of acetyl groups is catalyzed by different enzymes and the

Table 4
Modification strategies in anabolic pathway for L-arginine, L-ornithine, and L-citrulline production.

Strains	Substrate	Titer g l^{-1}	Product	Cultivation	Modulations	Reference
*C.glutamicum*ΔAPE6937	Glucose	13.6	Ornithine	Shake flask; batch	Two stages of adaptive evolution based on the growth selection	[69]
C. glutamicum Orn10	Glucose	20.8	Ornithine	Shake flask; batch	Insertion of a terminator in the upstream region *proB*	[205]
C. glutamicum ORN1	Glucose	12.1	Ornithine	Shake flask; batch	Removal of feedback inhibition through site-directed mutagenesis of ArgB	[67]
C. crenatum Cc-QF-4	Glucose	40.4	Ornithine	Bioreactor; batch	Deletion of *argF*; the heterologous expression of *argA* from *E. coli* and *argE* from *Serratia marcescens*	[161]
C. glutamicum SJ8074 (pEK-Ptrc::1469)	Glucose	0.32	Ornithine	Shake flask; batch	Insertion of a strong P_{trc} promoter in the upstream region of *ncgl1469* gene	[57]
C. glutamicum SJC8514 (pEK-CJBD$_{mut}$)	Glucose	12.48	Ornithine	Shake flask; batch	Co-expression of *ncgl0462* and *argCJBD*	[75]
C. glutamicum SO4	Glucose	26.8	Ornithine	Shake flask; batch	Insertion of a strong P_{tac} promoter in the upstream region of *cg3035*	[208]
C. glutamicum 1006△*argR-argJ*	Glucose	31.6	Ornithine	Shake flask; batch	Overexpression of *argJ*	[218]
C. glutamicum CO9	Glucose	6.1	Ornithine	Shake flask; batch	Insertion of a T4 terminator in the upstream region *argF*	[210]
C. glutamicum 1006Δ*argR*	Glucose	28.3	Ornithine	Shake flask; batch	Removal of feedback repression of ArgR by deletion of *argR*	[69]
C. glutamicum ΔAPE6937R42	Glucose	0.23	Ornithine	Shake flask; batch	Removal of feedback repression of ArgR by deletion of *argR*	[77]
C. glutamicum YW03	Glucose	17.3	Ornithine	Shake flask; batch	Removal of feedback repression of ArgR by deletion of *argR*	[69]
C. glutamicum Sorn10	Glucose	15.1	Ornithine	Shake flask; batch	Removal of feedback repression of ArgR by deletion of *argR*; the deletion of *argF*; the heterologous expression of *argCJBD*	[209]
C. crenatum pJC*tac-argJ*	Glucose	42.4	Arginine	Bioreactor; batch	Insertion of a strong P_{tac} promoter in the upstream region of *argJ*	[30]
C. crenatum SYPA-9039	Glucose	45.30	Arginine	Bioreactor; batch	Overexpression of *argCJBDFRGH* cluster; the inactivation of *argR*	[195]
C. crenatum Cc1	Glucose	53.2	Arginine	Bioreactor; fed-batch	Insertion of a strong P_{eftu} promoter in the upstream region of *argGH* and *argCJBDFR* operons; the inactivation of *argR*	[107]
C. crenatum H-7 GH	Glucose	67.92	Arginine	Bioreactor; fed-batch	Overexpression of *argGH* operon	[217]
C. glutamicum JML05	Glucose	51.96	Arginine	Bioreactor; fed-batch	Removal of feedback repression of ArgR and FarR	[203]
C. crenatum MT-M4	Glucose	12.3	Arginine	Shake flask; batch	Removal of feedback inhibition through site-directed mutagenesis of ArgB	[206]
C. crenatum SYPA-$argB_{EH3}$	Glucose	61.2	Arginine	Bioreactor; batch	Removal of feedback inhibition through site-directed mutagenesis of ArgB	[213]
C. glutamicum AR6	Glucose	92.5	Arginine	Bioreactor; fed-batch	Overexpression of *argGH* and *argCJBDFR* operons; the removal of feedback repression of ArgR and FarR; the insertion of a strong P_{sod} promoter in the upstream region of *carAB*	[46]
C. glutamicum CIT2	Glucose	5.43	Citrulline	Shake flask; batch	Deletion of *argR* and *argG*	[46]
C. glutamicum ΔAPE6937R42	Glucose	7.72[a]	Citrulline	Shake flask; batch	Co-expression of *argF* and $argB^{fbr}$	[31]
C. glutamicum 1006Δ*argR*	Glucose	8.51	Citrulline	Shake flask; batch	Overexpression of *argJ*	[46]

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

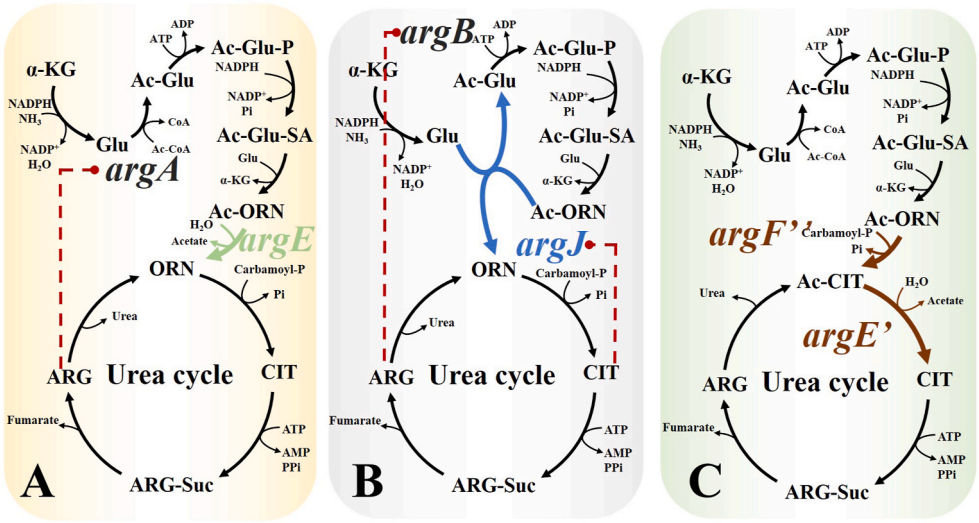

Fig. 11. Schematic diagram of three L-arginine anabolic pathways. A. The "linear" pathway; B. The "recycling" pathway; C. The "new" pathway. *argA*, encoding *N*-acetylglutamate synthase; *argE*, encoding N-acetylornithine deacetylase; *argF'*, encoding *N*-acetylornithine carbamoyltransferase; *argE'*, encoding *N*-acetylcitrulline deacetylase.

negative feedback is regulated by different genes [159]. Pathway I is the "linear" pathway, which has been found only in a few microorganisms such as *Myxococcus xanthus* [47] and *E. coli* [180]. In this pathway, acetylornithine (Ac-ORN) is deacetylated to yield L-ornithine by catalysis of *N*-acetylornithine deacetylase (NAOD, encoded by the *argE* gene), whereas *N*-acetylglutamate synthase (NAGS) is inhibited by L-arginine through a negative feedback regulation [44,180], which both differentiate the "linear" pathway from the "recycling" and "new" pathways. Pathway II is the "recycling" pathway, which is a major biosynthesis pathway. This pathway has been found in many prokaryotes, such as *C. crenatum* [30], *C. glutamicum* [61], *Thermotoga neapolitana* [108], *Geobacillus stearothermophilus* [108,143], *Pseudomonads* [178], *Neisseria gonorrhoeae* [109], and *Streptomyces coelicolor* [50]. This pathway is considered more economical than pathway I because the acetylation of L-glutamate and deacetylation of Ac-ORN are coupled through L-ornithine acetyltransferase (OATase, encoded by the *argJ* gene) [142,159]. OATase can be divided into monofunctional or bifunctional enzymes according to the species. For instance, bifunctional enzymes can accept both Ac-ORN and acetyl-CoA as acetyl group donors to acetylate L-glutamate and have been found in *G. stearothermophilus* [142] and *N. gonorrhoeae* [109]. In contrast, monofunctional enzymes can only accept Ac-ORN as a substrate, which has been found in various microorganisms, such as *S. coelicolor* [50], *C. crenatum* [30], and *C. glutamicum* [57]. In addition, another distinctive trait of the "recycling" pathway is that NAGK is inhibited by L-arginine as compared with the "linear" pathway [138,178,193]. Pathway III is the "new" pathway, which was newly identified in *Xanthomonas campestris* [119]. In this pathway, the formation of acetylcitrulline is catalyzed by acetylornithine carbamoyltransferase (encoded by the *argF'* gene) by transferring the carbamoyl group from carbamoyl phosphate to Ac-ORN. Meanwhile, acetylcitrulline is deacetylated by acetylcitrulline deacetylase (encoded by the *argE'* gene) to yield L-citrulline. However, the exhaustive details of this pathway have not been further explored as compared with the "linear" and "recycling" pathways [159].

It is worth noting that NAGS can be divided into four types: (1) the classical NAGS listed in the "linear" pathway; (2) the NAGS (bifunctional OATase, encoded by *argJ*) listed in the "recycling" pathway; (3) the short versions of NAGS (S-NAGS, encoded by the *Rv2747* gene from *Mycobacterium tuberculosis*) [35]; (4) ArgH-ArgA fusion types found in *Moritella abyssi* and *Moritella profunda* [197]. Both NAGS and OATase exist in *Geobacillus stearothermophilus* [143] and *Neisseria gonorrhoeae* [154], whereas the function of NAGS is considered to be an anaplerotic pathway to replenish the loss of acetylglutamate [24,154]. Novel types of NAGS have been continuously identified in recent years. For instance, a new class of NAGS (C-NAGS, encoded by the *cg3035* gene) from *C. glutamicum* has been discovered, which increases the diversity of NAGS [132].

The genes related to the biosynthesis of L-ornithine, L-citrulline, and L-arginine are listed as two operons, *argGH* and *argCJBDFR* [144], which are controlled by two transcriptional repressors ArgR [90,170] and FarR [45]. However, *cg3035* [73,132], *ncgl0462* (encoding Ncgl0462, displaying *N*-acetylornithine aminotransferase activity) [57], and *ncgl1469* genes (encoding Ncgl1469, displaying NAGS activity) [75] have also been found to play an unequivocal role in the anabolic pathway of L-ornithine, which is independent of the arg operons. ArgR is a global regulator that represses the transcription of *argC* and *argG*, which respond to intracellular L-arginine concentration [170]. In addition, FarR, as a global regulator, not only binds upstream of *argB*, *argC*, *argF*, and *argG* to control L-arginine biosynthesis [45,92], but also binds upstream of *gdh* and *pgl* [45,93].

5.1.2. *Modification strategies for developing L-ornithine, L-citrulline, and L-arginine-producing strains*

L-arginine and its derivatives, L-ornithine and L-citrulline, possess the same anabolic pathways in *C. glutamicum* and *C. crenatum*, indicating that uniform modification strategies can be applied to the development of the corresponding strains. The typical modification strategies related to anabolic pathways of L-ornithine, L-citrulline, and L-arginine for the construction of engineered strains are composed of four sections as follows: a. Removal of feedback inhibition of L-arginine on NAGK; b. removal of feedback repression of ArgR and FarR; c. overexpression of *argGH* or *argCJBDFR* operons; and d. blocking the competing and degradation metabolic pathways.

(1) Modification strategies focused on the anabolic pathway for L-arginine production

NAGK is a key rate-limiting enzyme that is inhibited by L-arginine in *C. glutamicum*. To obtain L-arginine high-producer strains, the removal of feedback inhibition through site-directed mutagenesis is a precondition. To obtain NAGK with high thermostability and specific activity, the variant NAGK EH3 (including E19Y, I74V, F91H, and K234T) was generated and introduced into *C. crenatum* SYPA5-5, which resulted in the recombinant strain SYPA-$argB_{EH3}$. The 96-h fermentation indicated

that L-arginine production reached 61.2 g/L, represents a 27.9% increase as compared with the control strain SYPA5-5 [213]. In addition, NAGK M4 with substitutions D311R, D312R, E19R, and H26E was generated and integrated into the chromosome of *C. crenatum* MT, resulting in a 26.2% increase in L-arginine production [206]. Furthermore, L-arginine-insensitive NAGK with substitutions H268 N or R209A [193] was introduced into the genome of *C. crenatum* SYPA5-5, resulting in the recombinant strains SYPA5-5-CcNAGK$_{H268N}$ and SYPA5-5-CcNAGK$_{R209A}$. Compared with the initial CcNAGK, the specific activities of CcNAGK$_{H268N}$ and CcNAGK$_{R209A}$ remained at 98.2% and 94.3% respectively in the presence of 15 mM L-arginine, whereas the NAGK enzyme activity in the control group declined to zero [217]. These studies suggested that feedback inhibition of CcNAGK could be removed by site-directed mutagenesis, which is a crucial strategy for developing L-arginine-producing strains.

ArgR and FarR are global repressor proteins in *C. glutamicum* and are responsible for the transcription inhibition for arg operon expression. Hence, the deletion of *argR* and *farR* is an appropriate approach for improving L-arginine accumulation. For instance, the *C. glutamicum* strain AR2 was formed by the double inactivation of *argR* and *farR*, based on strain AR1. The results of fed-batch fermentation indicated that the AR2 strain produced 61.9 g/L L-arginine, which was 82.6% higher than that produced by the control strain (33.9 g/L) [129]. Similarly, ArgR and FarR repressors were inactivated by removing *argR* and *farR* from the genome of *C. glutamicum* SNK 118, which generated the *C. glutamicum* strain JML05. After 72 h fed-batch cultivation, the L-arginine production titer of mutant strain JML05 reached up to 51.96 g/L, represents a 25.2% increase as compared with the parent strain [203]. Deletion of the above two repressors significantly improved L-arginine accumulation, indicating that stable expression of the arg operon promotes L-arginine anabolism. Overexpression of *argCJBDFR* and *argGH* operons is a common conventional strategy that is widely applied in L-arginine-producing strains. For instance, the *C. glutamicum* strain AR6 was constructed by replacing the native promoter of *argCJBDFR*. Fermentation results showed that AR6 produced 92.5 g/L L-arginine with a yield of 0.40 g/g, which are 12.8% and 14.3% higher than those of the control strain AR5 [129]. In addition, the *argGH* operon was overexpressed in *C. crenatum* strain H-7 by recombinant plasmid pDXW-10-*arg*GH, which resulted in the mutant strain H-7 GH. Then, the mutant strain produced 67.92 g/L L-arginine after 72 h fed-batch cultivation [217].

To further optimize L-arginine biosynthesis, increasing the supplementation of carbamyl phosphate facilitates the conversion of L-ornithine into L-citrulline. Carbamyl phosphate is converted from L-glutamine catalyzed by carbamoyl phosphate synthetase (encoded by *carAB*). Hence, overexpression of *carAB* by insertion of a P_{sod} promoter in *C. glutamicum* strain AR4 improved the biosynthesis of carbamyl phosphate, which resulted in the engineered strain AR5. The titer of L-arginine reached up to 82 g/L with a yield of 0.35 g/g by fed-batch cultivation of strain AR5, which was 14.3 and 12.9% higher than that of the start strain AR4 [129].

(2) Modification strategies focused on anabolic pathways for improving L-ornithine production

L-ornithine is an intermediate metabolite of the urea cycle, which can be catalyzed to form L-citrulline and L-arginine by ornithine carbamoyltransferase, argininosuccinate synthase, and argininosuccinate lyase. Thus, blocking the urea cycle by deletion of *argF* is necessary for the biosynthesis of L-ornithine. For instance, inactivation of *argF* in *C. glutamicum* S9114 generates the mutant strain Sorn1 that produced L-ornithine up to 7.97 g/L, representing 16-fold increase as compared to the parent strain (0.46 g/L) [209]. Similarly, ornithine carbamoyltransferase was inactive in *C. glutamicum* ATCC13032, which generated the mutant strain SJ8000, which produced approximately seven-fold more L-ornithine than the control strain [56]. Although L-ornithine-producing strains can be constructed by inactivation of ArgF, the accompanying problem of L-arginine auxotrophy requires exogenous addition of L-arginine, which not only increases the cost of fermentation production, but also leads to feedback inhibition of NAGK [210]. To avoid this drawback, attenuating the expression of *argF*, achieved by insertion of a T4 terminator, is an effective approach. Thus, *C. glutamicum* strain CO-9 was successfully constructed on the basis of strain CO-1, which not only downregulated the expression of *argF*, but also improved L-ornithine by 42.8% as compared with strain CO-1 harboring *argF* deletion [210].

Similar to L-arginine, the enzymes that catalyze the conversion of L-ornithine are coexpressed as an *argCJBDFR* operon, which is repressed by ArgR. Therefore, *argR* is deleted in almost all L-ornithine-producing strains [218]. Typically, *C. glutamicum* strain SJ8039 was constructed via deletion of *argR*, which increased the production titer of L-ornithine from 6.78 to 9.65 mg/g dry cell weight [56]. Additionally, deletion of *argR* in *C. glutamicum* strain ΔAPE6937 generated the mutant strain ΔAPE6937R42. The L-ornithine production titer of this mutant strain reached up to 17.3 ± 0.4 g/L, which was 27% higher than that of the control group [69]. Similar to the genetic modification of higher L-arginine-producing strains, enhancement of the L-ornithine biosynthesis pathway is an effective approach to improve L-ornithine production. Currently, two approaches are used to reinforce the L-ornithine biosynthesis pathway. The biosynthesis of L-ornithine could be enhanced by overexpression of the endogenous *argCJBD* operon. In particular, stable expression of *argJ* is the crucial step for the biosynthesis of L-ornithine in *C. glutamicum*. Thus, plasmid-based overexpression of *argJ* was introduced into strain 1006Δ*argR*, generating the mutant strain 1006Δ*argR*-*argJ*. During shake flask cultures, this mutant strain produced 31.6 g/L L-ornithine, suggests a 11.7% increase as compared to the initial strain [218]. In addition, *cg3*035 has a catalytic function in glutamate acetylation, which is similar to *argJ* [132]. Overexpression of the *cg3035* gene by insertion of the P_{tac} strong promoter increased the yield of L-ornithine by 12.6% [208]. Furthermore, plasmid overexpression of *ncgl0462*, encoding a class II aminotransferase, promotes L-ornithine production in *C. glutamicum* [75]. Coexpression of *ncgl0462* and mutated *argCJBD* genes in the *C. glutamicum* strain SJC8514 resulted in the recombinant strain SJC8514 (pEK-CJBD$_{mut}$). The fermentation results showed that the titer of the L-ornithine recombinant strain SJC8514 (pEK-CJBD$_{mut}$) was 12.48 g/L, exhibits 22.7% increase than that of the control group [75]. On the other hand, introducing an artificial "linear" pathway into *C. crenatum* can significantly promote L-ornithine accumulation. The propitious introduction of this novel artificial "linear" pathway requires highly active antifeedback inhibition of NAGS. After the experimental screening, heterologous expression of *argA* from *E. coli* and *argE* from *Serratia marcescens* was adopted. The fermentation results showed that the resulting strain *Cc*-QF-4 allowed the production of 40.4 g/L of L-ornithine, which was 48.1% higher than that obtained with the initial strain *Cc*-QF-1 (23.5 g/L) [161].

Although the inactivation of *argF* can block the conversion from L-ornithine into L-arginine and prevent the intracellular accumulation of L-arginine, supplementation of L-arginine in the medium is required for cell growth, which causes feedback inhibition of NAGK. Thus, L-arginine-insensitive NAGK, derived from *C. glutamicum* ATCC 21831 with the M64V mutation in *argB*, was introduced into the L-ornithine-producing strain, which significantly promotes L-ornithine accumulation [33,41]. Similarly, overexpression of NAGK, harboring E19R, H26E, and H268 N mutation, in strain ORN1 reached the production of 12.1 ± 0.5 g/L L-ornithine in CgXII medium with 4% glucose, which was 6.1% higher than that accumulated by ORN1(pEKEx3-*argB*) in the same medium [67].

(3) Modification strategies focused on anabolic pathways for L-citrulline production

L-citrulline is a mesostate of the L-arginine anabolic pathway. Theoretically, the genetic modification strategy for the construction of L-ornithine-high-producing strains can be completely applied to L-citrulline, and the genetic modification for L-citrulline-high-producing strains was as follows: First, blocking the conversion of L-citrulline into L-arginine. To avoid the conversion of L-citrulline into L-arginine, deletion of *argG* (encoding argininosuccinate synthetase, AS) is an essential measure for accumulation of L-citrulline [46,102]. For instance, *argG* was deleted in *C. glutamicum* ATCC13032, generating the recombinant strain CIT1, which produced 2.52 ± 0.10 g/L L-citrulline, representing 16.8-fold increase in the yield compared to the initial strain [46]. In addition, inactivation of the repressor ArgR not only increases L-ornithine and L-arginine production titer, but also improves L-citrulline production titer [46]. Deletion of *argR* in the mutant strain CIT1 resulted in the production of 5.43 ± 0.16 g/L L-citrulline, which was two-fold higher than that produced by the initial strain [46]. Furthermore, feedback inhibition is removed. The activity of the enzyme OATase (encoded by *argJ*) decreased by 50% in the presence of 30 mM L-citrulline, indicating that OATase was feedback inhibited by L-citrulline [46]. To facilitate the biosynthesis of L-citrulline, *argJ* was overexpressed in *C. glutamicum*, which improved the yield of L-citrulline by 57.2% [46].

In summary, genetic modifications, including removal of feedback repression by *argR* inactivation, the release of feedback inhibition by NAGK mutation, and enhancement of the anabolic pathway by arg operon overexpression, are universal strategies for developing L-arginine, L-ornithine, and L-citrulline (Fig. 12). The accumulation of L-ornithine and L-citrulline as intermediate metabolites of the urea cycle requires the deletion of downstream metabolic pathways. However, in practice, the "blocking" strategy results in insufficient biosynthesis of L-arginine, thus affecting cell growth, which requires excessive supplementation of L-arginine, which does not only cause feedback inhibition, but also increases the fermentation cost. To solve this problem, attenuating the expression of *argF* or *argG* by insertion of a terminator or screening for a feedback-resistance enzyme has been proven to be a feasible approach for improving L-ornithine or L-citrulline production.

5.2. Genetic modulation of the anabolic pathways for L-proline production

L-proline is an important amino acid that is widely applied in various industries, including agriculture, medicine, and food [100]. Currently, the annual production of L-proline reaches up to 350 tons through fermentation using *C. glutamicum* [60]. In *C. glutamicum*, the formation of L-proline processed via two pathways, which can be modulated to maximize the production of L-proline [68,212].

5.2.1. Anabolic pathways of L-proline and its regulation in C. glutamicum

In *C. glutamicum*, there are two anabolic pathways for the biosynthesis of L-proline that use L-glutamate as a precursor. The first pathway is the L-glutamate-derived anabolic pathway, which is composed of three enzymatic reactions including γ-glutamyl kinase (encoded by *proB*), L-glutamate-5-semialdehyde dehydrogenase (encoded by *proA*), and pyrroline-5-carboxylate reductase (encoded by *proC*), as well as one spontaneous reaction [1,68,214]. The activity of γ-glutamyl kinase is inhibited by L-proline, which regulates the intracellular L-proline concentration at a low level [166]. Alternatively, the second pathway is the

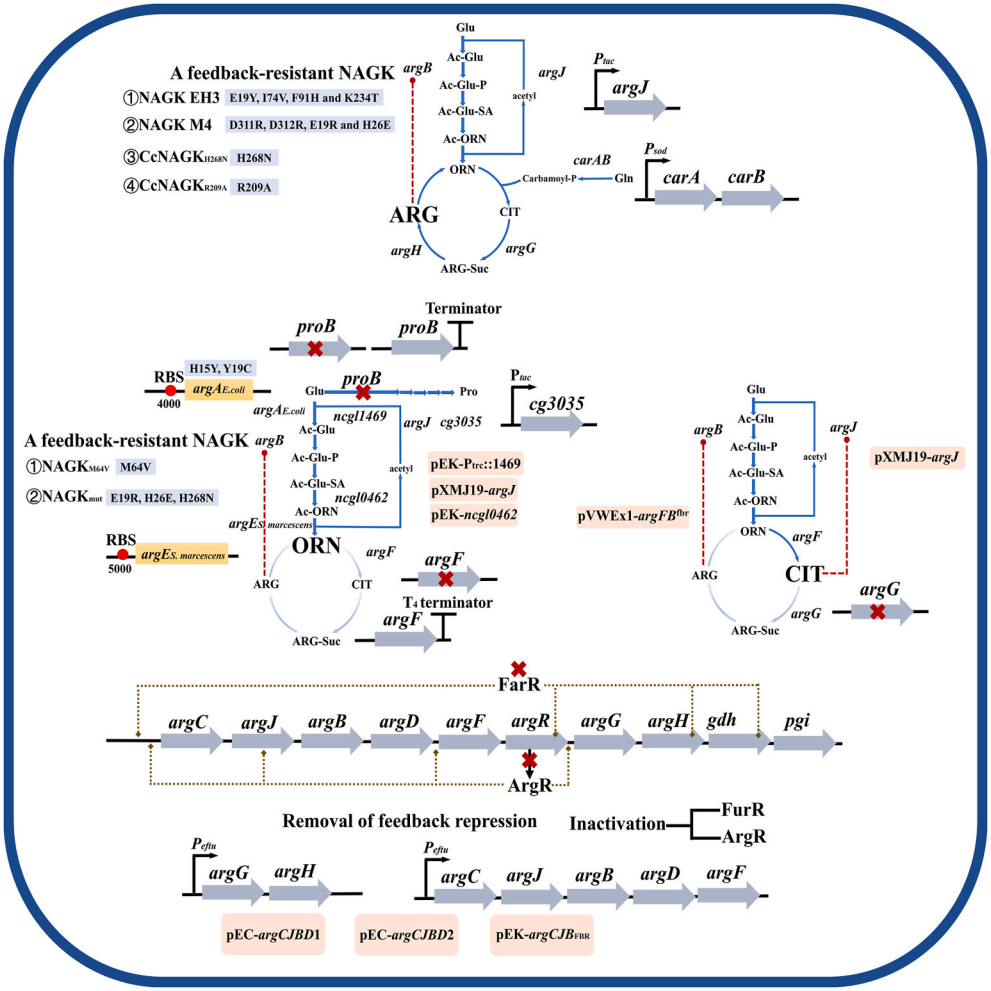

Fig. 12. Modification strategies for anabolic pathway of L-ornithine, L-citrulline, and L-arginine. *carAB*, encoding carbamoyl phosphate synthetase; *proB*, encoding γ-glutamyl kinase; *ncgl1469*, encoding Ncgl1469 displaying *N*-acetylglutamate synthase; *cg3035*, encoding Cg3035 displaying *N*-acetylglutamate synthase; *ncgl0462*, encoding Ncgl0462 displaying *N*-acetylornithine aminotransferase activity; *argR*, encoding a transcriptional regulator; NAGK, *N*-acetylglutamate kinase; FarR, a transcriptional regulator.

L-ornithine-derived anabolic pathway. In this pathway, L-ornithine, an intermediate metabolite of L-arginine biosynthesis, can be directly converted into L-proline through catalysis by ornithine cyclodeaminase (encoded by *ocd*) [68,89].

5.2.2. Modification strategies for the anabolic pathway of L-proline production

Based on these metabolic pathways, the corresponding genetic modification strategies were used to improve L-proline accumulation. The first strategy is to remove feedback inhibition of γ-glutamyl kinase. Thus, codon saturation mutation of G149 in γ-glutamyl kinase was carried out using Cpf1-assisted ssDNA recombineering, thereby generating the mutant strain ZQJY-1, which produced 4.47 ± 1.15 g/L L-proline, representing approximately four-fold increase compared to the model strain ATCC 13032 (1.35 ± 0.95 g/L) [212]. To increase the yield of L-proline, feedback-resistant γ-glutamyl kinases with the inducible promoter P_{tac} or the constitutive promoter P_{eftu} were processed to promote the biosynthesis of L-proline [212]. The second strategy was to block the degradation pathway of L-proline. Proline dehydrogenase and 1-pyrroline-5-carboxylate dehydrogenase (both encoded by *putA*) are responsible for catalyzing the conversion of L-proline into L-glutamate [120]. It has been reported that blocking this process can effectively improve L-proline production [214]. To promote the biosynthesis of L-proline, the *putA* gene was mutated, resulting in strain ZQJY-2, which produced L-proline at a titer of 6.05 ± 0.63 g/L, indicating a 35.3% increase as compared with the control strain ZQJY-1 (4.47 ± 1.15 g/L) [212]. The third strategy indirectly modifies the L-arginine anabolic pathway. It should be noted that this strategy is effectively carried out on the basis of L-ornithine higher producing strain. Overexpression of *ocd* can accelerate the conversion of L-ornithine into L-proline. For this purpose, heterologous expression of *ocd* from *P. putida* resulted in the recombinant strain JJ004 that produced L-proline at a titer of 10.0 ± 0.1 g/L, indicating a five-fold increase compared with the parent strain. Combining it with the overexpression of feedback-resistant NAGK resulted in strain JJ006 that produced L-proline at a yield of 12.7 ± 0.3 g/L—an increase of 27% compared with strain JJ004 [68]. The fourth strategy is the genome-scale metabolic model (GEMM)-assisted genetic modulation. A comprehensive GEMM of *C. glutamicum* ATCC13032 and *i*CW773, was applied to predict targets for the construction of high L-proline-producing strains. The predicted targets of *i*CW773 include the upregulation of ten genes, downregulation of one gene, and deletion of one gene. Then, those relevant targets were selected for genetic modification, generating strain Pro6, which produced 66.43 g/L L-proline during fed-batch fermentation, reaching a yield of 0.26 g/g glucose [68]. In conclusion, these genetic modification strategies are based on the L-proline anabolic pathway and L-arginine anabolic pathway, respectively (Fig. 13). Compared with these genetic modification strategies, modulating the L-proline anabolic pathway merely involves three enzymatic reactions and one spontaneous reaction, which is more economical and feasible for the biosynthesis of L-proline [68]. Simultaneously, to improve L-proline accumulation, genetic manipulations are required to remove feedback inhibition and block the degradation pathway, which enables L-proline production to reach up to 120.18 g/L at the 3 L fermentor level [212].

5.3. Genetic modulation of the anabolic pathways to produce GFAA derivatives

In recent years, the biosynthesis of GFAA derivatives based on the L-glutamate overproducing strain has attracted increasing attention. Here, the correlative metabolic pathways and genetic modification strategy of GFAA derivatives will be described in detail, including HYP, GABA, and 5-ALA (Table 5).

5.3.1. Anabolic pathway and metabolic engineering to produce GABA

GABA, a non-protein amino acid, plays an crucial role in the medical,

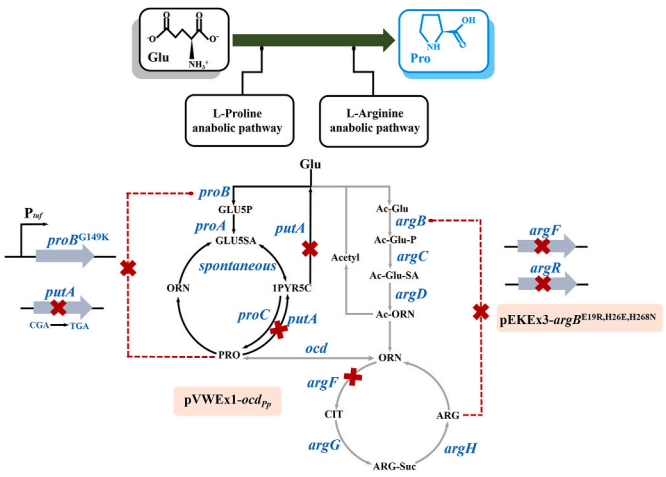

Fig. 13. Anabolic pathways of proline and its modification strategies in *C. glutamicum*. *proB*, encoding γ-glutamyl kinase; *proA*, encoding L-glutamate-5-semialdehyde dehydrogenase; *proC*, encoding pyrroline-5-carboxylate reductase; *putA*, encoding Δ1-pyrroline-5-carboxylate dehydrogenase; *ocd*, encoding ornithine cyclodeaminase.

pharmaceutical, food, feed, and chemical fields. Various studies have revealed that GABA has multiple physiological functions beneficial for human health, including a potential antitumor function [150], blood pressure-lowering function [49], treatment of neurological disorders [186], and improving the synthesis rate of brain protein [177]. In the chemical industry, GABA is a significant building block for the chemical synthesis of 2-pyrrolidone and bioplastic polyamide nylon 4 [130,146]. Thus, the production of GABA by microbial fermentation has great application value.

Traditionally, GABA production depends on *Lactobacillus* fermentation because of the presence of an inherent GAD [22]. However, *Lactobacillus* is not a native L-glutamate producer, requiring exogenous addition of L-glutamate to produce GABA. To address this problem, *C. glutamicum*, an ideal L-glutamate producer, can be engineered to convert endogenous L-glutamate to GABA. Currently, two metabolic pathways have been introduced into *C. glutamicum* to synthesize GABA (Fig. 14). Pathway I was established through the introduction of heterologous *gad* [7]. The bioconversion from the precursor L-glutamate into GABA can be catalyzed by GAD in a one-step enzymatic reaction. Pathway II was established via the putrescine route [71,72]. This is an alternative route for the biosynthesis of GABA, which requires the heterologous expression of *patA* (encoding putrescine transaminase) and *patD* (encoding γ-aminobutyraldehyde dehydrogenase) based on the putrescine-overproducing *C. glutamicum* strain. These metabolic pathways generated two genetic modification strategies for GABA production in *C. glutamicum*. The first strategy is the heterologous expression of *gad* from *E. coli* [7,22,127] or *Lactobacillus brevis* [155,156,182]. For instance, recombinant strains harboring *E. coli* GadB were constructed under the strong promoter H36 that produced 5.89 ± 0.35 g/L of GABA, which represents a breakthrough from nothing [22]. In addition, the heterologous expression of *gad* from *L. brevis* resulted in a recombinant *C. glutamicum* strain producing GABA at a titer of 29.5 ± 1.1 g/L [182]. The second strategy is the heterologous expression of the *patA* and *patD* genes from *E. coli*, which establishes an anabolic pathway of GABA in *C. glutamicum*. Introducing this modulation into the putrescine-producing *C. glutamicum* strain results in GABA production at a titer of 5.3 ± 0.1 g/L [71]. After further engineering by blocking the competitive metabolic pathway and removing the degradation pathway, the recombinant *C. glutamicum* strain GABA6C was successfully constructed, which produced GABA at levels up to 63.2 g/L [72]. Thus, the biosynthesis of GABA using pathway II had greater potential than pathway I. Considering that establishing pathway II was based on the

Table 5
Modification strategies in anabolic pathway for GABA, 5-ALA, and HYP production.

Strains	Substrate	Titer g/L	Product	Cultivation	Modulations	Reference
C. glutamicum pHG_{mut}	Glucose	14.0	GABA	Bioreactor; fed-batch	Insertion of strong promoter H36 in the upstream region of *gadB* from *E. coli*	[22]
C. glutamicum 13032-pDXW-8/*gadRCB2*	Glucose	1.45	GABA	Shake flask; batch	Expression of *gadRCB2* operon	[156]
C. glutamicum 13032/pDXW-10-*gadB1–gadB2*	Glucose	4.02	GABA	Shake flask; batch	Co-expression of *gadB1* and *gadB2*	[155]
C.glutamicum SNW201	Glucose	29.5	GABA	Bioreactor; fed-batch	Co-expression of *gadB1* and *gadB2*	[182]
C. glutamicum GABA4	Glucose	5.3	GABA	Shake flask; batch	Heterologous expression of *patA* and *patD* from *E. coli*	[71]
C. glutamicum GABA6C	Glucose	9.8	GABA	Shake flask; batch	Deletion of *argF argR* and *snaA*; the reverse mutation of *argF*; the deletion of *gabTDP* operon	[72]
C. glutamicum ALA7	Glucose	7.53	5-ALA	Bioreactor; fed-batch	Deletion of *pbp1a*, *pbp1b*, and *pbp2b*	[37]
C. glutamicum CgS1/pEC-SB-rhtA	Glucose	14.7	5-ALA	Bioreactor; fed-batch	Heterologous expression of *hemA* from *R. capsulatus* and *rhtA* from *E. coli*	[199]
C. glutamicum SA11	Glucose	0.827	5-ALA	Shake flask; batch	Co-expression of *hemL* from *E. coli* and *hemA* from *S. typhimurium*; replacing the sequence of natural RBS of *hemA* with the sequence of synthetic RBS with strengths of 5557.35 au; the deletion of *hemB*	[207]
C. glutamicum SEAL1	Glucose	1.79	5-ALA	Shake flask; batch	Co-expression of mutant $hemA^{M}$ and *hemL*; the insertion of degradation tag ASV into the C-terminus of HemB	[202]
C. glutamicum	Glucose	2.2	5-ALA	Shake flask; batch	Co-expression of *hemA* from *S. typhimurium* and *hemL* from *E. coli*	[139]
C. glutamicum ATCC13032/pEC-XK99E-p4hP	Glucose	0.106	HYP	Shake flask; fed-batch	Heterologous expression of *p4h* from *P. stutzeri*	[201]
C. glutamicum 14067P (pEKE_p4h1of)	Glucose	7.1	HYP	Bioreactor; fed-batch	Heterologous expression of *p4h* from *Dactylosporangium* sp. strain RH1	[36]
C. glutamicum Hyp-7	Glucose	21.72	HYP	Shake flask; batch	Optimization rare codons of *p4h* from *Dactylosporangium* sp. strain RH1; site-directed mutation of ProB (G446A); the deletion of *sucCD*; replacing the sequence of natural RBS of *p4h* with the sequence of synthetic RBS with strengths of 12000 au; construction of an expression cassette P_{tac}-^{RBS}*p4h-proB**	[215]

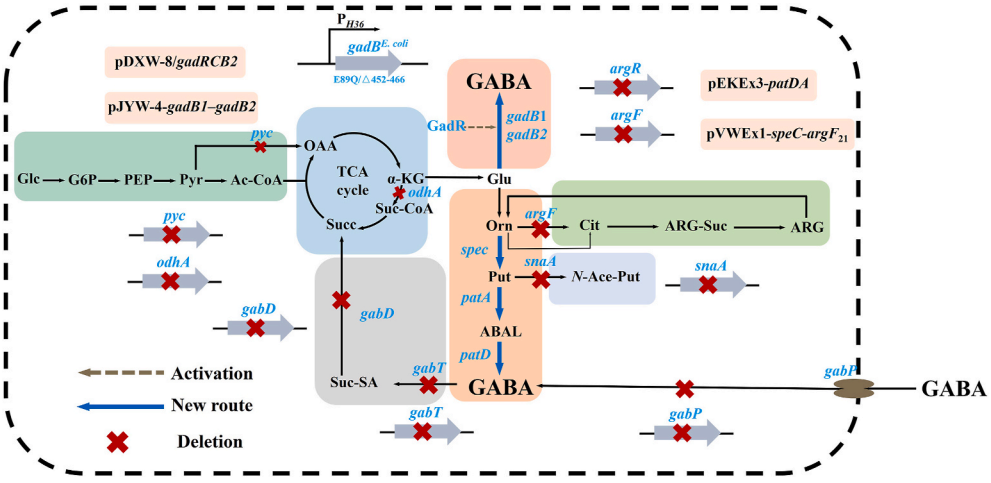

Fig. 14. Anabolic pathways of GABA and its modification strategies. *gadB1* and *gadB2*, encoding L-glutamate decarboxylase; *spec*, encoding L-ornithine decarboxylase; *patA*, encoding putrescine transaminase; *patD*, encoding γ-aminobutyraldehyde dehydrogenase; *snaA*, encoding spermi(di)ne *N*-acetyltransferase A; *gabT*, encoding γ-aminobutyrate aminotransferase; *gabD*, encoding succinatesemialdehyde dehydrogenase; *gabP*, encoding GABA-specific transporter; gadR, encoding a transcriptional regulator GadR; GadR, a transcriptional regulator; ABAL, γ-aminobutyraldehyde.

L-arginine anabolic pathway, disruption of the *argF* and *argR* genes is required to redistribute the carbon flux and remove feedback repression. In addition, producing GABA through pathway II involves the putrescine anabolic pathway, necessitating the overexpression of *spec*, *patA*, and *patD*. Furthermore, it is necessary to further increase production by knocking out *gabT*, *gabD*, and *snaA* to block the GABA degradation pathway and the competing pathway [71,72].

5.3.2. *Anabolic pathway and metabolic engineering for the production of 5-ALA*

5-ALA is an essential intermediate metabolite in the biosynthesis of vitamin B12, chlorophyll, and heme, which play an important physiological role in plants, humans, and animals [37]. Currently, the industrial production of 5-ALA depends mainly on chemical synthesis. However the environmentally unsustainable nature of this method has driven a shift toward more economical, environmentally friendly, and sustainable microbial fermentations [207]. Thus, various microbes, including *E. coli*, *C. glutamicum,* and *Rhodobacter sphaeroides*, have been extensively engineered for 5-ALA production. In *C. glutamicum*, the biosynthesis of 5-ALA has been performed by heterogeneous assimilation of two metabolic pathways, namely the C4 pathway derived from *R. sphaeroides* and the C5 pathway derived from *Salmonella typhimurium* [145] (Fig. 15). In the C4 pathway, 5-aminolevulinate synthase is responsible for condensing succinyl-CoA and glycine to form 5-ALA [37,

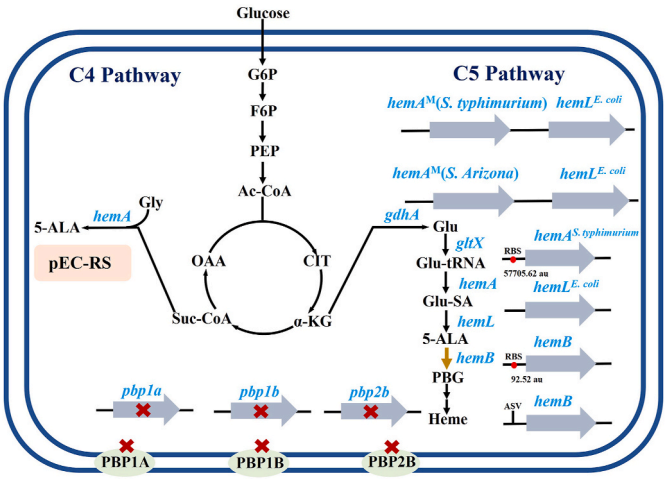

Fig. 15. Anabolic pathways of 5-ALA and its modification strategies. *hemA* (C4 pathway), encoding 5-aminolevulinate synthase; *gdhA*, encoding glutamate dehydrogenase; *gltX*, encoding glutamyl-tRNA synthetase; *hemA* (C5 pathway), encoding a NADPH-dependent glutamyl-tRNA reductase; *hemL*, encoding glutamate-1-semialdehyde aminotransferase; *hemB*, encoding 5-aminolevulinic acid dehydratase; *pbp1a*, encoding a high molecular-weight penicillin-binding protein 1A; *pbp1b*, encoding a high molecular-weight penicillin-binding protein 1B; *pbp2b*, encoding a high molecular-weight penicillin-binding protein 2B; PBG, porphobilinogen.

74]. The heterologous expression of *hemA* is a critical factor in the construction of the 5-ALA-producing strain via the C4 pathway. Thus, *hemA* from *R. sphaeroides* and *rhtA* (encoding 5-ALA transporter) from *E. coli* was introduced into *C. glutamicum*, which produced 5-ALA at a titer of 14.7 g/L [199]. However, glycine and succinate must be added into the fermentation medium to construct a 5-ALA-producing strain based on the C4 pathway, which was disadvantageous in terms of cost savings [207]. In the C5 pathway, the conversion of L-glutamate into 5-ALA was successively catalyzed by GltX (glutamyl-tRNA synthetase, encoded by *gltX*) [111], HemA (an NADPH-dependent glutamyl-tRNA reductase, encoded by *hemA*) [96,147], and HemL (glutamate-1-semialdehyde aminotransferase, encoded by *hemL*) [64]. To achieve the biosynthesis of 5-ALA in this pathway, *hemA* from *Salmonella enterica* serovar Typhimurium and *hemL* from *E. coli* were simultaneously overexpressed in *C. glutamicum* S9114, and optimization of the RBS resulted in recombinant strain SA11, which produced 5-ALA at a titer of 827 mg/L [207]. Similarly, coexpression of *hemA* and *hemL* generated a recombinant *C. glutamicum* strain that produced 5-ALA at a titer of 457 mg/L, representing a 25.9-fold increase over the parent strain (17 mg/L). An optimized fermentation medium and penicillin addition further improved 5-ALA production, reaching 2.2 g/L [139].

In conclusion, for the biosynthesis of 5-ALA, the C5 or C4 pathways can be introduced into *C. glutamicum*, each of which has its advantages and disadvantages. Although the construction of the 5-ALA anabolic pathway based on the C4 pathway only requires heterologous expression of *hemA* [37,199], 5-ALA production needs to be added to the fermentation medium, which is adverse [207]. The establishment of 5-ALA anabolic pathways based on the C5 pathway can overcome this disadvantage. This process involves the overexpression of *hemA* and *hemL* genes as well as the attenuation of *hemB* [139,202,207]. A comparison of 5-ALA production at the fermentor level indicated that the recombinant strain constructed based on the C4 pathway produced more 5-ALA than the C5 pathway [199].

5.3.3. Anabolic pathway and metabolic engineering for the production of HYP

HYP is a special chiral amino acid that naturally exists as a diastereomer, including two forms of 3-HYP and three forms of 4-HYP [201]. Among them, *trans*-HYP has important physiological functions owing to its abundance in mammalian collagen [215]. *Trans*-HYP and its derivatives have been widely used in the pharmaceutical, medical, cosmetic, and food industries. For example, HYP is considered a valuable chiral molecule for the pharmaceutical synthesis of etamycin, echinocandin, and actinomycin [5,53,157]. For a long time, the industrial production of *trans*-HYP mainly depended on the acid hydrolysis of mammalian collagen, which causes environmental pollution and increases the cost of downstream processing [63]. The increasing environmental issues of chemical processes and complicated downstream processing have promoted the development of eco-friendly *trans*-HYP production methods, such as microbial fermentation. Thus, appropriate genetic engineering strategies to breed recombinant strains with high yields of *trans*-HYP have become a research hotspot. A clear genetic background [62,73], adequate supply of precursors, and mature genetic engineering tools enable the *C. glutamicum* strain to serve as a superior HYP-producing strain, which requires heterogeneous expression of P4H. To construct the HYP biosynthesis pathway in *C. glutamicum*, *p4h* from various sources was screened and expressed, respectively. Fermentation results showed that the highest titer of *C. glutamicum* ATCC13032 expressing the *p4h* gene from *Pseudomonas stutzeri* reached up to 0.106 ± 0.002 g/L [201], indicating the feasibility of using recombinant *C. glutamicum* strains to produce HYP. Considering that the heterologous expression of *p4h* resulted in low HYP production titer, systematic metabolic engineering of *C. glutamicum* was employed to improve HYP production under the guidance of the GSMM. After various metabolic engineering modulations, including the overexpression of *p4h*, using mutated *proB*, redirecting the carbon flux of α-ketoglutarate node, and deletion of *sucCD* operon, the engineered strain Hyp-7 produced 21.72 g/L during the 60-h shaking flask fermentation [215].

6. Conclusion and prospect

In the current review, recent advances in the production of GFAAs in recombinant *C. glutamicum* strains, including physiological metabolism and genetic modification strategies, were summarized. Physiological metabolism involves metabolic pathways and metabolic regulation, including sugar uptake systems, glycolysis and the PPP, TCA cycle and related pathways, uptake of nitrogen sources, improvement in the intracellular environment, transport systems of amino acids, and the anabolic pathways of GFAAs. Constructing a robust strain requires reasonable and effective genetic modification strategies, which can be summarized as four two-word strategies including “fast uptake”, “rapid transport”, “unobstructed flux”, and “suitable configuration” (Figure 16), based on developed knowledge of the metabolic pathways and regulation of GFAA production. First, the “fast uptake” strategy involves controlling the pathway or speed of the nutrient uptake system. From another point of view, the concept of “fast uptake” involves not only the utilization of glucose or other alternative carbon sources, but also the uptake and metabolism of nitrogen sources, which can be regarded as a process that converts carbon sources and nitrogen sources into precursors that enter the anabolic pathways of target products. Second, the “rapid transport” strategy is to quickly transport the desired product from the intracellular to the extracellular environment. Meanwhile, blocking the reabsorption system or degradation pathway has been proven to be effective for improving the production titer of the desired product. Third, the “unobstructed flux” strategy is to redirect metabolic flux to the product biosynthesis pathway by regulating key nodes, specifically including removal of feedback inhibition and repression, blocking the competitive pathways and regulation at the posttranscriptional level. Fourth, the “suitable configuration” is to introduce GFAA biosynthesis pathways into cells to construct high-producing strains. Many factors should be considered in the establishment of new metabolic pathways, such as the simplicity of genetic modification, the balance between cell growth and product accumulation, the coordination between precursor formation and transformation

rates, the screening of synthase with high activity, and the avoidance of substrate addition during fermentation. The implementation of the above strategies is flexible and varies in accordance with different features to produce GFAAs. Among these, the "unobstructed flux" strategy is the most important for the construction of robust strains because it is closely related to the synthesis of GFAAs.

Although GFAAs and their derivatives produced by recombinant *C. glutamicum* strains have made great achievements in production titer, various factors should be considered in the future design of genetic modification strategies for building microbial cell factories.

(1) Systematic analysis of different metabolic modules and the design of optimal pathways are crucial for improving the efficiency and simplicity of breeding robust strains. The single module modification is suitable for the local optimization of the metabolic system, but an imbalance between different modules still exists, which will cause system instability. Modularization analysis of metabolic networks can help us understand the structure and function of these networks. Systems biology for the analysis of *C. glutamicum* metabolism has attracted a great deal of attention, especially the accurate engineering of *C. glutamicum* under the guidance of GSMM [54,215]. For example, the TCA cycle and the HYP anabolic pathway are coupled through metabolic flux and robustness analyses to balance biomass accumulation and product biosynthesis [215]. Therefore, the division, optimization, integration, and assembly of metabolic modules through systems biology analysis and GSMM prediction can achieve the goal of global optimization of intracellular metabolic networks and stable operation of the system.

(2) Optimizing the industrial properties of key enzymes contributes to establishing efficient cell factories for target product biosynthesis. Natural enzymes with low catalytic efficiency and poor industrial properties must be transformed into efficient biocatalysts through rational engineering and directed evolution. In view of this, it is necessary to establish an efficient biocatalyst library, in which a multitude of efficient catalysts with excellent industrial properties can be obtained from the mutant library through directed evolution and high-throughput screening. The enzymes obtained significantly improved substrate tolerance, thermal stability, soluble expression, and coenzyme reproducibility, which significantly promoted the accumulation of the desired products.

(3) The low-value production of GFAAs can be transformed into the biosynthesis of high-value chemicals by establishing microbial cell factories. GABA, 5-ALA, and HYP, which serve as GFAA derivatives, can be synthesized in *C. glutamicum*. The development of high-value compounds related to 5-ALA based on existing metabolic pathways is also feasible. For instance, the microbial synthesis of hemoglobin is a potential research topic. *C. glutamicum*, as a GRAS strain subject with a clear genetic background and mature molecular tools, also serves as a potential platform for hemoglobin biosynthesis.

(4) The application of dynamic regulation strategies can maximize the production capacity of microbial cell factories. Static regulation strategies based on deletion or overexpression of genes inevitably result in many issues, such as an imbalance in metabolic and energy flux, growth arrest, redox imbalance, and accumulation of toxic intermediates, which limit the production capacity of cell factories [153]. Biosynthetic pathways have been successfully optimized by improved understanding of dynamic regulation mechanisms and the emergence of a series of regulatory elements, including riboswitches, biosensors, and protein degradation tags. Dynamic regulatory strategies have been applied for the precise distribution of cell growth and metabolic flux, including those based on metabolite response, quorum sensing, environmental parameter response, and protein-level regulation. Thus, dynamic regulation strategies alleviate growth arrest and metabolic flux imbalance caused by static regulation strategies, providing a new effective method for increasing the yield of target products [28,168].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships thatcould have appeared to influence the work reported in this paper.

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