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Review

Advances in Synthetic Biology Techniques and Industrial Applications of *Corynebacterium glutamicum*

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Abstract: *Corynebacterium glutamicum* is a Gram-positive bacterium (non-spore-forming) that has been wildly used for amino acid production. Due to its stable protein secretion, low extracellular hydrolase activity, and non-toxicity, the application field of *C. glutamicum* has been greatly expanded. Currently, gene editing technology based on synthetic biology has great potential for synthetic biology research and genetic modification in *C. glutamicum* because of its ability to efficiently regulate the physiological and metabolic networks of the strain. Therefore, we summarize the gene editing tools and strategies of *C. glutamicum* from the aspects of genetic modification and expression elements, and we also describe the effects of gene editing techniques on a variety of products such as amino acids and their derivatives, recombinant proteins, and functional sugars, which provide a certain theoretical basis for the research on the modification of *C. glutamicum* strains and industrial applications. Finally, we prospect the design and industrial application of *C. glutamicum* genetic modification from multiple perspectives based on gene editing techniques.

Keywords: *Corynebacterium glutamicum*; genetic modification; gene editing techniques; expression elements; application

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1. Introduction

C. glutamicum is a Gram-positive microorganism with a genome GC content of 53.8%. It exhibits tolerance to various inhibitors, has high environmental adaptability, fast metabolism and growth rates, and has been deemed safe by the US FDA [1,2]. After Kinoshita et al. discovered in the 1950s that *C. glutamicum* could produce glutamic acid, it became a major production strain for various amino acids in the global fermentation industry [3,4]. This has also stimulated numerous researchers to explore the biosynthesis of amino acids and their derivatives in *C. glutamicum*. Since then, research on the production of various amino acids has thrived, resulting in significant increases in yields and dramatic cost reductions. Therefore, it is even more crucial to develop industrially high-yielding strains [5,6]. With the continuous advancement of science and technology, gene editing techniques are being progressively developed and applied to precisely engineer *C. glutamicum*. Currently, engineered *C. glutamicum* is increasingly utilized for producing various chemical substances, such as parabens, natural plant products such as eriodictyol, recombinant proteins, industrial enzymes including maltooligosyltrehalose synthase, biofuels, and biochemicals that comprise nutritional products such as organic acids and functional sugars. Its applications are widespread across industries, including chemicals, food, medical, cosmetics industry,

and animal feed, as shown in Figure 1 [7–11]. *C. glutamicum* has emerged as a favored host for industrial-scale production.

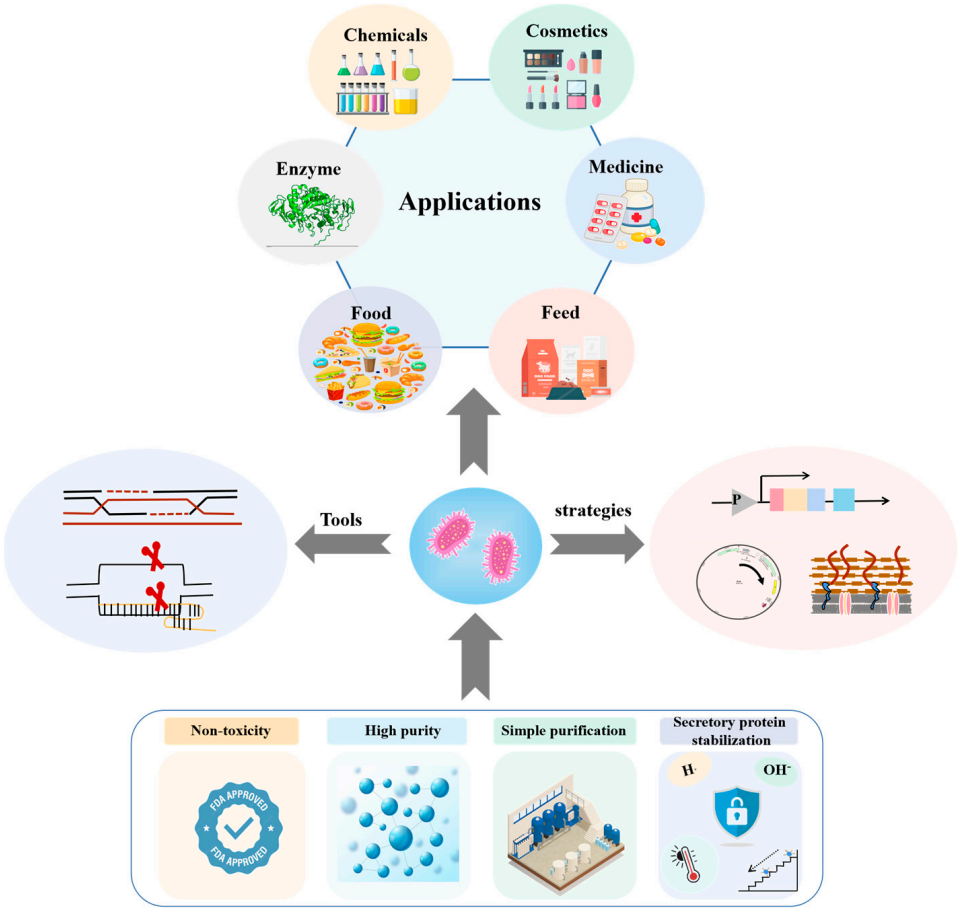

Figure 1. Advantages of *C. glutamicum* chassis cell, gene editing tools and strategies, and their diverse applications.

At present, research has been conducted on genetic modification in model strains, such as *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* [12–15]. Compared to these model strains, *C. glutamicum* exhibits unique physiological characteristics. These include non-toxicity and host cell protein contamination, a well-established protein secretion system that includes Sec and Tat pathways, high extracellular target protein purity, simple protein purification, and stable secretion of proteins. Additionally, the strain exhibits resistance to phage infection during the culture process and boasts numerous advantages, including high-density cultivation and simultaneous utilization of multiple carbon sources [16–18]. Thus, *C. glutamicum* is a highly promising microbial strain for sustainable biosynthesis, which has garnered significant attention from researchers seeking to explore its full potential. In the late 20th century, the isolation of plasmid DNA from *C. glutamicum* and the development of first-generation gene editing technology were significant milestones in engineering *C. glutamicum* [19]. At the beginning of the 21st century, German and Japanese scholars jointly published the complete genome sequence of *C. glutamicum* ATCC 13032, which served as a cornerstone for the genetic engineering of *C. glutamicum* [20]. With the emergence of the genome era, novel gene editing technologies have been developed, and the publication of *C. glutamicum*'s genome sequence has accelerated the research and application of gene editing technology in this organism. Recent advancements in molecular biology, molecular genetics, and synthetic biology have accelerated significant progress in the research on the genetic engineering of *C. glutamicum*. Microbial strains, which are designed and constructed using genetic editing technology, can biosynthesize numerous high-value

products while avoiding growth defects and environmental intolerance issues commonly associated with strains obtained through traditional mutagenesis in culture [21]. In practical applications, the development of *C. glutamicum* with superior performance and efficient green biotechnology can serve as an ideal cell factory for industrial production purposes.

In this review, we summarize the comprehensive research and application of synthetic biology tools and techniques in *C. glutamicum*, from the aspects of gene editing strategies as well as gene transcription and expression regulation. We will also introduce practical applications through product production examples. Finally, we prospect the design and industrial application of *C. glutamicum* from four perspectives: developing efficient synthetic biology tools, constructing high-efficiency and stable exogenous expression vectors, studying microbiological genetic mechanisms in-depth, and utilizing artificial intelligence.

2. Gene Editing Techniques for the *C. glutamicum*

2.1. Traditional Gene Editing Techniques

With the advancements in genomics and molecular biology, there are now many tools available that can be used to engineer microbial strains. These tools can be broadly categorized into two groups: traditional gene editing technologies and CRISPR technologies. Traditional gene editing technologies refer to the introduction of target genes into the host cell through homologous recombination, reverse screening with screening markers, and then editing target genes through an allelic exchange. Currently, traditional gene editing techniques applied to *C. glutamicum* can be categorized into non-replicating plasmids (suicide plasmids) and replicating plasmids-mediated homologous recombination, Cre/loxP-mediated site-specific recombination, and RecT-mediated single-stranded recombination based on the method of recombination [22–24].

2.1.1. Homologous Recombination Mediated by Non-Replicating Plasmids

Non-replicating plasmids, also known as suicide plasmids, can be categorized based on their mediated homologous recombination into single-exchange and double-exchange recombination. Single exchange is a method of inactivating the target gene by introducing resistance markers into the host strain. Since the introduction of the resistance gene may alter some physiological properties of the host strain or influence the gene expression or inactivation of other genes, another allelic exchange is performed on top of this to discard other parts of the gene editing process, and this method is called double exchange [25].

In the 1990s, Jäger et al. [26] developed a gene knockout method for *C. glutamicum* by utilizing the *sacB* gene. Since then, the pk18*mobsacB* system, a secondary exchange homologous recombination mechanism mediated by a suicide plasmid, has become the most widely used gene editing system for *C. glutamicum* to date. The *sacB* gene in this system functions as a negative screening marker gene by encoding a secreted sucrose fructanase. This enzyme catalyzes the hydrolysis of sucrose into glucose and fructose while also facilitating the synthesis of fructose into a polymeric fructan that is lethal to the strain. By applying a reverse screening strategy, the appropriate colonies can be obtained when cultivating the suicide plasmid in a medium containing sucrose. Zhang et al. [27] used the pk18*mobsacB* system to adjust the metabolic pathway associated with 4-hydroxyisoleucine biosynthesis in *C. glutamicum* by manipulating gene expression through knockout or overexpression strategies. Currently, the classical pk18*mobsacB* system is a commonly used tool for researchers to engineer strains. However, the practical application still presents some challenges, such as prolonged recombination time, complex operation, and susceptibility to false positives [28,29]. In response to issues with the pk18*mobsacB* system, certain scholars have proposed alternative anti-selection markers and nuclease systems. Kim et al. [30] used the *rpsL* gene (streptomycin resistance) as a counter-screening marker for *C. glutamicum*, which not only improved the editing efficiency but also improved the growth of the cells. Ma et al. [31] used the *upp* gene as a reverse screening marker in combination with the I-SceI-mediated recombination system to reduce the likelihood of false positive strains and achieve efficient mutant screening. Wang et al. [22] attempted to

mutate the *rpsL* gene encoding small ribosomal protein S12P in *C. glutamicum*, resulting in streptomycin resistance and significantly enhancing counter-selection efficiency during suicide plasmid-mediated genome editing. This approach simplifies strain screening and improves overall efficiency.

2.1.2. Site-Specific and Single-Stranded Recombination

Cre Recombinase (Cyclization recombinase) was first identified in 1981 from P1 phage and is a site-specific recombinase that exhibits catalytic activity. Cre recombinase mediates specific recombination between two LoxP sites (a palindromic sequence of 34 base pairs), which enables the deletion or recombination of gene sequences located between the LoxP sites. Compared with homologous recombination methods, this technique has been applied to gene knockout, exogenous gene integration, and screening of efficiently expressed motifs due to its high efficiency, specificity, and accuracy. As such, it is a powerful tool for gene editing technology [24,32].

Suzuki et al. [24] developed a Cre-loxP-mediated deletion system to achieve the deletion of large fragments without affecting the normal growth of *C. glutamicum* and improved the efficiency of gene knockout. However, the knockout efficiency of this method is unsatisfactory due to prolonged secondary recombination time, reversible LoxP site mediated by Cre, and gene residues present in the Cre/loxP system. Therefore, the RecT recombination system was subsequently introduced in a later study by the researchers. RecT is a single-stranded DNA (ssDNA) annealing protein (SSAP) that utilizes the RecT recombination system derived from the phage Rac's *recT* gene to facilitate single-stranded recombination, which is both straightforward and independent of DNA sequence and length [29]. In 2013, the first RecT-based recombinant system was developed and applied in *C. glutamicum*, which was combined with nanosensing technology to achieve rapid and efficient strain isolation [33]. Later on in the research, RecT-mediated single-stranded recombination was gradually applied to engineer strain. However, it was found that this system was inadequate for editing multiple genes simultaneously in *C. glutamicum*. Therefore, researchers have developed gene editing systems such as the RecET-Cre/loxP system and the CRISPR/Cpf1-RecT system, which have been applied in *C. glutamicum*, further enriching the arsenal of microbial gene editing tools [34–36].

2.2. CRISPR/Cas Gene Editing Techniques

2.2.1. CRISPR/Cas9 System

CRISPR technology was first proposed in the 1990s and has since been widely used for gene editing across various fields, including life sciences, medicine, and agriculture. Among these applications, the CRISPR/Cas9 system stands out as a simpler yet highly effective tool that is currently favored by researchers worldwide [37]. CRISPR/Cas9 technology includes two fundamental processes: gRNA-guided Cas9-targeted DNA cleavage and subsequent DNA repair. Additionally, DNA repair mechanisms include non-homologous end joining (NHEJ) and homology-directed repair (HDR).

Since non-homologous end repair is absent in *C. glutamicum* and homologous end repair is inefficient, the researchers introduced a CRISPR-based base editing system into the organism. Base editing technology is constructed by fusing the expression of Cas9 nuclease and deaminase protein, which enables targeted mutations without inducing DNA double-strand breaks, exogenous DNA templates, or homologous recombination repair mechanisms [38]. There are two primary categories of base editors, namely the CG-to-TA cytosine base editor (CBE) and the AT-to-GC adenine base editor (ABE), both of which have been applied in *C. glutamicum* [39–42]. Among them, Wang et al. [39] fused CRISPR/Cas-based Cas9 and cytidine deaminase (AID)-based cytosine base editor (CBE) tools to achieve multi-locus gene editing in *C. glutamicum*. The BETTER technology was subsequently developed by the researchers based on the base editing system, which allows for simultaneous editing of up to 10 genes with varying expression levels, thereby facilitating gene editing diversification [43]. A study revealed that the leakage of the

Cas9 toxin posed challenges in transferring plasmids containing the *Cas9* gene into *C. glutamicum* [44]. Therefore, to mitigate the toxicity of Cas9, the researchers integrated the *Cas9* gene and rec-ET-encoded recombinase into the genome. They also constructed plasmids carrying donor DNA and sgRNA expression cassettes to mitigate Cas9-induced toxicity. Ultimately, they successfully produced a mutant strain of *C. glutamicum* that was highly efficient in producing L-homoserine [45]. And other studies are shown in Table 1. The development and application of these gene editing technologies offer novel approaches for multi-locus gene editing in *C. glutamicum*, expedite the genetic engineering of *C. glutamicum*, and provide additional tools for fundamental gene editing.

2.2.2. CRISPR/Cpf1 System

Compared to CRISPR/Cas9, the CRISPR-Cpf1 system has attracted widespread attention as a novel gene editing tool with minimal off-target effects and no cellular toxicity. Cpf1 (Cas12a) is a type V, class II CRISPR effector protein with specific double-stranded ribonucleic acid endonuclease activity. It was initially characterized in *Francisella novicida* U112 [46]. Compared to Cas9 protein, Cpf1 protein exhibits several advantages: it can recognize and cleave the AT-rich proto-spacer adjacent motif (PAM) without requiring tracrRNA; its own RNase activity enables processing of CRISPR sequences into mature crRNA; its smaller molecular weight facilitates easier entry into tissues and cells [46,47]. In addition, the Cpf1 protein generates sticky ends upon cleavage, while the Cas9 protein produces blunt ends, as illustrated in Figure 2a,b. The presence of sticky ends can facilitate the insertion of target genes into specific loci through non-homologous recombination. Given the advantages mentioned above, the CRISPR/Cpf1 system indicates excellent potential for application in the field of synthetic microbial biology. Therefore, researchers have invested a lot of manpower and material resources in research and development to improve, some of the results of the research progress are shown in Table 1.

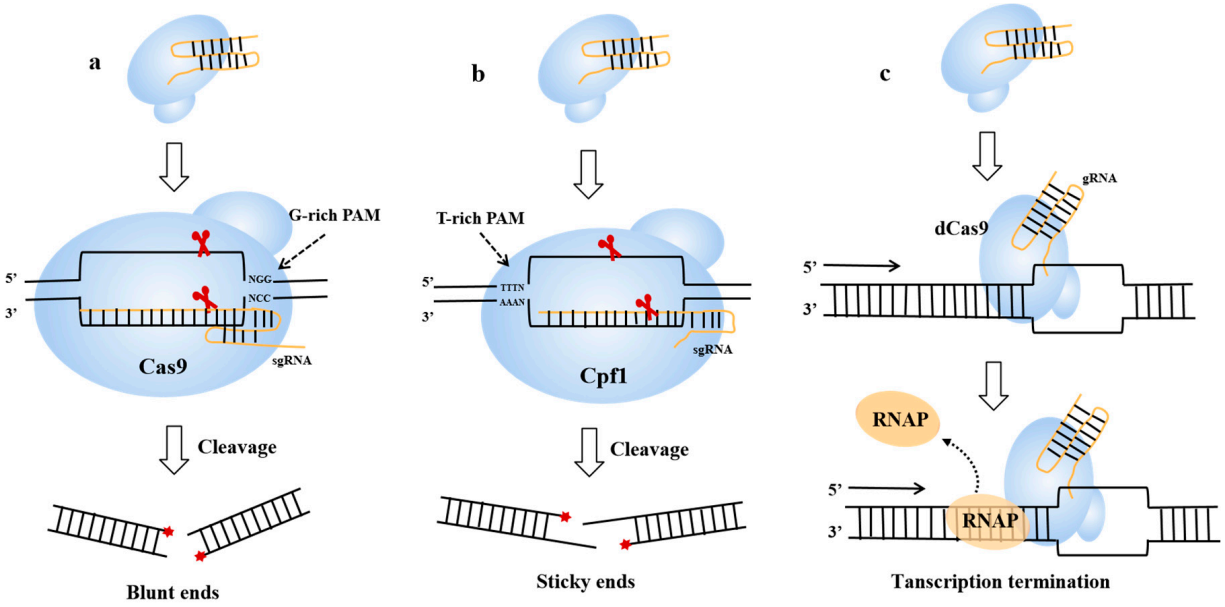

Figure 2. CRISPR- Cas9/Cpf1, CRISPRi gene editing characteristics. (**a**) Cas9 protein shears to form blunt ends; (**b**) Cpf1 protein shears to form sticky ends; (**c**) the dCas9/gRNA complex binds to target genes to suppress gene expression. RNAP: RNA polymerase.

Table 1. Research progress in the gene editing techniques of CRISPR/Cas and CRISPR/Cpf1 systems in *C. glutamicum*.

Editing System	Features	Editing Efficiency	Applications	Ref
CRISPR/Cas9	Cas9 protein codon optimization	20% knockout efficiency	Obtained γ-aminobutyric acid production strain.	[44]
CRISPR/Cas9	Dual Plasmid System	Knockout rate of *porB* gene up to 100%	Enhanced expression of GFP [1] in strains lacking specific genes.	[48]
CRISPR/Cas9	Co-expression of Cas9 and gRNA	Gene knockout efficiency was 30.8–60%, and gene integration efficiency was 16.7–62.5%	Obtained strains of *C. glutamicum* ATCC 13032 and ATCC 13869.	[49]
CRISPR/Cpf1	Optimized the original PAM sequence, spacer length, and repair template type	Gene knockout efficiency of about 32%	Improvement of isobutyric acid production in *C. glutamicum.*	[50]
CRISPR/Cpf1	Multi-locus editing, targeted deletion of large DNA, and insertion of possible	The simultaneous editing efficiency of two genes was 91.6%, while the knockout efficiencies for DNA fragments of 1 kb, 5 kb, and 20 kb were 79.6%, 91.3%, and 36.4%, respectively	Obtained a strain of *C. glutamicum* ATCC 14067.	[36]
CRISPR/Cpf1	ssDNA recombination binding, in situ saturation point mutation	Small fragment gene editing efficiency of 86–100% efficiency	Obtained high-yielding strains resistant to L-proline inhibition.	[51]
CRISPR/Cpf1	Using CRISPR-induced DNA double-strand breaks as counter-selection	Knockout efficiency > 80%, gene integration efficiency > 76% (gene length within 4 kb)	High-yielding D-pantothenic acid (vitamin B5) producing strains were constructed.	[52]

[1] GFP: Green fluorescent protein.

2.3. CRISPR Interference Technology

CRISPR interference (CRISPRi) is a highly specific silencing of transcripts based on the CRISPR/Cas system of RNA. The typical CRISPRi system is found in the type II CRISPR machinery of *Streptococcus pyogenes*. The system comprises two components, catalytically inactivated Cas9 (dCas9) and guide RNA (gRNA). Upon binding to the target DNA, the dCas9/gRNA complex exerts a spatial site-blocking effect on the transcriptional machinery, resulting in gene expression repression [53], as shown in Figure 2c.

Cleto et al. [54] increased the amino acid productivity of *C. glutamicum* by dCas9 downregulation of genes *pgi*, *pck*, and *pyk*. The researchers introduced mutations to the *Cas9* gene in the genome and constructed a double plasmid system with *mCherry* as a reporter gene. This resulted in the downregulation of *mCherry* expression, demonstrating the effective suppression of gene expression by the CRISPR-dCas9 system [55]. Gauttam et al. [56] applied CRISPR interference to downregulate the *ArgH* and *Pgi* genes. They demonstrated that in *C. glutamicum*, the *ArgH*-encoding gene catalyzes the conversion of argininosuccinate lyase to L-arginine, and the Pgi-encoding gene heterodimerizes fructose-6-phosphate to glucose-6-phosphate. Therefore, CRISPRi technology is now utilized for the characterization of functional genes such as cell growth and environmental tolerance, reduction in toxic intermediates, blocking competing pathways in strain fermentation, etc. This is due to its efficient and rapid gene suppression ability in a stable manner without causing gene deletion or mutation [56–58].

3. Elements of Gene Expression and Regulatory Strategies

3.1. Promoters

A promoter is a DNA sequence located upstream of the 5′ end of a structural gene that specifically activates RNAP to bind precisely to template DNA for transcription initiation. As the promoter serves as the initiation point for controlling gene transcriptional expression, it stands as a crucial element in enhancing the yield of the target product. To date, compared to *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae*, there are relatively few mature promoters available for *C. glutamicum*, which limits both the use of strategies to precisely regulate the expression levels of *C. glutamicum*-associated genes via promoters and the development of various synthetic biology tools that rely on promoters. More studies in *C. glutamicum* focus on inducible promoters, such as IPTG-inducible (isopropyl-β-D-thiogalactopyranoside) promoter tac [59], arabinose-inducible promoter araBAD [60], etc.

Most of the inducible promoters currently characterized are stronger than endogenous constitutive promoters. However, their use requires expensive inducers, which increases costs, and some of these inducers may be toxic or unsuitable for industrial applications. As a result, the use of constitutive promoters has been increasingly favored in recent studies [61–65]. In addition to screening for more robust and more efficient promoters, replacing or modifying existing promoters with efficient alternatives represents a direct and effective approach to enhancing target product expression. In subsequent research, a series of synthetic promoters were gradually developed to achieve precise regulation of microbial metabolism. In summary, there are three main sources of promoters currently used in *C. glutamicum*: (1) endogenous promoters derived from itself, (2) promoters obtained from other hosts, and (3) promoter libraries constructed through random mutations, as shown in Table 2.

Table 2. Commonly used promoters in *C. glutamicum*.

Promoter	Type	Representation	Hosts	Effect	Ref
P_{tac}	IPTG-inducible	LysE expression	*C. glutamicum* S9114	The yield of l-ornithine obtained was 25 g/L.	[66]
P_{T7}	IPTG-inducible	eYFP [1]	*C. glutamicum* MB001 (DE3)	The fluorescence level is 3.3 times greater than that of P_{tac}.	[67]
P_{trc}	IPTG-inducible	synthesis of poly-hydroxybutyrate (PHB)	*C. glutamicum* ATCC14067	The strain was found to accumulate approximately 2–14% of PHB in both LBG medium [2] and l-glutamic acid production media.	[68]
P_{A256}	Ethanol induction	sfGFP [3]	*C. glutamicum* CGMCC 1.15647	The ultimate yield was approximately 2.5 times greater than that achieved with the potent promoter P_{H36}.	[69]
P_{iolT1}	Myo-inositol induction	GFP	*C. glutamicum* ATCC 13032	The yield of 5-aminolevulinic acid (5-ALA) was enhanced to 0.73 g/L.	[70]
P_{araBAD}	Arabia sugar-inducible	Succinic acid yield	*C. glutamicum* ATCC 13032	Under the condition of arabinose as the sole carbon source, a yield of 41.0% was achieved to the theoretical maximum yield of succinic acid.	[71]
$P_{cat\text{-}B}$	Benzyl alcohol induced	EGFP [4]	*C. glutamicum* CGMCC1.15647	The NT-proBNP yield was tenfold higher compared to that of the P_{tac} strain.	[72]

Table 2. *Cont.*

Promoter	Type	Representation	Hosts	Effect	Ref
P_{tacM}	Constitutive	Glutamate decarboxylase (GAD) enzyme activity	*C. glutamicum* SH	The activity of GAD was significantly increased, resulting in the production of more than 25 g/L of gamma-aminobutyric acid (GABA).	[73]
P_{CP_2836}	Constitutive	GFP	*C. glutamicum* CP	The exponential and stable phases of fluorescence intensity accounted for 75% and 28% of P_{tuf}, respectively.	[74]
P_{pgk}	Constitutive	β-Galactosidase	*C. glutamicum* ATCC 13032	The enzyme activity is approximately 1.5-fold higher than that observed at the P_{tac} transcriptional level.	[61]
P_{sod}	Constitutive	The activity of 2,3-butanediol dehydrogenase.	*C. glutamicum* CGS9	The strain increased the yield of 2,3-butanediol to reach 16.58 g/L.	[75]
P_{70}	Synthetic	GFP	*C. glutamicum* ATCC 13032	Its strength of it exceeds that of the P_{tac} by 121%.	[76]
P_{H36}	Synthetic	Accumulation of L-histidine	*C. glutamicum* ATCC 13032	The histidine biosynthesis pathway was upregulated, resulting in the production of 5.0 g/L l-histidine and 3.9 mg/L l-creatine.	[77]
$P_{brnFE\ 7}$	L-isoleucine sensing promoter	The activity of threonine dehydrogenase	*C. glutamicum* WM001	The strain increased the yield of L-isoleucine by 36.1%.	[78]

[1] eYFP: Enhanced Yellow Fluorescent Protein; [2] LBG medium (g/L): peptone 10 g, yeast powder 5 g, NaCl 10 g, glucose 5 g; [3] sfGFP: Superfolder Green fluorescent protein; [4] EGFP: Enhanced Green Fluorescent Protein.

3.2. Non-Coding Regions in the 5′ Untranslated Region (UTR)

Promoter optimization is a widely used approach for optimizing transcriptional strategies. However, screening for strong promoter-5′ UTR complexes to achieve precise regulation of metabolic pathways in microorganisms has been applied to numerous model strains, including *E. coli* and *B. subtilis*. The 5′ untranslated region (UTR) is a non-coding segment located at both ends of the messenger RNA (mRNA) molecule, and its secondary structure plays a crucial role in regulating the efficiency of internal ribosome binding site (RBS) recognition and start codon identification [79]. The initiation of translation in the 5′ UTR region depends on recognition by 16S rRNA of the RBS ribosome binding site, and the efficiency of this interaction affects gene expression levels. Therefore, optimizing the non-coding 5′ UTR region, developing robust complexes in the 5′ UTR, and improving RBS binding efficiency are crucial strategies for improving cellular efficacy.

Li et al. [18] chose 90 potentially promoter-5′ UTR-containing sequences of *C. glutamicum* species and obtained 17 strong PUTRs by screening with fluorescent proteins as reporter genes. Yim et al. [80] found a 3.2-fold increase in ScFv secretion from *C. glutamicum* upon substitution of the original Shine–Dalgarno (SD) sequence with tpi-SD. Feng et al. [73] optimized the RBS sequence and the spacing between the start codon and RBS core sequence based on a series of natural promoters, which ultimately resulted in an 82% increase in γ-aminobutyric acid (GABA) production. Similar to expression elements such as promoters, in most cases, RBSs are often incompatible across different hosts. Therefore, it is important and necessary to develop RBS elements that can be universally applied across species. After constructing a library of RBS mutants and characterizing them individually in *C. glutamicum* and *E. coli*, the researchers found that 18 RBSs exhibited similar intensities between the two species. After analyzing the correlation of RBS intensities between the two hosts, it was finally determined that these 18 RBSs could be used across species [81]. In addition, some sequences, including GAAAGGCGA, GAAAGGA, AGAGGGG, GTTATTAG,

and AAAGGGA, were also identified as strong RBS sequences for the expression of target genes in *C. glutamicum* [61,82,83].

3.3. Signal Peptides (SPs)

SPs, typically composed of 20–40 amino acids, facilitate the translocation of secreted proteins to the extracellular space and are subsequently cleaved by signal peptidase (SPase) during or after transmembrane transport [84]. In *C. glutamicum*, secreted proteins are transported through two distinct pathways: the Sec pathway for unfolded proteins and the Tat pathway for folded proteins [17], as shown in Figure 3b,c. According to different transport pathways, SPs can be classified into Sec pathway and Tat pathway SPs. The SPs of proteins in the Sec or Tat pathway exhibit similar characteristics but also have their specificities, as illustrated in Figure 4. A range of SPs, including cg1514, CspB2, PorB, CspA, cgR1176, and cgR2070, have been successfully isolated and identified in *C. glutamicum* [85–89]. To enhance the production of extracellular secreted proteins, the screening of an optimal signal peptide (SP) is a primary and equally crucial consideration. Different production strains may exhibit varying levels of secretory expression for target proteins with different SPs, and the best choice for one strain may not necessarily be optimal for another. Of course, previous studies have demonstrated that the optimal SP varies for different secreted proteins [90,91].

Currently, the limited knowledge of SP properties required for target secreted proteins and the unpredictability of optimal SPs for specific secreted proteins necessitate the construction and screening of high-volume SP libraries combined with high-throughput screening. This strategy is by far the most popular and successfully applied method for identifying optimal SPs [90,92]. Jin et al. [91] constructed an SP library based on previously validated SPs to enhance enzyme secretion efficiency. Subsequently, they identified the optimal Cg2196 SP for secretion in *C. glutamicum* through SDS-PAGE and Western blot analysis. Other researchers have effectively utilized the Sec SP library of *B. subtilis* to enhance protein secretion in *C. glutamicum* [93,94]. Using keratinase as a model enzyme, Hemmerich et al. [94] studied the effect of *B. subtilis* Sec SPs and process conditions on the performance of heterologous protein secretion in *C. glutamicum* and demonstrated that optimizing biological process conditions could improve the efficiency of heterologous protein secretion.

In industrial applications, *C. glutamicum* presents a highly promising option for the expression of exogenous proteins due to its safety profile. However, the tough and thick cell wall of *C. glutamicum* poses challenges in industrial applications due to difficult fragmentation and increased cost, as shown in Figure 3a. Although the addition of SPs for extracellular secretory expression vector construction has shown some potential in *C. glutamicum*, further research is needed before industrialization can be achieved. Therefore, altering the structure of the cell wall and enhancing its permeability is crucial in addressing this issue. This not only resolves the challenge of cell wall fragmentation but also facilitates extracellular secretion of the target protein to a certain extent, as shown in Figure 3.

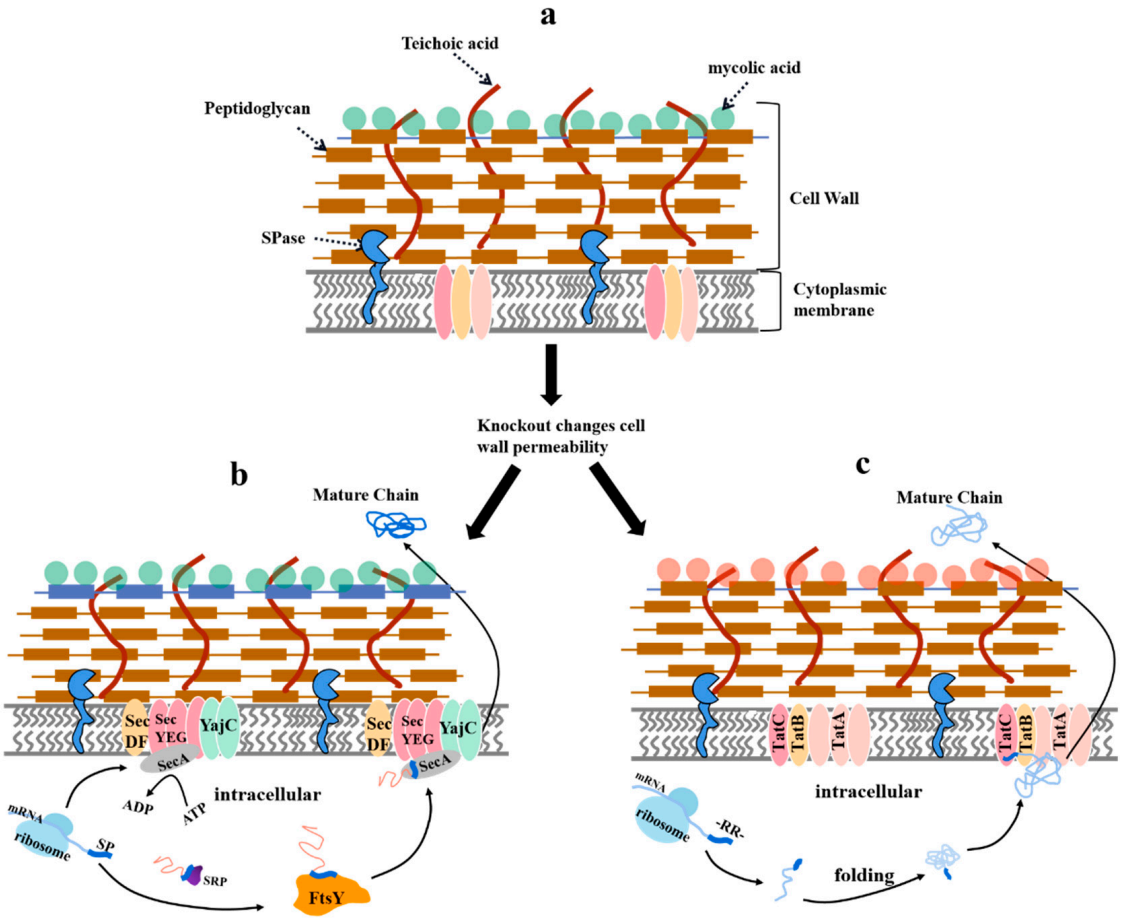

Figure 3. The cell wall structure of *C. glutamicum* and its protein transmembrane secretion pathway. (**a**) Cell wall structure of *C. glutamicum*. The cell wall is composed of an outer layer primarily consisting of mycolic acid and an inner layer mainly comprising peptidoglycan. (**b**) Sec secretion pathway. The SecA protein facilitates ATP hydrolysis to generate energy and interacts with the Sec-YEG transporter complex, and Sec-YEG is composed of three subunits: SecY, SecE, and SecG. SecDF (consisting of SecD and SecF) forms a transporter protein-associated complex, while YajC participates in forming this complex. Additionally, YidC is involved in the integration and folding of membrane proteins. The signal recognition particle (SRP) binds to the SP at the initiation of translation, followed by the translocation of SRP and nascent protein to membrane protein FtsY. The unfolded protein then associates with SecA and is secreted extracellularly via SecYEG. (**c**) Tat secretion pathway. The protein export channel is composed of TatA, TatB, and TatC. The folded protein binds to the complex formed by TatB and TatC, translocates with the assistance of a TatA-targeted chaperone that is cleaved by SPase after translocation, and ultimately secretes the mature protein into the extracellular space [95]. (**a**)→(**b**)\(**c**) indicates the disruption of either the mycolic acid or peptidoglycan layer in the cell wall, which leads to the change in cell permeability and facilitate the secretion of protein into the extracellular.

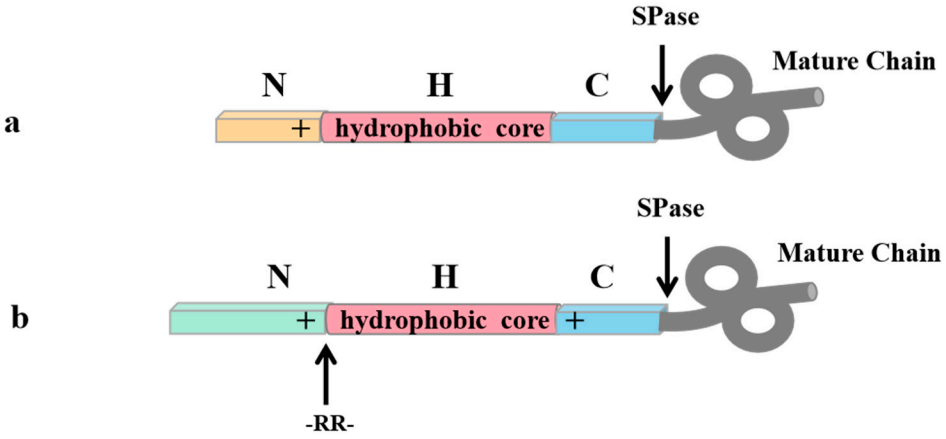

Figure 4. (**a**) Structural characteristics of Sec pathway signal peptide. (**b**) Structural characteristics of Tat pathway signal peptide. Both contain an N-region, a hydrophobic region, and a C-region. Compared to Sec-type SPs, Tat-type SPs possess a longer N-region, a highly conserved arginine residue "-RR-" near the N-terminus, and a C-region that typically contains positively charged amino acids. These characteristics prevent the mislocalization of Tat substrates to the Sec pathway [96].

When the synthesis of mycolic acid or peptidoglycan layer on the cell wall is impaired or absent, it leads to an increase in cell wall permeability and extracellular secretion of target products [97,98]. In a previous study, researchers treated *C. glutamicum* with Triton X-100. Although it increased cell wall permeability and improved cell activity, the non-food grade nature of Triton X-100 limited its industrial application for producing food-grade tagatose [99]. The researchers later disrupted the mycolic acid layer to inhibit cell wall synthesis and increase cell wall permeability, but this also significantly impeded strain growth and division [100]. Without affecting cell growth, Liu et al. [101] knocked down the *ponA* gene to induce structural changes in the peptidoglycan layer and partially inhibit cell wall synthesis. This led to a nearly five-fold increase in the efficiency of electro-conversion of exogenous DNA due to the resulting change in cell wall permeability. Because the host's proteases degrade or misfold proteins (including heterologous protein), Liu et al. [102] delete the endogenous protease *CLPS* gene from the genome of *C. glutamicum*, resulting in an increase in heterologous protein secretion. Although enhancing cell wall permeability and suppressing endogenous protein-degrading enzymes can boost extracellular secretion of the protein, further research is still required to apply this approach to *C. glutamicu*'s extracellular secretion production. In future industrial application research, the strategy of modifying the cell wall structure of the chassis can be combined with SP engineering to construct microbial cells with enhanced permeability and extracellular secretion capacity.

3.4. Terminators

The terminator, situated downstream of the 3′ regulatory sequences of the target gene, plays a crucial role in regulating gene expression along with the promoter. The primary function of this is to effectively terminate transcription, which prevents interference with downstream genes and enhances the metabolic competition pathway of microorganisms. In addition, it is crucial in maintaining the stability of gene transcription products [103]. Currently, the study of terminators has not been as extensive as that of promoters. However, as expression systems become increasingly complex and effectively terminating transcription becomes more important to avoid interference with downstream regions, research on terminators is gradually advancing [104].

The metabolic pathway from glutamic acid to proline competes with that from ornithine for precursors. To address this issue, researchers constructed two mutant strains of *C. glutamicum* S9114: orn9, in which a terminator was inserted upstream of gene *ncgl2228*, and orn10, in which a terminator was inserted upstream of gene *proB*. The findings indicate that both mutant strains upregulated ornithine expression and improved the status of the

two competing metabolic pathways [66]. The gene *argF* encodes ornithine carbamoyltransferase, which is a crucial enzyme in the degradation pathway of L-ornithine. However, the disruption of this gene to increase L-ornithine production results in growth defects within the strain. Thus, Zhang et al. [105] introduced a terminator (rrnB) between the *arg D* and *arg F* genes, resulting in an 11.2-fold increase in L-ornithine production (6.1 g/L) compared to the original strain and a 42.8% increase compared to the *argF* knockout strain (4.27 g/L). Additionally, growth levels were increased by 10.6% compared to the *argF* knockout strain. The strategy of incorporating terminators to attenuate gene expression and enhance metabolic pathways has also been applied in the production of other compounds, such as amino acids [106]. This approach obviates the need for gene knockouts that impact target gene expression, simplifying experimental procedures and avoiding physiological and metabolic repercussions on host cells. It represents a straightforward and convenient strategy for engineering chassis cells.

3.5. Expression Vectors

The transcriptional expression levels of genes are not solely determined by the expression elements but are also influenced by their respective expression vectors. Currently, the primary expression vectors utilized in *C. glutamicum* include its endogenous vector, as well as *E. coli–C. glutamicum* and *B. subtilis–C. glutamicum* shuttle vectors. Further research is necessary to optimize expression vectors of *C. glutamicum* for industrial-scale production [107,108].

Currently, the majority of expression vectors used for efficient overexpression of target genes in *C. glutamicum* require the supplementation of IPTG and antibiotics, which are unsuitable for large-scale industrial production. Optimizing a specific element or combination of elements based on the aforementioned factors can enhance the yield of the target product. However, this process is time-consuming, laborious, and has limited applicability. Therefore, Sun et al. [59] developed a bicistronic protein expression vector that enhances the performance of inducible promoters and enables efficient production of various recombinant and exogenous proteins. Moreover, the research team has previously demonstrated that a bicistronic expression system in *C. glutamicum* provides distinct advantages compared to its monocistronic system [109].

In a subsequent investigation, Li et al. [65] devised an antibiotic-free *E. coli–C. glutamicum* shuttle expression vector pLY-4, which exhibits the advantage of stable replication in nutrient-deficient host strains without resorting to antibiotics. This led to a 33.96% increase in L-methionine production via heterologous expression of the target gene. In addition, increasing the plasmid copy number represents a feasible strategy for constructing efficient expression vectors. Choi et al. [110] conducted laboratory adaptive evolution experiments to screen a high-copy-number strain and successfully enhanced the endo-xylanase secretion in *C. glutamicum*. However, it has been demonstrated that high copy number plasmids are unstable, which increases the likelihood of coding sequence mutations. Additionally, the maintenance of high copy number plasmids in bacteria imposes a significant metabolic burden on the host organism, leading to poor cellular growth and reduced production of recombinant proteins [111]. Subsequently, Battling et al. [112] developed an industrial strain carrying the genome without plasmids, which has been successfully utilized for producing 5-keto-fructose by *C. glutamicum*. This breakthrough provides a powerful tool for constructing expression vectors in industrial strains.

4. Summary and Prospect

With advancements in genetic engineering and synthetic biology, gene editing technology has evolved from traditional homologous recombination-based methods to more efficient, precise, and rapid CRISPR technology. Additionally, the development of CRISPR-based base editors has further facilitated the engineering of *C. glutamicum*. Meanwhile, novel expression elements that apply to *C. glutamicum* have been developed progressively, further enriching the synthetic biology toolbox. Recently, CRISPR gene editing technology

has emerged and been implemented in *C. glutamicum*, significantly enhancing the efficiency of genetic engineering and providing a favourable tool for engineering *C. glutamicum*. However, compared to traditional model strains such as *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae*, the strain of *C. glutamicum* is limited in terms of genetic modification tools, exhibits low efficiency for genetic modification, and has unclear physiological metabolism and gene expression mechanisms. These factors significantly impede the development of this microorganism as a viable chassis. For this reason, scientists have been investigating the genetic metabolic mechanisms and culture conditions of their strains, providing a theoretical basis for the industrial production of high-value compounds such as shark inositol, streptococcus lactis, siderophores, and terpenoids.

Although some progress has been made in studying and developing production applications using *C. glutamicum* as a chassis cell in recent years, further systematic research is necessary to fully exploit the potential of *C. glutamicum* and construct high-functioning, high-yield, and stable strains that can compete effectively with other industrial model production strains. In the future, the research focus should contain four aspects: Firstly, to develop the synthetic biology toolbox of *C. glutamicum*, we will continue to develop and optimize gene editing tools to improve the efficiency of gene editing (including single and multi-locus editing as well as short and long fragment gene editing) while combining high-throughput screening techniques to efficiently and rapidly obtain better production strains to achieve the widespread and universal use of *C. glutamicum* in practical industrial applications. Secondly, we will develop stable and efficient exogenous expression vectors that are compatible with *C. glutamicum*, thereby facilitating rapid and efficient genetic engineering operations. Thirdly, we will further delve into our research on synthetic biology, molecular biology, and genomics and explore the physiological metabolism, genetic mechanisms, and gene expression of *C. glutamicum* to provide the theoretical basis for researchers. Fourth, we will employ the application of emerging artificial intelligence technology to construct intelligent and efficient fermentation process detectors, which can automatically detect and optimally regulate the fermentation process. This will lead to an increased yield through the optimization of fermentation conditions and genetic modification of the strain.

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