Advances on systems metabolic engineering of *Bacillus subtilis* as a chassis cell

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A B S T R A C T

The Gram-positive model bacterium *Bacillus subtilis*, has been broadly applied in various fields because of its low pathogenicity and strong protein secretion ability, as well as its well-developed fermentation technology. *B. subtilis* is considered as an attractive host in the field of metabolic engineering, in particular for protein expression and secretion, so it has been well studied and applied in genetic engineering. In this review, we discussed why *B. subtilis* is a good chassis cell for metabolic engineering. We also summarized the latest research progress in systematic biology, synthetic biology and evolution-based engineering of *B. subtilis*, and showed systemic metabolic engineering expedite the harnessing *B. subtilis* for bioproduction.

Introduction

Nowadays, using microorganisms to produce chemicals has become a global development trend. Biobased production can be used to overcome challenges of traditional chemical synthesis processes, such as high cost, high energy consumption and difficult to remove by-products, etc. [1]. So far, some microorganisms have been used as chassis cells for industrial production, whereby most research focused on model strains such as *E. coli* and *Saccharomyces cerevisiae*. Nevertheless, they also have some shortcomings. The codon preference of the *E. coli* gene is very obvious. When expressing foreign proteins, the continuous appearance of rare codons will hinder protein synthesis and codon mismatches will occur, affecting the correct and rapid expression of genes. *Saccharomyces cerevisiae* has excessive glycosylation of foreign gene expression. At the same time, it produces ethanol during the growth process, and long-term accumulation will affect the growth of the bacteria, so it is difficult to carry out high-density culture. Compared with these microorganisms, *B. subtilis* is more appropriate as a chassis cell due to its non-pathogenicity and excellent protein secretion ability. In addition, *B. subtilis* is generally regarded as safe (GRAS) [2]. It also has the advantages of no pathogenicity, good environmental compatibility, being unlikely to generate resistance, and being able to secrete a variety of enzymes and antibiotics. Moreover, it also has high adaptability of genetic engineering, no obvious codon bias, and can be grown using easy and cheap culture methods in large-scale

fermentation [3]. Moreover, it has a good fermentation basis and has a wide range of uses including medicine, agriculture and industry [4].

Systems metabolic engineering simultaneously uses synthetic biology, systematic biology and evolution-based engineering [5]. It integrates omics analyses and genome-scale computational simulation of systematic biology, the gene editing tools of synthetic biology, and effective mutagenesis methods of evolution-based engineering [6]. Strains can be transformed using systematic metabolic engineering to obtain mutant strains that meet production needs.

As a model strain, *B. subtilis* has a good expression and secretion system. Thus, systematic metabolic engineering can be used to transform *B. subtilis* to be more suitable for factory production. Systematic biology aims to fully understand the endogenous characteristics of *B. subtilis* by analyzing its genome, transcriptome, proteome, and metabolome. Synthetic biology aims to construct new synthetic pathways in *B. subtilis* or to optimize existing biosynthesis pathways to improve the production of target chemicals. Evolution-based engineering aims to screen adaptive or randomly generated *B. subtilis* mutants to select desired phenotypes [7]. By combining systematic biology, synthetic biology and evolution-based engineering, a better biosynthetic pathway for *B. subtilis* can be constructed which meets the requirements of the research community and industry (Fig. 1).
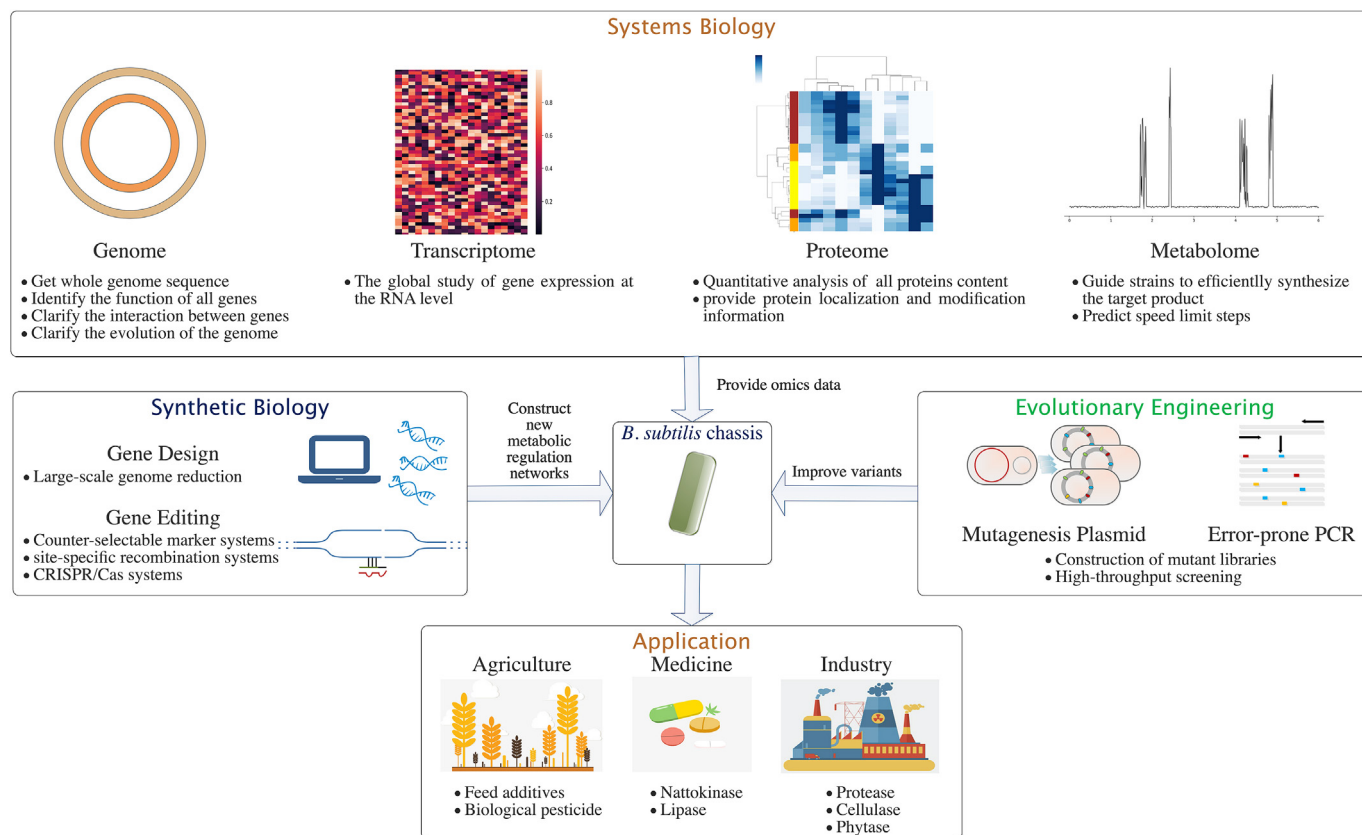
Fig. 1. Construction of *B. subtilis* chassis cells using systems biology, synthetic biology, and evolution-based engineering.

2. Systematic biology advances in *B. subtilis*

2.1. Bioinformatics of B. subtilis

Kunst et al. [8] performed genome-wide sequencing and genome annotation of *B. subtilis*, which provided guidance for the genetic modification of *B. subtilis* [4]. Buescher et al. introduced integrated analysis and model-based data analysis of statistically assisted multi-level omics data to infer regulatory interactions [9]. Through the data analysis of the transcriptome [10], proteome [11,12] and metabolome [13] of *B. subtilis*, its endogenous characteristics were comprehensively understood, providing a basis for rational metabolic engineering.

B. subtilis is present in the soil under natural conditions, so it must adapt to changing environments. The omics data were integrated with genomic sequence data to create several *B. subtilis*-related databases, including BioBrick Box [14], SporeWeb [15], DBTBS [16], MetaCyc database [17] and SubtiWiki [18]. BioBrick Box provides integrated vectors, reporter, and epitope tags of *B. subtilis*, as well as offering strength assessments for promoters and ribosome binding sites [14]. SporeWeb concentrates all the developmental stages of spore formation in *B. subtilis*, helping to fully understand the dynamics of the complete sporulation process [15]. DBTBS provides detailed information on the *B. subtilis* transcription system, including transcription factor recognition sequences and regulatory genes [16]. The MetaCyc database includes data on enzymes and metabolic pathways which is useful for a variety of fields [17]. The SubtiWiki database publishes all genes and proteins, including gene expression, metabolism and protein-protein interactions [18]. These databases provide timely, comprehensive biological information that can be used as a reference for metabolic engineering (Table 1).

2.2. Regulatory factors of B. subtilis

The goal of systems biology is to use transcriptomic data to construct models of transcriptional regulation. Understanding the *B. subtilis* transcriptome is in need of the identification of individual regulators and regulatory factors that control gene expression, including transcription factors (TFs) and σ factors. Transcription factors (TFs) can be distinguished by their structure, biochemical and genetic properties [19]. The structural characteristics of TFs can be found on Pfam, a database of protein domain families [20]. Ishii et al. [21] used the information from Pfam to construct a database (DBTBS), which consists of a compilation of experimental promoters and transcription factors including σ factors. A global transcriptional regulatory network with all known transcriptional regulators (sigma and TF) can be constructed by using the *B. subtilis* gene transcription datasets published [9,22].

B. subtilis has 6 global transcription factors: CcpA, TnrA, CodY, ComK, Spo0A and AbrB [23]. CcpA is not only a common repressor in catabolite repression (CR), but also a positive regulator of excess carbon excretion related genes [24]. For example, the carbon catabolism repression of citrate synthase and aconitase genes depends on CcpA [25]. TnrA, as a transcription factor for nitrogen metabolism genes, is active during nitrogen limitation [26], activating and inhibiting many genes [27]. CodY is an important transcription factor in *B. subtilis* and has many functions. It is a transcriptional regulator of branched-chain amino acid response [28]. In addition, CodY can regulate the transcription of quiescent and sporulation genes, thereby adapting cells to nutritional restrictions [29]. ComK, as a competence transcription factor (CTF) [30] regulates DNA binding, uptake and transcription of related genes during the formation of *B. subtilis* competent state. Transcription regulator Spo0A is a multi-effect regulatory protein that can regulate spore formation [31] and biofilm formation [32]. AbrB is a negative regulator of biofilm formation [33] and is also involved in the inhibition of catabolics [34].

Table 1
Biological information from the *B. subtilis* databases.

B. subtilis Database	Biological Information	Reference
BioBrick Box	integrated vectors, reporter, epitope tags, the strength of promoters and ribosome binding sites	[14]
SporeWeb	spore formation	[15]
DBTBS	transcription factor recognition sequences and regulatory genes	[16]
MetaCyc database	enzymes and metabolic pathways	[17]
SubtiWiki	gene expression, metabolism, regulatory pathways, and protein-protein interactions	[18]

2.3. Post-translational modification in B. subtilis

Post-translational modifications play an important role in regulating the function of many proteins. In *B. subtilis*, phosphorylation is a common post-translational modification. The Mrp family protein SalA is only active if it is phosphorylated by PtkA, which indirectly promotes the production of AprE by inhibiting SalC [35,36]. Furthermore, AbrB, as a global transcriptional regulator [37], its phosphorylation is particularly important. Kobir et al. [38] determined that Hanks kinase phosphorylates AbrB, thereby interfering with its affinity for DNA binding, which stimulates exoprotease production and inhibits sporulation. Biofilms help protect *B. subtilis* in variable environments. Kiley et al. [39] demonstrated that the formation of *B. subtilis* biofilm is also associated with phosphorylation. Overall, phosphorylation plays a vital role in the metabolism control of *B. subtilis*. Systematic biology can be used to predict the phosphorylation site and provide a theoretical basis for the regulation of metabolic pathways [40].

In addition to phosphorylation, acylation modifications are also common post-translational modifications. Kim et al. [41] found 332 lysine acetylation sites in 185 proteins of *B. subtilis*, which are mainly involved in cell internal functions. Gardner et al. [42] proposed that acuABC operon can control the activity of acetyl-CoA synthetase by encoding AcuA and AcuC. Acetyl-CoA synthetase can catalyze the synthesis of acetyl-CoA in the central metabolism process. Its acetylation and deacetylation directly affect the concentration of acetyl-CoA in the organism, affecting the normal progress of the metabolic process and metabolic flow. In addition, recently it has been reported that TufA translation and activity are regulated by acetylation and succinylation [43]. Reverdy et al. [44] demonstrated that lysine acetylation is very important bfor the formation of *B. subtilis* biofilm. What's more, MreB acetylation controls cell diameter by restricting cell wall growth [45]. Ogura et al. [46] demonstrated that adding glucose to the medium may result in increased levels of acetylated CshA.

2.4. Metabolic optimization of B. subtilis

Systematic biology can provide guidance for the optimization of metabolic pathways. In order to maximize the production of targeted small molecules, it is necessary to control the expression of heterologous pathways through modified control systems to achieve the desired production. Accumulation of metabolites may cause feedback inhibition or toxicity and decreased cell growth. The identification of key metabolic intermediates and control of gene expression levels can prevent the accumulation and relieve the negative impact of intermediates, as well as drive the flux through the pathway to achieve higher yields or productivity [47]. What's more, through dynamic monitoring of metabolic pathways, speed limit steps can also be identified. Using metabolic flux to dynamically monitor the entire metabolic flow, thereby editing the relevant genes purposely and increasing the expression of the target product [48]. The PsrfA-mediated cell density-coupled autoregulation system is affected by glucose addition, and the final cell density can be increased by adding glucose [49].

Metabolic engineering can be extended from the gene to the genome scale through sRNA and CRISPR-Cas9 systems. Bikard et al. [50] used Cas9 nuclease mutants which called 'dead' Cas9 (dCas9) to regulate the transcription. Specifically, dCas9 can be combined with DNA to inhibit the transcription. On the other hand, dCas9 can be bound to the ω subunit of RNAP to enhance transcription. In addition, sRNA regulates gene expression by combining with target mRNA. This method does not need to construct a strain library, which is more convenient than traditional gene knockout [51].

2.5. Spore formation in B. subtilis

Spore is a special molecular biological feature of *Bacillus*, and its regulation has received extensive attention in recent years. It is of great significance to study the germination of spores. For example, spores can be served as a surface display system [52] or as a carrier for mucosal immunity [53]. In harsh environments, *Bacillus* can sporulate and enter to a dormant state [54]. The transformation of *B. subtilis* from vegetative growth to spore formation is controlled by the main transcription regulator Spo0A [55]. The phosphorylation process of Spo0A mainly involves five kinds of kinases. The acid transferases Spo0F and Spo0B transfer the phosphate group on the histidine kinase to Spo0A, forming Spo0A ~ P. Spo0A ~ P directly regulates the expression of about 121 genes, including those required for spore formation [31]. The level of Spo0A ~ P determines whether sporulation is triggered. Lower Spo0A ~ P levels can promote biofilm formation, while higher Spo0A ~ P levels can promote spore formation [56]. During spore formation, two copies of the chromosome form axial filaments connecting the two poles of the cell (RacA locates the chromosome at the two poles of the cell by binding to GC-rich inverted repeat sequences near the replication origin [57] and interacting with DivIVA [58]). Asymmetric segmentation is regulated by sporulation-specific σ^F and σ^E (the spatiotemporal specific expression of sigma factor leads to spatiotemporal specificity of gene expression). This stage has two characteristics. The first is the asymmetric membrane, the middle membrane is transformed into an asymmetric membrane, and the division of cells into mother cells and prospores is a morphological feature of spore formation. The second feature is the asymmetric distribution of chromatin. During the asymmetric separation process, the prespores have only 1/3 of the chromatin. The DNA translocation enzyme SpoIIIE pumps 2/3 of remaining chromatin into the prespores [59]. Next, the end diaphragm bends around the prespore, similar to mother cells "phagocytosing" prespore cells [56]. Including the thinning of the diaphragm wall, swallowing membrane movement and peptidoglycan synthesis process [60]. Regulation by prespore-specific σ^G and mother cell-specific σ^K. The spores are released and the mature spores are wrapped by two concentric shells: the spore coat and the cortex [61]. The spore coat is the outer shell and is composed of about seventy different proteins and is used to resist the harsh environment of the outside world [62]. The cortex is the inner shell and is composed of specific peptidoglycans including the inner layer embryo, cell wall and outer layer cortex [56].

2.6. Application of system biology in B. subtilis

With the help of information science and computers, the interaction between functional genomes, proteomes, transcriptomes and metabolomes is integrated, making the development of biological research from descriptive science to quantitative prediction science-system biology. The application of multi-omics strategy makes it easier to

Table 2
Growth and advantages of different degrees of reduction in the *B. subtilis* genome.

Percentage of genome reduction	Growth compared to wild type	Advantages compared to wild type	Reference
7.7%	the same	lacks the BsuM restriction-modification system	[77]
20%	lower	Cellulase and protease productivity increase	[78]
25%	Slightly reduced	Cellulase productivity increase	[79]
36%	Slightly reduced	Significant increase in proteins involved in the biosynthesis and assembly of iron-sulfur clusters	[70]

obtain biological big data related to cell physiology and metabolism. These data are used to transform and optimize production bacteria. Systems biology can annotate the gene function of *B. subtilis* through omics analysis. Metabolomics analysis of *B. subtilis* EA-CB0575 revealed that some genes can promote plant growth [63]. Through genetic manipulation of these genes, agriculture will be greatly developed. Surfactant is a very versatile industrial product, and its production has attracted people's attention. At present, using glycerin as a carbon source to produce surfactants is the most economical way. A transcriptome study of *Bacillus velezensis* BS-37 found that the gene expression level associated with surfactant production is high, indicating that it can be used as a chassis cell for surfactant production [64]. A major role of systems biology is that omics data can be used to build metabolic models. Massaiu et al. [65] used kinetic and omics data to construct a model that predicts wild-type and growth-type metabolic flux. Nattokinase secreted by *B. subtilis* can dissolve fibrin and has a significant thrombolytic effect. However, nattokinase is immunogenic, so it is particularly important to produce nattokinase with low antigenicity. By predicting amino acids which are immunogenic and mutating them, low-immunity mutants are obtained. Meanwhile, modeling mutants and predict their stability, which is important for producing safer nattokinase [66]. Pullulanase has broad application in industry, but the amount of *B. subtilis* produced is low. Using transcriptome data to find the strong promoters and to increase the amount of pullulanase produced by *B. subtilis* [67]. Overall, systematic biology can be used to establish metabolic models, predict gene function and protein structure, and guide metabolic engineering.

3. Synthetic biology advances in *B. subtilis*

3.1. Genomic design and large-scale genome reduction of B. subtilis

The ideal chassis is a well-characterized cell that contains only the minimum genes required for cell maintenance, growth, and producing the synthetic target product. Furthermore, reduction of the host genome size reduces the number of interactions between the heterologous pathway and the host metabolic network, facilitating the production of the target product [68]. There are two ways to reduce the genome, one is bottom-up and the other is top-down [69]. The bottom-up way aims to synthesize minimal cells using the essential genes encoding key proteins, while the top-down approach aims to produce mutants with smaller genomes by identifying and deleting non-essential genes in the microorganism. Necessary genes are essential for the growth and development of strains, and their identification is important for building minimal cells that can still survive [70]. There are many methods for identifying and studying essential genes, such as hypersaturated transposon mutagenesis [71], transposon-directed insertion-site sequencing [72], targeted gene deletion [73], random

transposon insertion mutagenesis [74], and genome-wide RNAi screening [75]. Moreover, Commichau et al. [76] commented on the essential genome dynamics of *B. subtilis*, and the latest information on essential genes in this organism can be found on the "Essential Genes" page on *Subti*Wiki [68].

Westers et al. [77] knocked out 7.7% of the *B. subtilis* genome and eliminated 332 genes. Importantly, the growth and development of the strain was not affected [77]. The ability of *B. subtilis* MBG874 with 20% deleted genome to produce extracellular cellulases and proteases was significantly enhanced [78]. The growth of a 25% genome-reduced *B. subtilis* in enzyme production medium was slightly reduced, but there were no significant morphological changes, and the enzyme activity was increased [79]. Moreover, 146 successful mutant strains were generated on complex media [80]. Reuss et al. [70] constructed the smallest genome of *B. subtilis*, which is approximately 36% smaller than the original. In addition, they found that the translation resources occupied by the necessary genes were significantly more than the remaining unknown genes [70]. It can be seen that there are enormous differences in gene count and resource allocation. Therefore, by studying the dispensability of *B. subtilis* genes, methods for further reducing the genome can be proposed [70] (Table 2).

3.2. Genome editing tools for B. subtilis

B. subtilis has the ability to absorb linear DNA, and its high efficiency of homologous recombination makes it possible to redesign the genome [81]. Furthermore, using gene editing technology can make *B. subtilis* more suitable for factory production [82]. Genome editing operations typically involve gene deletions, insertions, and mutations [4]. The development of advanced genome editing tools is the basic requirement for developing *B. subtilis* as a chassis organism. Currently, established genome editing tools include counter-selectable marker systems [83], site-specific recombination systems [4] and CRISPR/Cas systems [84] (Table 3).

The counter-selectable marker system is used as a common gene editing tool for *B. subtilis* due to its no marker left. The genes currently used as selection markers include hew1 [85], upp [86], blaI [87], ysbC [88], araR [89] and mazF [90]. Wang et al. [85] used a temperature-sensitive promoter PR to control the expression of hewl. The PR promoter is repressed at 30 °C and the repression is lifted at 42 °C. The toxic effect of hewl is turned on or off by temperature. Fabret et al. [86] first used the upp gene to achieve point mutations and deletions. What's more, Brans et al. [87] used blaI to achieve the inactivation of single genes and the introduction of target genes. In short, using marker genes to perform gene editing on *B. subtilis* is simple and effective, and it is a good gene editing tool.

Currently, both the Cre/loxP system and the Xer/dif system have been used to develop resistance marker elimination systems. Yan et al.

Table 3
Genome editing tools for *B. subtilis*.

Genome editing tools	Advantage	Disadvantage	Reference
counter-selectable marker systems	no marker left	limited antibiotic selection markers	[83]
site-specific recombination (SSR) systems	eliminate the selection marker	leave remnant sequences	[4]
CRISPR/Cas systems	simple and efficient	the off-target effects	[84]

[91] used a temperature-sensitive plasmid to express Cre enzyme to promote the recombination of two loxP recognition sites in the same direction, thereby removing the marker gene. The recombinase of the Xer/dif system developed by Bloor et al. [92] can be replaced by RipX and CodV of *B. subtilis*, without the need to introduce exogenous recombinase. The high recombination frequency of site-specific recombination eliminates the need to include counter-selectable marker genes.

CRISPR-Cas gene editing technology has significant advantages such as high efficiency, specificity and simple design, bringing revolutionary breakthroughs to gene editing technology [93,94]. David Liu's lab reported the "base editor" based on the fusion of cytosine deaminase and CRISPR/Cas9, which can achieve safer, more efficient, and accurate without introducing DNA double-strand breaks or recombining repair templates. Base editor, and the efficiency is much higher than the HDR repair method caused by DSB [95]. Zhang et al. [96] used the CRISPR-Cas system to construct *B. subtilis* which is more suitable for industrial fermentation. However, CRISPR-Cas9 technology has off-target effects. In order to solve this problem, Ran et al. [97] used two sgRNAs to direct Cas9n to two adjacent targets to improve the specificity of gene targeting.

3.3. Evolutionary engineering advances in B. subtilis

Evolutionary engineering is based on the selection or screening of cells with a desired phenotype from an adaptive or randomly generated pool of mutated cells [98]. High-throughput screening or adaptive evolution of strain libraries using random mutagenesis is a well-known method for obtaining mutants with desirable features [40]. Zhang et al. [99] reported a new directed evolution method that combines super-powered cells with plasmids. Averesch et al. [100] used adaptive evolution to produce mutants that can metabolize xylose and compared the pABA production capacity from different carbon sources. Han et al. [101] increased the promoter strength of *B. subtilis* by approximately 3-fold by constructing mutant libraries. Chakravorty et al. [98] developed the rational thermostable mutation prediction tool RankProt, which provides a theoretical basis for evolution-based engineering. Compared to random methods of directed evolution, it ensures a shorter development time.

4. Perspectives

There have been many studies on the synthetic biology of *B. subtilis* as a chassis organism, and various technical methods are becoming more and more mature. With the development of systems biology, the application of multi-omics technology is more extensive, which is conducive to the development of metabolic engineering. Meanwhile, evolutionary engineering based on directed evolution and random mutation provides mutants which meet production needs. The systematic metabolic engineering that integrates synthetic biology, systematic biology, and evolution-based engineering is the future development direction of engineered strains. Although the research on *B. subtilis* is relatively mature, there are still some ideas to propose.

At present, metabolic regulation usually only focuses on one level of regulation, the most common is the promoter or RBS. In fact, it is possible to control on multiple levels at the same time to achieve a more refined control process. Develop a system that regulates both on transcription and translation levels to precisely control key points in the metabolic pathway [102]. *B. subtilis* can be used as a biosensor and has a wide range of applications. On the other hand, biosensors have relatively high sensitivity requirements, so it is particularly important to develop high-sensitivity biosensors. Simultaneously transform cells at various control levels to cascade amplify the signal to improve sensitivity [103]. The determination of rate-limiting steps in metabolic pathways is an important task of metabolic engineering. Through transcriptome analysis of related genes in metabolic pathways, the rate-limiting steps can be determined [104]. Using multi-omics technology to identify potential gene manipulation and flux regulation targets is of great significance to metabolic engineering. Therefore, more omics data and catalytic rate of metabolic enzymes need to be integrated to further improve the accuracy and precision of prediction. At present, directed evolution first builds a mutant library, and after high-throughput screening, mutants with greatly improved target are obtained. However, this method is expensive and time-consuming, sometimes it is difficult to achieve the purpose. Using machine learning to guide directed evolution can not only save time, but also save costs and reach a higher level of adaptability [105]. Through continuous exploration and research on system metabolic engineering, *B. subtilis* will become a more excellent engineered strain.

5. Conclusions

B. subtilis is an important industrial organism because it is non-pathogenic, secretes a variety of enzymes and antibacterial substances, and has a good fermentation base. In addition, it can form competent cells to take up exogenous genes, and has a good expression and secretion system. In this paper, we discussed the metabolic engineering from the aspects of systematic biology, synthetic biology and evolution-based engineering. Systematic biology can provide genome-scale information on *B. subtilis*, which is an important theoretical basis for synthetic biology to design and simplify genes. At the same time, the computational tools of systematic biology simplify the design and optimization of metabolic pathways. Furthermore, simplified genetic systems created using synthetic biology approaches will supply systematic biology with an in-depth understanding of the basics of natural gene regulation. In particular, evolution-based engineering provides mutants which meet production needs. Overall, systems biology increases the understanding of *B. subtilis* using omics, synthetic biology can be utilized to construct new metabolic regulation networks, and evolution-based engineering enables the adaptive evolution of *B. subtilis*. Together, these approaches greatly expand the scope of metabolic engineering to efficiently produce a variety of proteins and chemicals.

CRediT authorship contribution statement

Mengjie Xiang: Conceptualization, Writing - original draft, Writing - review & editing, Visualization. **Qian Kang:** Writing - review & editing. **Dawei Zhang:** Supervision.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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