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Construction of an ultra-strong PtacM promoter via engineering the core-element spacer and 5′ untranslated region for versatile applications in *Corynebacterium glutamicum*

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ABSTRACT

As one of the most important synthetic biology elements in transcriptional regulation, promoters play irreplaceable roles in metabolic engineering. For the industrial microorganism *Corynebacterium glutamicum*, both the construction of a promoter library with gradient strength and the creation of ultra-strong promoters are essential for the production of target enzymes and compounds. In this work, the spacer sequence (both length and base) between the −35 and −10 regions, and the 5′-terminal untranslated region (5′UTR) were particularly highlighted to investigate their contributions to promoter strength. We constructed a series of artificially induced promoters based on the classical tac promoter using *C. glutamicum* ATCC13032 as the host. Here, we explored the effect of sequence length between the −35 and −10 regions on the strength of the tac promoter, and found that the mutant with 15 nt spacer length (PtacL15) was transcriptionally stronger than the classic Ptac (16 nt); subsequently, based on PtacL15, we explored the effect of the nucleotide sequence in the spacer region on transcriptional strength, and screened the strongest PtacL15m-110 (GAACAGGCTTTATCT), and PtacL15m-87 (AGTCGCTAAGACTCA); finally, we investigated the effect of the length of the 5′-terminal untranslated region (5′UTR) and screened out the optimal PtacM4 mutant with a 5′UTR length of 32 nt. Based on our new findings on the optimal spacer length (15 nt), nucleotide sequence (AGTCGCTAAGACTCA), and 5′UTR (truncated 32 nt), an ultra-strong PtacM, whose transcriptional strength was about 3.25 times that of the original Ptac, was obtained. We anticipate that these promoters with gradient transcriptional strength and the ultra-strong PtacM will play an important role in the construction of recombinant strains and industrial production.

1. Introduction

Engineered microorganisms have been widely used in the industrial production of chemicals and enzymes. In recent years, metabolic engineering is becoming one of the most important frontiers in constructing and optimizing the metabolic pathways of microbial cells to achieve the goal of producing bulk and fine compounds.[1–4] The regulation of cellular metabolism is intricate since it includes multiple dimensions such as transcription, translation, and post-translational modification.[4] Among these strategies, promoters are the most important element in the regulation of cellular metabolism at the transcriptional level.[5] Over the past decades, metabolic engineering has relied heavily on the discovery and characterization of convenient and efficient promoters.[1] Microbial endogenous promoters, however, have certain limitations, such as insufficient strength for target genes to initiate transcription. To solve the above-mentioned drawbacks, promoter engineering has emerged as a powerful tool. Promoters with different strengths can be created to enhance comprehensive transcriptional regulation and precise control of target genes.[6] Promoter engineering has been widely used in *Escherichia coli*,[7] *Bacillus subtilis*,[8] *Corynebacterium glutamicum*,[9] *Saccharomyces cerevisiae*,[10] and other model organisms.[4]

C. glutamicum is an important GRAS (Generally Recognized as Safe) workhorse in industrial white biotechnology, and it is widely used in the production of amino acids, fuels, and high-value-added compounds.[5] With the rapid development of synthetic biology, an increasing number of promoters in *C. glutamicum* have been progressively discovered and

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created, with the two main types being constitutive promoters and inducible promoters.[11] The endogenous constitutive promoters that have currently been identified include Psod, Ptuf, Pfba *etc.*[12] To achieve fine-tuning of target genes, many constitutive promoter libraries have also been constructed for *C. glutamicum*.[11] Shang et al. characterized the relative strengths of 16 endogenous promoters of *C. glutamicum*,[12] besides, Zhang et al. constructed a synthetic constitutive promoter library based on the core region sequence of *C. glutamicum* with a wide range of transcriptional strengths.[9] The exogenous inducible promoters applied to *C. glutamicum* mainly include β-isopropylthiogalactoside (IPTG)-inducible Ptac[13] and Ptrc,[14] arabinose-inducible ParaBAD[15] and tetracycline-inducible Ptet.[16] The application of ParaBAD is limited in *C. glutamicum* due to the lack of arabinose transporter protein, while the application of Ptet is also greatly limited by the fact that its inducer is an antibiotic compound. Currently, IPTG-inducible Ptac and Ptrc are still widely used promoters in *C. glutamicum*.

Tac promoter is a hybrid promoter constructed from the trp promoter and the lacUV5 promoter, which can be repressed by the lac repressor and induced by IPTG.[17] Ptac is now extensively used for protein expression and synthesis of valuable chemicals in *C. glutamicum*.[18,19] Previous works, focusing on core region sequence mutation studies of the tac promoter in *C. glutamicum*, have accomplished the construction of tac promoter mutants with enhanced strength.[20,21] At the same time, more extensive mutation studies have been conducted on *E.coli*, covering the spacer region between the −35 region and −10 region of the tac promoter.[22,23] However, in *C. glutamicum*, little research has been reported on tac promoter mutations other than studies focused on the core region. In order to enrich the synthetic biology elements of the model organism *C. glutamicum* and fulfill the needs of fine-tuning different genes, it is necessary to develop a tac promoter library with a wide range of transcription strengths.

In this study, we engineered the tac promoter by changing the length and base composition of the spacer sequence between the −35 region and −10 region as well as the length of the 5′ untranslated region (5′UTR) to construct a tac promoter mutant library of *C. glutamicum* ATCC13032. By exploring the effect of the above factors on transcription strength, we constructed a series of promoters with gradient strengths. Through combining the discovered optimal conditions, we constructed the ultra-strong PtacM, and successfully applied it in protein expression and morphological engineering.

2. Materials and methods

2.1. Plasmids and strain construction

The plasmids and strains used in this study are listed in Table S1. The primers used in this study are listed in Table S2. The starting plasmid pEC-Ptac-GFP used for Ptac mutants screening was constructed previously by substituting the original trc promoter with tac promoter amplified from the plasmid pXMJ19 and inserting the fluorescent protein gene *gfpuv* as screening reporter following the tac promoter.[24] To optimize the spacer length between the −35 region and −10 region, eight plasmids (e.g. pEC-PtacL12-GFP) were constructed. To optimize the spacer sequence, the promoter libraries pEC-PtacL15m-GFP were constructed by randomizing the nucleotides between the −35 and −10 conserved regions. To optimize the length of 5′-UTR between lacO and RBS region, 19 plasmids (e.g. pEC-PtacM1-GFP) were constructed. The gene *lacZ* from *E. coli* BL21 was amplified, digested (restriction sites are shown in Table S1), and ligated into pEC-Ptac and pEC-PtacM resulting in the recombinant plasmids pEC-Ptac-β-gal and pEC-PtacM-β-gal, respectively. The gene *ftsZ* from *C. glutamicum* was amplified and ligated into pEC-Ptac-GFP, pEC-PtacM-GFP and pEC-PtacL15-GFP to construct the recombinant plasmids pEC-Ptac-GFP-FtsZ, pEC-PtacM-GFP-FtsZ and pEC-PtacL15-GFP-FtsZ. PCRs were performed using Phanta Max Master Polymerase purchased from Vazyme (Nanjing, China). All mutant fragments were fused by Gibson Assembly Kit purchased from Clonesmarter Technologies (Scottsdale, AZ, United States). The kits used for plasmid extraction and gene purification were purchased from OMEGA (Norcross, GA, United States). All plasmids were sequenced by GENEWIZ (Suzhou, China).

E. coli Trans10 (Transgene) was used for plasmid construction. The wild-type *C. glutamicum* ATCC13032 was used for mutated Ptac screening and identification.

2.2. Culture conditions

E. coli Trans10 and the derivatives were routinely grown at 37 °C in LB medium (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L). The seed culture of *C. glutamicum* was LB medium containing 20 g/L glucose and 25 μg/mL kanamycin. The flask culture of *C. glutamicum* contained 20 g/L corn syrup powder, 30 g/L $(NH_4)_2SO_4$, 40 g/L glucose, 1 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 10 mg/L $FeSO_4{\cdot}7H_2O$, and 10 mg/L $MnSO_4{\cdot}7H_2O$. The medium was sterilized by autoclaving at 121 °C for 20 min. Before inoculation, filtrated kanamycin and glucose solutions were added.

A single colony of the recombinant strain was cultured overnight in LB medium at 30 °C with agitation of 200 rpm. For GFP analysis, a 2.5% v/v of seed culture and 1 mM IPTG were inoculated into 500 mL flasks containing 50 mL fermentation medium and then cultured at 30 °C with agitation of 200 rpm.

2.3. Fluorescence assessment

The recombinant *C. glutamicum* strains containing various Ptac mutants were assembled with the GFP to evaluate the promoter strength, as described previously.[25] After being cultured for 24 h, the strains were harvested and resuspended in deionized water to $OD_{600} = 1.0$. The GFP fluorescence value was measured by a TECAN Infinite M200 PRO microplate reader (Männedorf, Switzerland). Extinction and emission wavelengths were set as 385 nm and 509 nm, respectively. Experiments were performed in triplicate from three independent cultures.

2.4. Quantitative PCR (qPCR) analysis

After being cultured for 12 h, the recombinant strains were harvested by centrifugation at 12,000 rpm for 10 min at 4 °C. The total RNA was extracted using a Bacterial RNA Kit (OMEGA, MA, United States). After measuring the concentration of RNA using the NanoDrop 1000 (Thermo Scientific, United States), cDNA was synthesized using 1st synthesis Kit by adding the same amount of total RNA in all samples. The GFP transcriptional level was quantified by qPCR using a SuperReal Premix SYBR Green Kit (Vazyme, Nanjing, China) and ABI 7300 real-time PCR system (Applied Biosystems, United States). The GFP transcriptional level in recombinants was normalized to that of the 16S RNA. All reactions were performed in triple.

2.5. β-Galactosidase activity assay

β-Galactosidase activity was determined as previously described.[26] Briefly, cultured cells were collected by centrifugation at 13,000 rpm for 5 min. The cell pellets were washed with Z-buffer (60 mM $Na_2HPO_4{\cdot}12H_2O$, 40 mM $NaH_2PO_4{\cdot}2H_2O$, 10 mM KCl, and 1 mM $MgSO_4{\cdot}7H_2O$), treated with lysozyme (15 mg/mL final concentration) at 37 °C for 10 min, and then added Triton X-100 (0.1% final concentration) to obtain the sample for β-galactosidase activity assay. To analyze the β-galactosidase activity, 1.9 mL ZB-buffer (Z-buffer with 20 μL/mL 2-Mercaptoethanol), 400 μL 4 mg/mL O-NPG solution (2-Nitrophenyl-β-D-galactopyranoside dissolved in Z-buffer) and 100 μL of lysed cell suspension were mixed immediately and incubated at 28 °C for 45 min, and then 1 mL 1 M Na_2CO_3 solution was added to stop the reaction. The specific activities of the β-gal reporters were expressed in Miller units/mL. All experiments were performed in triplicate.

2.6. Observation of cell morphology

The recombinant *C. glutamicum* strains overexpressing *ftsZ* gene with different Ptac mutants were used to confirm the strength of promoters via cell morphology. After 24 h of culture, cells were washed with PBS buffer and then harvested by centrifugation. The glutaraldehyde solution was added for fixation for 1 h. After sample preparation, the cell morphology of *C. glutamicum* was observed via scanning electron microscope (SEM, FEI Quanta 200). The HV was set as 20 kV and WD was set as 27.9 mm.

3. Results

3.1. The effect of the spacer length between the −35 and −10 core regions on the strength of the tac promoter

Firstly, we regulated the strength of the tac promoter by changing the length of the spacer between the −35 and −10 regions. We analyzed the spacer sequences of promoters in *C. glutamicum* and bacteriophage and found that most of them were between 12 nt and 19 nt, as shown in supporting information in Table S1. Accordingly, we designed the spacer length of Ptac in this work (12–20 nt). Furthermore, we analyzed the promoter spacer sequences of sigA and sigD factors of *C. glutamicum* and compared them with Ptac, as shown in Table S2. We found that there was an AT-rich region in all of the spacer sequences which were highlighted in red and we added 1–4 bases to the 5′-end of Ptac by referring to the upstream sequences of the AT-rich region in the promoter of sigA and sigD factor to create the Ptac mutants. Illustrated in Fig. 1A, the spacer length of the original tac promoter is 16 nt. Using GFP as the reporter to measure the transcriptional strength of different promoters, our experimental design comprised a total of 8 promoter mutants with different spacer lengths (Fig. 1B). The results in Fig. 1C showed that, compared to the original tac promoter (16 nt), the transcriptional strength of most promoter mutants was significantly lower. For example, the transcriptional strength of PtacL12∼14 and PtacL19∼20 was only about one-tenth of that of Ptac, and PtacL17∼18 retained only a quarter of the transcriptional strength of Ptac. Surprisingly, the transcriptional strength of PtacL15 was higher than the original Ptac (16 nt) by approximately 20%. In summary, we found that the tac promoter with a 15 nt spacer length was particularly stronger than the original Ptac (16 nt) in *C. glutamicum*.

3.2. The effect of the spacer nucleotide sequence between the −35 and −10 core regions on the strength of the tac promoter

Based on the above results, we proposed that the nucleotide sequence of the spacer also affected the strength of the tac promoter. As shown in Fig. 2A, we constructed a promoter mutation library based on PtacL15 by designing a random spacer nucleotide sequence with length of 15 nt. Using GFP as the reporter, the transcriptional strength of each promoter was initially characterized by deep-well plate fermentation. On the basis of preliminary screening, 18 strains with strong fluorescence intensity were selected. We further sequenced the promoters and analyzed their base composition. To verify the experimental results, we subjected these strong fluorescent intensity strains to a shake flask culture. Compared with PtacL15, the selected 18 promoters had significantly increased *gfp* gene transcription (Fig. 2B). Fig. 2C revealed the sequencing results of the four promoters with the strongest fluorescence intensity, from which we found that the intensity of mutant PtacL15m-110 was 1.76 times that of the original Ptac. Furthermore, we analyzed from Fig. 2D the 1–15 bases of the top four strongest PtacL15 mutants with enhanced fluorescence intensity. The most conserved sites were the 13th and 14th positions, where the 13th position was all T base, and the 14th position was all C base. This was followed by position 11, where 75% of promoters were A and 25% were T. In addition, the 3rd, 4th and 12th positions were also highly conserved. Thus, to contribute to strengthening the tac promoter, we found an optimal spacer sequence 5′-GAACAGGCTTTATCT-3′ (PtacL15m-110), while the original sequence was 5′-ATTAATCATCGGTCG-3’.

3.3. The influence of 5′UTR length on the strength of the tac promoter

As reported previously, the 5′UTR region of the promoter might also significantly affect its transcriptional strength. In this study, we defined the sequence between the lacO operator and the ribosome binding site (RBS) as 5′UTR (Fig. 3A), and investigated the effects of the 5′UTR lengths of Ptac in *C. glutamicum*. First, by gradually truncating the 5′UTR region downstream of the tac promoter in the pEC-XK99E plasmid in units of 4 nt, we synthesized 13 mutants with different 5′UTR lengths named M1-M13. Promoter mutants of different 5′UTR lengths showed a variation of gradient strength, among which PtacM4-7 were stronger than the original promoter, and PtacM5 was the strongest (Fig. 3B). Further, we gradually truncated M4 until the 5′UTR length reached 28 nt (corresponding to the length of M6) in units of 1 nt. Fig. 3C illustrated the relative strength of promoters with a 5′UTR length of 28–36 nt, we observed that these nine promoters showed a gradient of strength, and

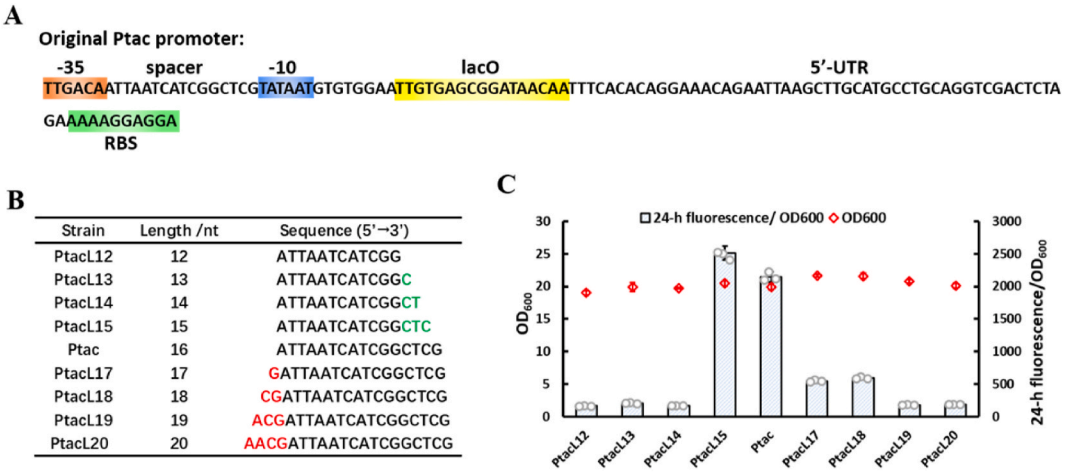
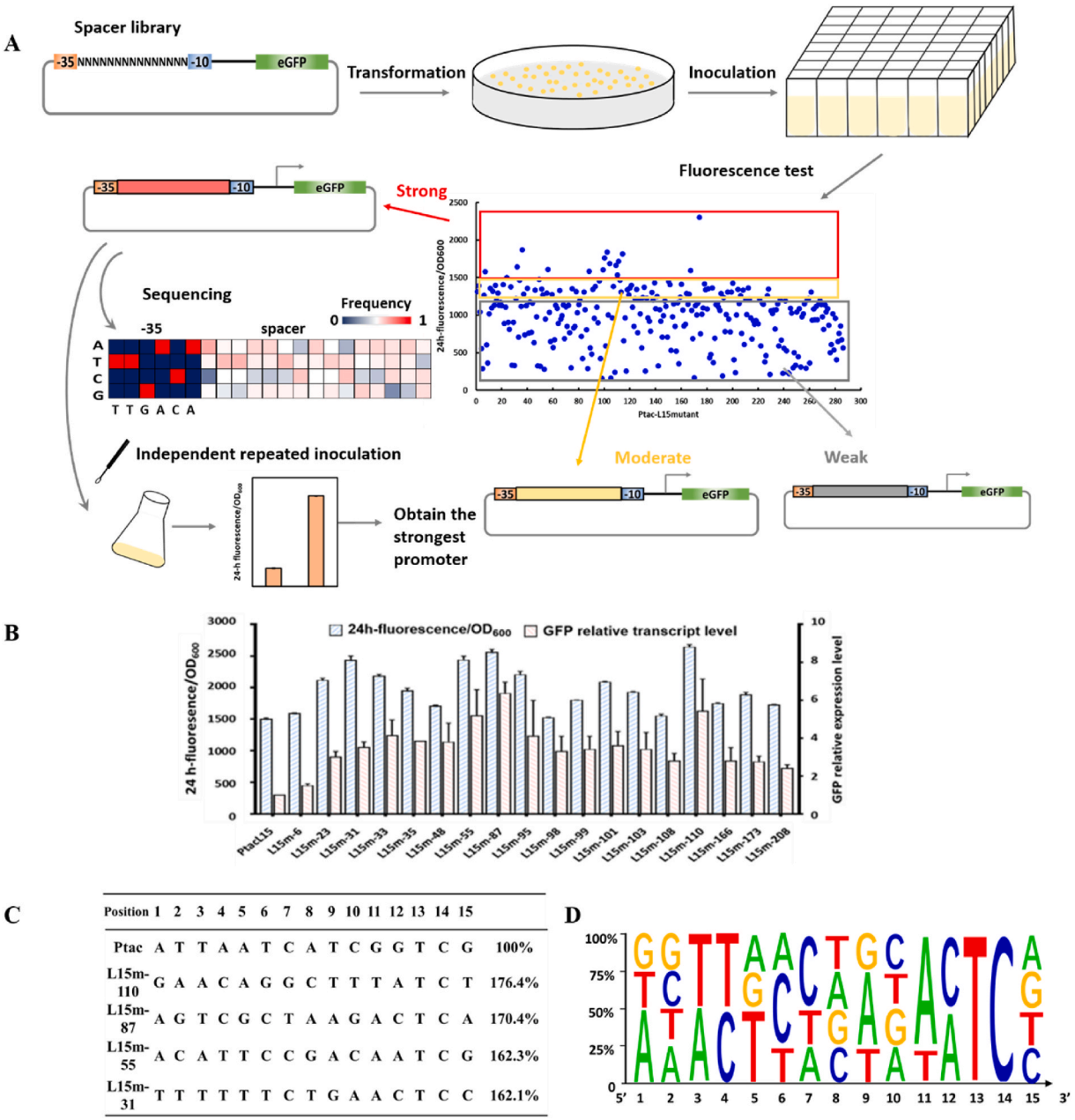


Fig. 1. Length optimization of spacer between the −35 and the −10 regions of the tac promoter in *C. glutamicum*. A. Sequence of the original tac promoter in pEC-XK99E plasmid used for gene overexpression in *C. glutamicum*. B. Design of spacer length optimization. C. GFP produced by different lengths of Ptac spacer in *C. glutamicum*.



Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Ptac	A	T	T	A	A	T	C	A	T	C	G	G	T	C	G	100%
L15m-110	G	A	A	C	A	G	G	C	T	T	T	A	T	C	T	176.4%
L15m-87	A	G	T	C	G	C	T	A	A	G	A	C	T	C	A	170.4%
L15m-55	A	C	A	T	T	C	C	G	A	C	A	A	T	C	G	162.3%
L15m-31	T	T	T	T	T	T	C	T	G	A	A	C	T	C	C	162.1%

Fig. 2. Nucleotide sequence optimization of spacer between the −35 and the −10 regions of the tac promoter in *C. glutamicum*. A. Workflow of random spacer optimization. B. Independently repeated cultivation of promoters stronger than the original Ptac. C. Spacer sequence recognition of the Top 4 promoters. D. Conserved sequence logo of the spacer region in the Top 4 promoters.

M5 was the strongest promoter. Then, by combining the 5′UTR region of PtacM5 and the spacer region of PtacL15m-87, we constructed a new PtacM promoter. Using GFP as the reporter, we found that PtacM is 3.25 times stronger than the original Ptac, making it an artificially constructed ultra-strong promoter.

3.4. Application of PtacM in β-galactosidase expression in C. glutamicum

C. glutamicum is an important industrial microorganism widely used in protein expression and production of chemical compounds. Insufficient endogenous promoter strength in *C. glutamicum* hinders further enhancement of target enzyme or compound yield. We applied the constructed super-strong PtacM to the expression of the model enzyme β-galactosidase (β-gal, Fig. 4). The SDS-PAGE results verified the overexpression function of PtacM (Fig. 4A). The qPCR results showed that PtacM significantly increased transcription of the *lacZ* gene, and the relative transcriptional level of the *lacZ* gene in *C. glutamicum* (PtacM-β-gal) was over two times that of *C. glutamicum* (Ptac-β-gal) (Fig. 4B). Moreover, as shown by the enzyme activity assay, the β-gal activity of the *C. glutamicum* (PtacM-β-gal) strain was 1.5 times that of the control group (Fig. 4C and D). These experimental results confirmed that the PtacM mutant can significantly increase the expression of protein/enzyme in *C. glutamicum*, which is of significant value in biomanufacturing.

3.5. Application of PtacM in morphological engineering of C. glutamicum

Cell morphology is a determining factor for the performance of industrial microorganisms. Microbial cell morphological engineering is achieved by modulating some genes relating to the regulation of the

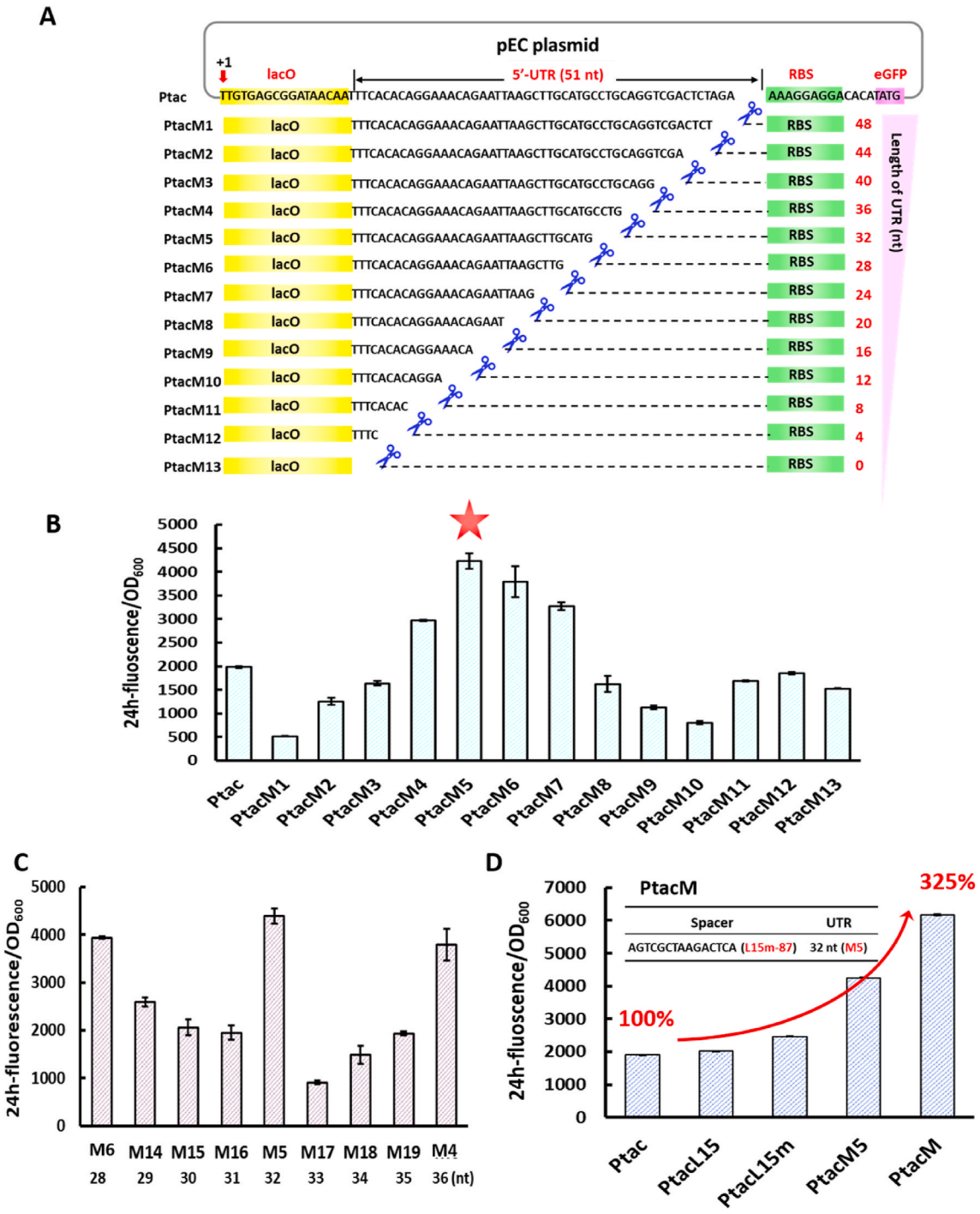


Fig. 3. Length optimization of 5′UTR between lacO and RBS of the tac promoter in *C. glutamicum*. A. Design of 5′UTR length optimization. B. GFP fluorescence produced by Ptac variants constructed via 4 nt-stepping truncation in *C. glutamicum*. C. GFP fluorescence produced by Ptac variants constructed via 1 nt-stepping truncation from 28 nt to 35 nt of 5′UTR length. D. GFP fluorescence produced by Ptac variants in *C. glutamicum*.

elongasome and divisome to change cell morphology, allowing cells to morph from rods to fibers, large spheres, or mini-cells.[27,28] Changes in cell morphology can enable the enhanced accumulation of intracellular products and inclusion bodies.[29] Notably, the FtsZ protein is involved in the formation of the divisome by assembling into Z-ring structures at the site of cell division.[30] In *C. glutamicum*, the overexpression of the *ftsZ* gene allowed the shape of cells to become long and thick rods with a remarkably enlarged single-cell surface area.[31] In this research, we expressed the *ftsZ* gene using three promoters with different strengths, Ptac, PacL15, and PtacM, and observed the changes in cell morphology under the same inducing condition. After overexpression of the *ftsZ* gene, mutants with different promoters driving the *ftsZ* led to different cell morphologies (Fig. 5). Obviously, an increase in promoter strength resulted in the elongation of the cell, followed by an increase in cell surface and volume. Suppose that the cell is an ideal geometry, with two hemispheres at both terminals and a cylinder in the middle, the parameters relating to cell size are shown in Table 1. For the *C. glutamicum* (PtacM-FtsZ) strain, the cell length was increased from 1.29 μm to 2.82 μm, and the surface area and volume of a single cell also increased by 118% and 146% to the wild-type *C. glutamicum*.

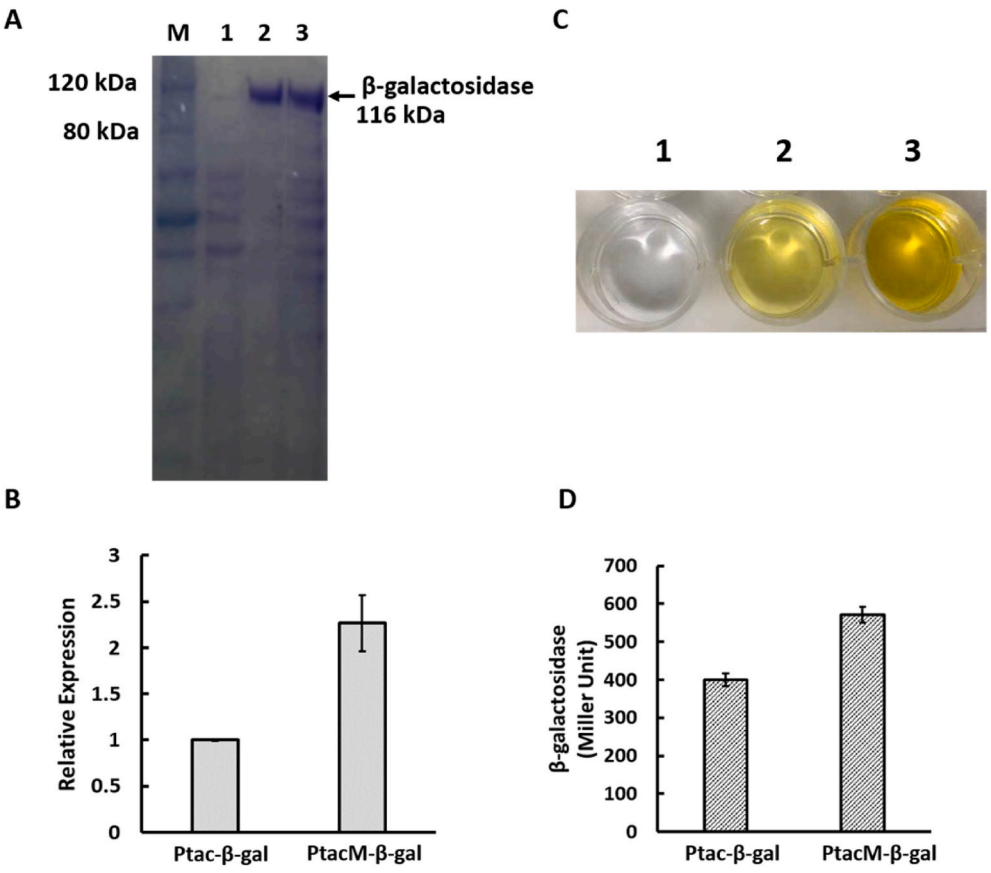


Fig. 4. Application of the mutant promoter PtacM to β-galactosidase expression in *C. glutamicum*. A. SDS-PAGE results of β-galactosidase overexpression driven by the original promoter Ptac and the mutant promoter PtacM in *C. glutamicum*. (M: marker, Lane 1: blank, Lane 2: Ptac, Lane 3: PtacM) B. Relative transcription level of *lac*Z driven by Ptac and PtacM promoters. C. Visualized comparison of β-galactosidase activity of *C. glutamicum* (Ptac-β-gal) and *C. glutamicum* (PtacM-β-gal) at 24 h (Lane 1: blank, Lane 2: Ptac, Lane 3: PtacM) D. Quantification of Ptac and PtacM driving β-galactosidase (β-gal) activity at 24 h.

4. Discussion

For biomanufacturing that applies chassis-microorganisms such as *C. glutamicum* to produce target products, promoters are usually regarded as the most important regulatory element at the transcriptional level. Identifying more basic rules in promoter sequence-function relationships, further enriching promoter libraries with gradient transcriptional strength, and also constructing artificial promoters with ultra-high driving strength are quite essential to fine-tune diverse metabolic modification nodes and improve the yield of target compounds. The mechanism of transcriptional regulation of promoters is very complex and is influenced by multiple factors, such as the sequence of the −35 and −10 core region and the conserved identifying sequence of the sigma factor of RNA polymerase.

As we know, the contribution of the −35 and −10 core region sequences to the promoter strength has been profoundly studied in diverse microbial strains for efficient biomanufacturing.[5,32,33] But the contribution and evolution of other elements, such as the core-region spacer and 5′UTR sequences, were less investigated except for basic research; also, most studies were focused on *E.coli* host. For example, the important function of the length of the spacer sequence between the −35 and −10 regions on the transcriptional strength of the promoter was highlighted by Mulligan et al.[22] William R. McClure et al. studied 112 promoters in *E. coli*, of which 56 had a spacer length of 17 nt, while only 22 ones in 16 nt or 18 nt.[34] They further investigated the effect of linear tac promoters (TAC16, TAC17, TAC18) with spacer lengths of 16, 17, and 18 nt on RNA polymerase of *E.coli* activity *in vitro*.[22] They used K_B for describing the rapid binding of RNA polymerase to a promoter to form an inactive closed complex, and k_2 for isomerization to form an active open complex. The results indicated that the promoter strength was mainly affected by the initial binding step (K_B). The value of K_B for the TAC17 spacer was ten times that of the 16 nt and 18 nt, and k_2 was slightly lower. Thus, in terms of linear tac promoter and *E. coli* RNA polymerase binding *in vitro*, 17 nt spacer length was optimal. But for *in vivo* experiments, the transcriptional strength of TAC17 and TAC18 was 90% and 65% that of TAC16, respectively.[23] That is, 16 nt spacer length is regarded as the best for an *in vivo* stronger Ptac in *E.coli*. D. Johnson et al. confirmed that the sigma factor of RNA polymerase in *E.coli* could sense the spacer between the −35 and −10 regions, and thus served as a molecular ruler.[35] They promoted a model in which the sigma factor spanned the distance of the spacer between the core regions, while at the same time making productive contacts at both regions. When the length of the spacer was too long, the sigma factor was not able to reach the gap, and when it was too short, the sigma factor was not able to adjust its own structure to accommodate the spacings.[35] In recent years, the evolution of the spacer and 5′UTR sequence in *E. coli* was highlighted in different promoters, with the development of synthetic biology, obtaining stronger promoters successfully.[36,37] But for other important industrial microorganisms, corresponding studies are still relatively rare.

In this work, *C. glutamicum* but not *E.coli* was focused, because of the continuously increased interest in it in the field of biomanufacturing. We first explored the effects of varying spacer length on the transcriptional strength of the classic tac promoter in *C. glutamicum*. To meet the demand for industrial biotechnology applications, we performed *in vivo* studies directly. For the first time, we found that the Ptac with a spacing of 15 nt had the strongest transcription initiation efficiency in *C. glutamicum*, which differed from the results in *E. coli*. We deduced that this difference was arising from the distinctions in the RNA polymerase (sigma factor) of the two microorganisms. This new finding will also enlighten novel ideas for the evolution of the other promoters in *C. glutamicum* and even in other microorganisms.

In the subsequent studies on the spacer nucleotides, we further identified the significance of the base composition. In *E. coli*, the base

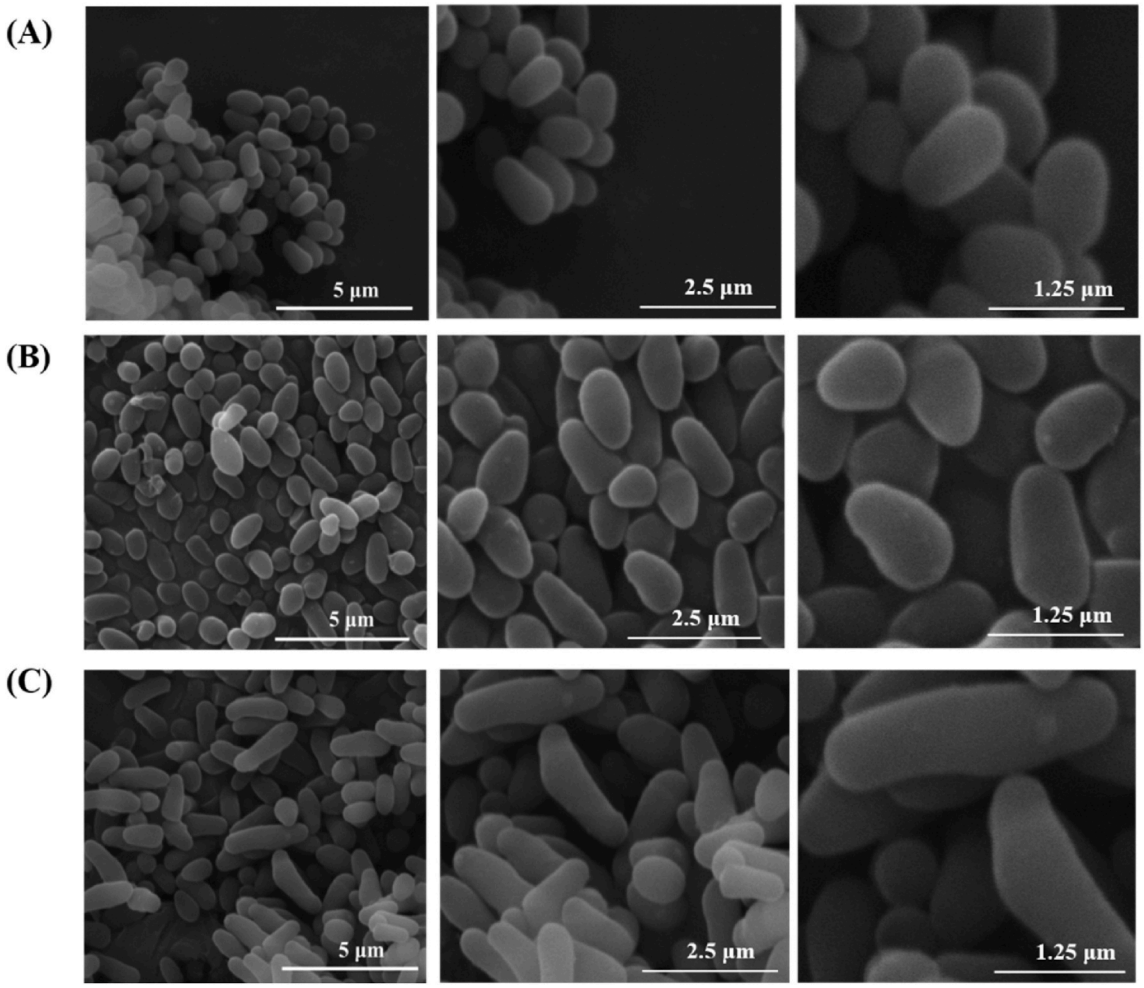


Fig. 5. The application of Ptac mutant to microbial morphology engineering. SEM images for *C. glutamicum* strains containing different plasmids. A: wild-type *C. glutamicum* ATCC13032; B: C. *glutamicum* ATCC13032 transformed with pEC-Ptac-GFP-ftsZ plasmid; C: *C. glutamicum* ATCC13032 transformed with pEC-PtacM-GFP-ftsZ plasmid.

Table 1
The parameters relating to the cell morphology of *C. glutamicum*.

Parameters	Wild type	Ptac-FtsZ	PtacM-FtsZ
Cell length (μm)	1.29 ± 0.11	1.74 ± 0.10	2.82 ± 0.27
Cell surface (μm^2)	2.76 ± 0.23	3.71 ± 0.22	6.01 ± 0.57
Cell volume (μm^3)	0.41 ± 0.04	0.58 ± 0.04	1.01 ± 0.10

composition of the spacer sequence is not conserved and therefore is not thought to affect transcriptional initiation.[35] Nevertheless, we found that the base composition could also affect the transcriptional strength of the promoter to some extent after random mutations of the spacer region. In the promoter library we constructed, the PtacL15m-110 mutant (GAACAGGCTTTATCT) was 1.76 times stronger than PtacL15. In addition, we also found that the bases at certain positions (e.g. 3rd, 4th, 13th, 14th) were significantly conserved, mostly T or C. We believed that in the process of forming a complex between RNA polymerase and promoters, both constituents were allosteric simultaneously, resulting in a complex structure. The base composition of the spacer region could potentially affect the process of promoter transitions, thus leading to changes in the stability of the complex and affecting the transcriptional strength of the promoter.

The regulation of transcriptional strength by changing the sequence of the promoter's 5′UTR also belongs to the metabolic regulation at the transcriptional level. Among *C. glutamicum* promoters, some strong endogenous PUTRs (promoter untranslated regions) have been identified, such as Psod-UTR and Ptuf-UTR.[38] In this study, we constructed a 5′UTR library by progressively truncating the 5′UTR between lacO sequences and RBS sequences in *C. glutamicum*. In this library, the tac 5′UTR region of different lengths exhibited gradient variations in transcriptional strength, and for the first time, we found that the truncated 5′UTR with a 32 nt length showed the highest transcription strength with respect to the original 54 nt length.

Combining the optimal spacer length (15 nt), nucleotide sequence (PtacL15m-87, AGTCGCTAAGACTCA), and 5′UTR (32 nt), we constructed an ultra-strong PtacM promoter. Using green fluorescent protein as a reporter, the strength of 4 promoters during construction compared with the wild-type Ptac were shown in Fig. 3D. We found that the transcriptional strength of this super-strong promoter was 3.25 times higher than that of Ptac, and it was much stronger than other constitutive strong promoters in *C. glutamicum*.[24] We hypothesized that this improvement might arise from their differences in mRNA secondary structure. As shown in Fig. 6, we forecasted the mRNA secondary structure of both the original tac promoter and PtacM and predicted the free energy of this segment of the mRNA sequence through the RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). Transcriptionally, the two promoters produce significantly different mRNA structures, with PtacM having less neck-loop structure compared to Ptac. David M. Mauger et al. demonstrated that less secondary structure formed by the first ten codons of the UTR + CDS region

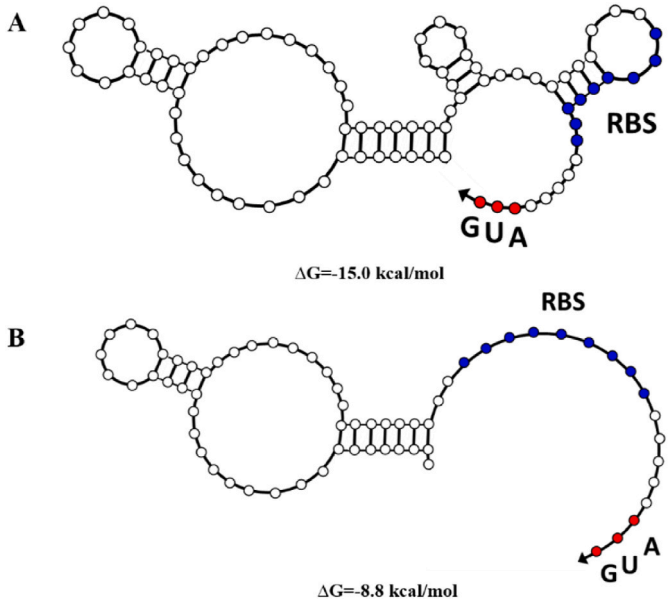


Fig. 6. Secondary folding structure and minimal free energy of mRNA transcribed by Ptac (A) and PtacM (B).

at the 5′ end of the mRNA, resulted in higher expression of the encoded protein.[39] This explained the stronger intensity of PtacM compared to Ptac.

Ultimately, we applied the synthetic PtacM to protein/enzyme expression and morphological engineering of *C. glutamicum*. Results showed that PtacM significantly increased the transcriptional level of the *lacZ* gene. Furthermore, at the translation level, the expression of β-gal is also significantly enhanced by PtacM, and the enzyme activity reached 1.5 times compared to Ptac. In cell morphology regulation, gradient-strength promoters can be applied to the controlled regulation of cell size. We mediated the overexpression of the *ftsZ* gene with tac promoter mutants of different transcriptional strengths and found that the morphology of *C. glutamicum* showed different degrees of alteration. Therefore, we resolved that a larger cell volume could accommodate more intracellular products and thus provided more attachment space for membrane proteins.

To sum up, we investigated the effects of varied spacer length, nucleotide composition, and 5′UTR-length on the transcriptional strength of Ptac in *C. glutamicum*. Based on new findings on the optimal spacer length (15 nt), base composition, and 5′UTR (truncated 32 nt), an ultra-strong PtacM was obtained and successfully applied to enhanced expression of enzymes and enlarging regulation of cell morphology. The super-strong PtacM promoter is expected to be widely used in versatile product synthesis by *C. glutamicum*.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

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References

1 Blazeck J, Alper HS. Promoter engineering: recent advances in controlling transcription at the most fundamental level. *Biotechnol J*. 2013;8(1):46–58.
2 Cazier AP, Blazeck J. Advances in promoter engineering: novel applications and predefined transcriptional control. *Biotechnol J*. 2021;16(10).
3 Jin L-Q, et al. Promoter engineering strategies for the overproduction of valuable metabolites in microbes. *Appl Microbiol Biotechnol*. 2019;103(21-22):8725–8736.
4 Xu N, Wei L, Liu J. Recent advances in the applications of promoter engineering for the optimization of metabolite biosynthesis. *World J Microbiol Biotechnol*. 2019;35(2).
5 Chai M, et al. Synthetic biology toolkits and metabolic engineering applied in *Corynebacterium glutamicum* for biomanufacturing. *ACS Synth Biol*. 2021;10(12):3237–3250.
6 Nesvera J, Patek M. Tools for genetic manipulations in *Corynebacterium glutamicum* and their applications. *Appl Microbiol Biotechnol*. 2011;90(5):1641–1654.
7 Hwang HJ, Lee SY, Lee PC. Engineering and application of synthetic nar promoter for fine-tuning the expression of metabolic pathway genes in *Escherichia coli*. *Biotechnol Biofuels*. 2018;11.
8 Liu D, et al. Construction, Model-based analysis, and characterization of a promoter library for fine-tuned gene expression in *Bacillus subtilis*. *ACS Synth Biol*. 2018;7(7):1785–1797.
9 Zhang S, et al. Model-based reconstruction of synthetic promoter library in *Corynebacterium glutamicum*. *Biotechnol Lett*. 2018;40(5):819–827.
10 Portela RMC, et al. *Synthetic Core Promoters as Universal Parts for Fine-Tuning Expression in Different Yeast Species*. Figshare; 2016.
11 Rytter JV, et al. Synthetic promoter libraries for *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol*. 2014;98(6):2617–2623.
12 Shang X, et al. Native promoters of *Corynebacterium glutamicum* and its application in L-lysine production. *Biotechnol Lett*. 2018;40(2):383–391.
13 Ravasi P, et al. Design and testing of a synthetic biology framework for genetic engineering of *Corynebacterium glutamicum*. *Microb Cell Factories*. 2012;11.
14 Yim SS, et al. Isolation of fully synthetic promoters for high-level gene expression in *Corynebacterium glutamicum*. *Biotechnol Bioeng*. 2013;110(11):2959–2969.
15 Zhang Y, et al. Development and application of an arabinose-inducible expression system by facilitating inducer uptake in *Corynebacterium glutamicum*. *Appl Environ Microbiol*. 2012;78(16):5831–5838.
16 Lausberg F, et al. A tetracycline inducible expression vector for *Corynebacterium glutamicum* allowing tightly regulable gene expression. *Plasmid*. 2012;68(2):142–147.
17 Deboer HA, Comstock LJ, Vasser M. The tac promoter - a functional hybrid derived from the trp and lac promoters. *Proc Natl Acad Sci USA Biol Sci*. 1983;80(1):21–25.
18 Sun M, et al. Enhanced production of recombinant proteins in *Corynebacterium glutamicum* by constructing a bicistronic gene expression system. *Microb Cell Factories*. 2020;19(1).
19 Peters-Wendisch P, et al. Metabolic engineering of *Corynebacterium glutamicum* for L-serine production. *Appl Environ Microbiol*. 2005;71(11):7139–7144.
20 Xu D, et al. An improved shuttle vector constructed for metabolic engineering research in *Corynebacterium glutamicum*. *Plasmid*. 2010;64(2):85–91.
21 Xu J-Z, et al. Rational modification of tricarboxylic acid cycle for improving L-lysine production in *Corynebacterium glutamicum*. *Microb Cell Factories*. 2018;17.
22 Mulligan ME, Brosius J, McClure WR. Characterization invitro of the effect of spacer length on the activity of *Escherichia coli* RNA-polymerase at the tac promoter. *J Biol Chem*. 1985;260(6):3529–3538.
23 Brosius J, Erfle M, Storella J. Spacing of the -10 and -35 regions in the tac promoter - effect on its *in vivo* activity. *J Biol Chem*. 1985;260(6):3539–3541.
24 Cheng F, Yu H, Stephanopoulos G. Engineering *Corynebacterium glutamicum* for high-titer biosynthesis of hyaluronic acid. *Metab Eng*. 2019;55:276–289.
25 Cheng F, et al. Biosynthesis of chondroitin in engineered *Corynebacterium glutamicum*. *J Microbiol Biotechnol*. 2019;29(3):392–400.
26 Wang M, et al. Single-gene Regulated Non-spore-forming *Bacillus Subtilis*: Construction, Transcriptome Responses and Applications for Producing Enzymes and Surfactin. *Metab Eng*. 2020.
27 Guo L, et al. Engineering microbial cell morphology and membrane homeostasis toward industrial applications. *Curr Opin Biotechnol*. 2020;66:18–26.
28 Wang Y, et al. Microbial engineering for easy downstream processing. *Biotechnol Adv*. 2019;37(6).
29 Huo K, et al. Morphology engineering: a new strategy to construct microbial cell factories. *World J Microbiol Biotechnol*. 2020;36(9).
30 Letek M, et al. Cell growth and cell division in the rod-shaped actinomycete Corynebacterium glutamicum. *Int J Gen Mol Microbiol*. 2008;94(1):99–109.
31 Zheng Y, et al. Enhancing single-cell hyaluronic acid biosynthesis by microbial morphology engineering. *Synth Syst Biotechnol*. 2020;5(4):316–323.
32 Casa PL, et al. Beyond consensual motifs: an analysis of DNA curvature within *Escherichia coli* promoters. *Biologia*. 2022;77(4):1095–1102.
33 Fu G, et al. An operator-based expression toolkit for *Bacillus subtilis* enables fine-tuning of gene expression and biosynthetic pathway regulation. *Proc Natl Acad Sci USA*. 2022;119(11).
34 Hawley DK, McClure WR. Compilation and analysis of *Escherichia coli* promoter DNA-sequences. *Nucleic Acids Res*. 1983;11(8):2237–2255.

35 Dombroski AJ, et al. The sigma subunit of *Escherichia coli* RNA polymerase senses promoter spacing. *Proc Natl Acad Sci USA*. 1996;93(17):8858–8862.
36 Kang S-Y, Heo KT, Hong Y-S. Optimization of artificial curcumin biosynthesis in *E. coil* by randomized 5 '-utr sequences to control the multienzyme pathway. *ACS Synth Biol*. 2018;7(9):2054–2062.
37 Zhou Y, et al. Biosynthesis of adipic acid by a highly efficient induction -free system in *Escherichia coli*. *J Biotechnol*. 2020;314:8–13.
38 Li N, et al. Obtaining a series of native gradient promoter-5 '-UTR sequences in *Corynebacterium glutamicum* ATCC 13032. *Microb Cell Factories*. 2020;19(1).
39 Mauger DM, et al. mRNA structure regulates protein expression through changes in functional half-life. *Proc Natl Acad Sci USA*. 2019;116(48):24075–24083.