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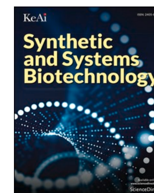

Original Research Article

Engineering artificial cross-species promoters with different transcriptional strengths

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ABSTRACT

As a fundamental tool in synthetic biology, promoters are pivotal in regulating gene expression, enabling precise genetic control and spurring innovation across diverse biotechnological applications. However, most advances in engineered genetic systems rely on host-specific regulation of the genetic portion. With the burgeoning diversity of synthetic biology chassis cells, there emerges a pressing necessity to broaden the universal promoter toolkit spectrum, ensuring adaptability across various microbial chassis cells for enhanced applicability and customization in the evolving landscape of synthetic biology. In this study, we analyzed and validated the primary structures of natural endogenous promoters from *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, and *Pichia pastoris*, and through strategic integration and rational modification of promoter motifs, we developed a series of cross-species promoters (P_{sh}) with transcriptional activity in five strains (prokaryotic and eukaryotic). This series of cross species promoters can significantly expand the synthetic biology promoter toolkit while providing a foundation and inspiration for standardized development of universal components The combinatorial use of key elements from prokaryotic and eukaryotic promoters presented in this study represents a novel strategy that may offer new insights and methods for future advancements in promoter engineering.

1. Introduction

Transcriptional regulation plays a crucial role in controlling the expression and concentration of intracellular proteins [1]. Especially, promoter as the basic regulatory element has been deeply studied and many natural promoters for model organisms for instance *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae* and *Pichia pastoris* have been identified, characterized and applied for constructing microbial cell factories. In view of the comparatively simple structure of prokaryotic promoters, many rational approaches have been developed for engineering synthetic strong and stress-responsive promoters for *E. coli* [2,3], *B. subtilis* [4]and *C. glutamicum* [5,6]. In particular, by training a convolutional neural network with high throughput DNA sequencing data, Brempt and colleagues successfully constructed a library of orthogonal sigma factor-specific promoters [7].

With the development of synthetic biology tools and the deep understanding of the complex structure of eukaryotic promoters, many strong and minimized artificial yeast promoters, especially for *S. cerevisiae* [8–11] and *P. pastoris* [12–15], have been designed and constructed. To improve the multi-gene co-expression capability, a library of 168 synthetic bidirectional promoters were generated, enabling the rapid optimization of metabolic pathways (taxadiene, β-carotene) [16]. Moreover, to facilitate the screening of suitable chassis hosts and construction of efficient microbial cell factories towards enzymes or metabolites of interest, we have constructed a list of broad-spectrum promoters for *E. coli*, *B. subtilis*, and *S. cerevisiae* by integrating the consensus motifs (TTGACA and TATAAT) into the synthetic minimal yeast promoter [11]. Recently, the promoters for both brewing *S. cerevisiae* and *E. coli* have also been developed [17]. However, no

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robust cross-species promoters that covering *P. pastoris* have been reported. In recent years, *P. pastoris* has been considered as one of the ideal industrial hosts for producing natural products [18,19] or enzymes [20,21]. Moreover, *C. glutamicum* has also been engineered as cell factories for producing various compounds [22,23].

However, although promoter engineering for specific cells has been extensively investigated, cross-species promoter studies applicable to multiple hosts are still not well-developed, thereby making it challenging to screen or characterize the expression construction process of genes in multiple hosts [24]. To address these issues, in recent years, Rahmi et al. constructed activation subsystems that are active in seven types of microbial cells by randomly screening 200-nt DNA sequences proved the availability of obtaining universal promoter sequences by random mutation [25]. Additionally, in some shuttle plasmids, different promoters are fused or connected in parallel to form analogous cross-species promoters to utilize the same screening tag in different species, and these shuttle plasmids have been matured for the production of compounds or proteins. Examples include the pGAPZ-(alpha) series of *E. coli-P. pastoris* shuttle plasmids [26,27], and the pYES2 series of *E. coli-S. cerevisiae* shuttle plasmids [28].

In our previous research [29], broad-spectrum promoters P_{bs} across three hosts were developed. However, there is still room for improvement in the strength and breadth of this promoter. In order to further expand the spectrum of the cross-species promoter in different hosts, in this study, we created an upgraded version of the P_{bs} promoter, the P_{sh} promoter. This series of promoters creatively produced strong activation activity in five microbial cells, which is conducive to further expanding the toolbox of cross-species components of synthetic biology and broadening the breadth of cross-species. In addition, the combined use of key elements of prokaryotic and eukaryotic promoters proposed in this study represents a novel strategy that may provide new insights and approaches for the future development of promoter engineering.

2. Materials and methods

2.1. Strains and plasmids construction

All the strains and plasmids used in this study are listed in Table S1. The primers used for recombinant plasmid construction and DNA-seq are listed in Table S2. All plasmids in this study were constructed by Gibson assembly and verified by DNA sequencing. All primers were synthesized by GENEWIZ (Suzhou, China). The P_{st} promoter sequence was synthesized by GENCEFE Biotech (Wuxi, China). Primer UP1–N was used to construct promoter UP elements and UP mutation libraries. Primer GAP-F/R, GCW14-F/R, Aox1core-F/core-R, TDH3-F/R/core-R/core-F was used to construct the yeast promoter full sequence and core sequence activity detection GFP-expression plasmid. Plasmid pEBCP-gfp provided the URA sequence of *S. cerevisiae* from plasmid pY26, the *P. pastoris* HIS4 integration site from plasmid pAO815, and the *B. subtilis* and *E. coli* replicon and terminator from pEBS. All fragments were constructed by Gibson assembly and verified by DNA sequencing.

2.2. Medium and culture conditions

Luria–Bertani (LB) broth (10.0 g/L tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl); yeast extract peptone dextrose (YPD) medium (10.0 g/L yeast extract, 20.0 g/L tryptone, constant volume to 900 mL and after sterilization, 100 mL 2 % glucose was added); minimal dextrose (MD) medium (per liter 100 mL 20 % glucose, 100 mL yeast nitrogen base without Amino Acids (YNB), 2 mL 500 × Biotin, 2 % agar); synthetic drop-out medium (SD) ura^- (50 mg/L leucine, 50 mg/L histidine, 50 mg/L tryptophan was added on the basis of MD medium); brain heart infusion (BHI) medium (91 g/L sorbitol, 37 g/L brain heart infusion powder). Solid media were prepared by adding 2.0 g/L agar to the media. When required, ampicillin (100 mg/L) or kanamycin (50 mg/L) or chloramphenicol (25 mg/L) were added to the LB media, 600 mg/L geneticin (G418) were added to the YNB media. Determining AOX1 promoter (*P. pastoris*) activity, glucose in YPD medium was replaced with 1 % (v/v) methanol. Induction medium: Cells were cultured in YPD (glucose) medium for 24 h, centrifuged and collected, washed twice with sterile normal saline, and inoculated in YPD (methanol) medium at 2 % by volume (v/v). Fluorescence was determined after induction culture for 24 h.

E. coli JM109 and *B. subtilis* 168 was cultured in LB broth, 37 °C and 220 rpm. *S. cerevisiae* CEN.PK2–1C and *P. pastoris* GS115, was cultured in YPD. SD medium was used for selection *S. cerevisiae* and MD medium was used for selection *P. pastoris*, all yeast strains were cultured at 30 °C in an incubator. BHI medium was used to grow *C. glutamicum* at 30 °C and 220 rpm.

2.3. Fluorescence assay

Cells recombinants were picked into YPD medium in 24-well Deep Well Plates and cultivated at 30 °C with shaking at 900 rpm. The GFP fluorescence intensity was quantified with the plate reader Infinite 200 PRO (Tecan, Austria) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. OD_{600} was also detected. The gain value was set to 60. The cells were washed in phosphate-buffered saline for two times for fluorescence detection. Promoter activity was indicated by the ratio of GFP reporter fluorescence intensity to OD_{600}.

2.4. Flow cytometry screening

Single-cell fluorescence was analyzed with the BD FACSAria III (BD Biosciences) flow cytometer. GFP was excited at a wavelength of 488 nm, and the fluorescence signal was recovered with a 529 (28)-nm band pass filter. The *E. coli*, were cultured overnight and inoculated into the corresponding medium at 1 % of the inoculated amount. After culture to the middle stage of index, the cells were washed twice in phosphate buffered saline and diluted with 0.01 M phosphate buffered saline for 1:100. The top 0.1 % cells of control fluorescence intensity were recovered.

3. Results

3.1. Construction of UP element for improving P_{bs} promoter activity in *E. coli*

The UP element is a sequence from −45 to −60 bp upstream of the transcriptional start site, which can bind to the RNA polymerase alpha subunit carboxy-terminal domain (αCTD), promote the recognition of RNA polymerase and promoter sequence, and enhance promoter activity [30,31]. As a consequence, to improve the P_{bs} activity, herein, we firstly introduced six UP sequences at the upstream of P_{bs} promoter (Fig. 1a). Specifically, UP1, UP5 and UP6 were originated from the upstream sequence of genes *rrnD*, *rybB* and *rpoE* [30]. UP2 was a consensus sequences identified through in vitro selection [32], while UP3 and UP4 were synthetic sequences from the sequence consistency rule [33]. These six UP sequences have been reported to enhance promoter activity. We found introduction of UP4 and UP6 sequences significantly increased promoter activities, which were 1.4 and 1.6 times that of the P_{bs} promoter [29].

Base on the sequences of UP4 and UP6 (Fig. 1b), we created a UP element library as 5′-NNANATGANGATCAAAAANANANNCNNNNN-3′ (Fig. 1b) and created a mutant library for flow cytometry screening (Fig. 1c, Fig. S1). After further cultivating on 96-well plate (Fig. 1c) and selecting with fluorescence intensity, we identified 30 UP mutant sequences, which activities covered from 190 % to 300 % intensity comparing to P_{bs} (Fig. 1d). Eventually, UP-G1, UP-G2 and UP-G3 with the highest activities (Fig. 1e) were used for following experiments. The results confirmed that in addition to altering the spacer sequences between the conserved −35 and −10 boxes [34], optimization of the UP

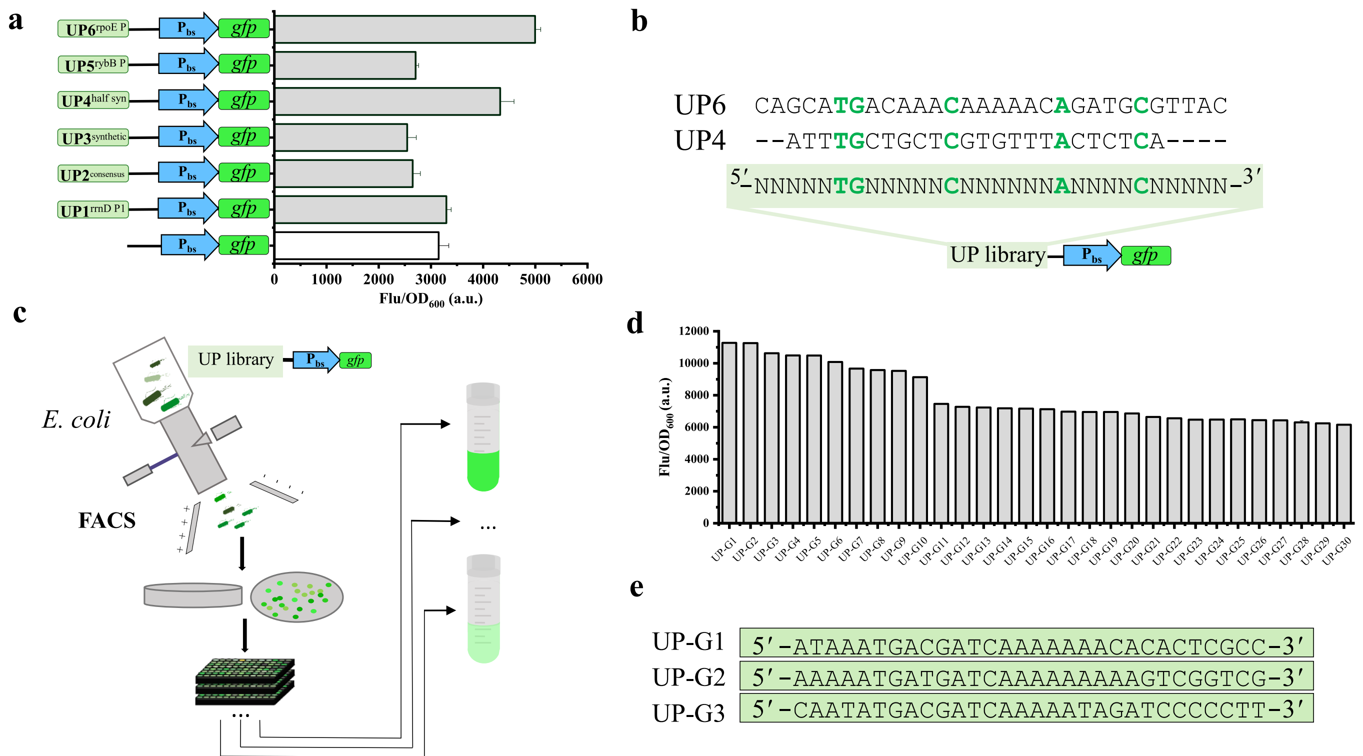

Fig. 1. Construction of a library for the UP sequence of prokaryotic promoter elements. (a) UP1 originated from rrnD P and UP2 was a comprehensive sequence [32], UP3 is an artificial synthetic sequence and UP4 is a semi-synthetic sequence [33], UP5 and UP6 were derived from rybB and rpoE, respectively. Activity testing was performed on the known 6 UP sequences, and it was found that UP1/4/6 showed better enhancement of promoter activity. **(b)** The sequences of UP1/4/6 were aligned, and bases that appeared two or more times were considered as consensus bases, resulting in a consensus sequence. **(c)** The obtained consensus UP sequences were constructed upstream of the P_{bs} promoter for flow cytometry screening. The screened cells were then picked and inoculated into a 96-well plate, followed by fluorescence retesting after cultivation. **(d)** The top 30 transformed colonies with the highest fluorescence intensity were selected, and plasmids were extracted for sequencing, yielding a series of optimal UP sequences. **(e)** The sequences of the three best UP elements.

sequences would be also an effective strategy for engineering strong promoters. These new sequences of UP elements we obtained significantly enhanced the strength of P_{bs} promoters, so we used these UP elements as part of a cross-species promoter.

3.2. Upgrading P_{bs} promoter skeleton by integrating σ factor binding site engineering

Integration of σ factor binding sites to engineer cross-species

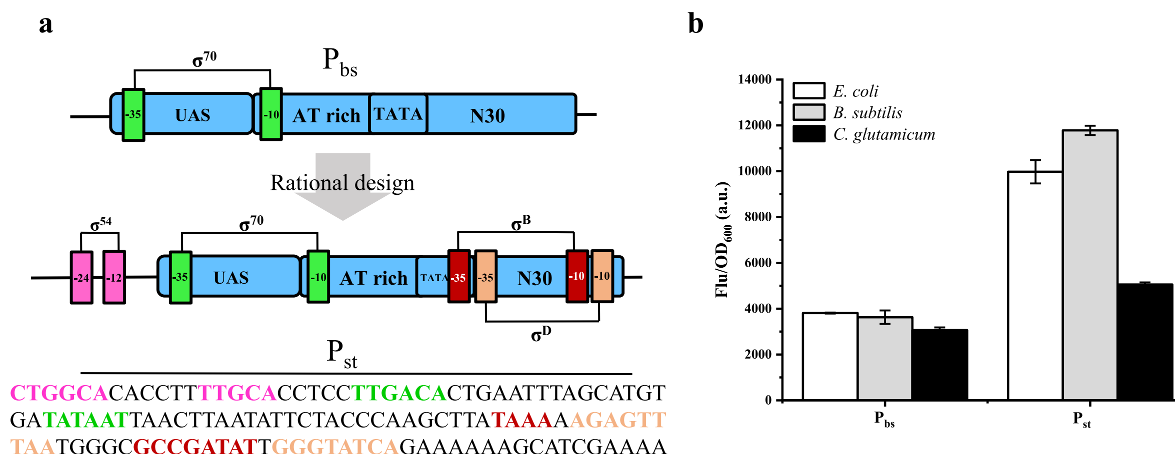

Fig. 2. Rationally design the insertion of σ factor binding sites to form a new cross-species promoter framework. (a) The $σ^{54}$ from *E. coli*, $σ^B$ and $σ^D$ from *B. subtilis* were inserted into the promoter P_{bs} framework to form the Pst promoter. The −24 region, −12 region, and the spacer sequence (underlined) of $σ^{54}$ 5′-CTGGCA-CACCTTTTGCAT-3′ were introduced upstream of the UAS1 sequence. The original TATA region of the P_{bs} sequence and the N30 region 5′-TATAAAA-GAGCACTGTTGGGCGTGAGTGGAGGCGCCGG-3′ were mutated or altered to introduce the conserved recognition sequences of $σ^B$ and $σ^D$, changed to 5′-TATAAAAAGAGTTTAATGGGCGCCGATATTGGGTATCAG-3’. The recognition sequence for sigma 54 is depicted in berry purple, with the spacer of $σ^{54}$ underlined, while the recognition sequence for $σ^B$ is shown in red, and the recognition sequence for $σ^D$ is shown in orange. **(b)**The strength of the modified P_{st} promoter was improved in *E. coli*, *B. subtilis* and *C. glutamicum*. The highest strength increase was 300 % in *B. subtilis* and the lowest was about 160 % in *C. glutamicum*. The background fluorescence value of the wild-type strain was subtracted from the calculation of fluorescence intensity. Data are presented as the mean ± standard deviation (n = 3).

promoter skeleton to improve the prokaryotic elements of P_{bs} promoter. Different σ factors in bacteria are responsible for recognizing different promoter sequences. Taking *E. coli* as a reference, [35] σ^{70} is involved in the major growth stages of the cell and is known as the housekeeping sigma factor, while σ^{54} is involved in the transcription of genes related to environmental regulation, its classification as another significant family of sigma factors. Despite variations, the recognition sequences of σ factors in different bacterial strains share some degree of similarity and compatibility [36]. For instance, the conserved recognition sequence of *E. coli* σ^{70} can be recognized by *B. subtilis* [37].

By effectively utilizing these sigma factors, it is expected that broad and effective promoter sequences for bacterial transcription can be obtained. We found that the recognition sequences of σ^B and σ^D matched the original sequence of P_{bs} promoter. In addition, the addition of the σ^{54} recognition sequence helps to expand the family of σ factors on the promoter sequence. Therefore, by matching with the promoter skeleton sequence of P_{bs}, the binding sites of σ^{54}, σ^B and σ^D are finally added. The recognition sequence of σ factors used in this study is sorted out in (Table S3). The σ^{54} recognition sequence is derived from *E. coli*, and the σ^B and σ^D recognition sequences are derived from *B. subtilis*. The design sequence and structure are shown in (Fig. 2a). Using the promoter P_{bs} as the template, primers were used to introduce the conserved sequence of the σ factor at specific sites, which was then integrated into the promoter P_{bs} through stepwise PCR reactions. Based on the sequence characteristics, the σ^{54} recognition site was placed upstream, and the σ^B and σ^D sites were inserted into the TATA-N30 region of the P_{bs} promoter. A new promoter, P_{st}, was designed. Compared with the promoter P_{bs}, the designed promoter P_{st} has significantly enhanced the activity level in *E. coli*, *B. subtilis* and *C. glutamicum*. Notably, the relative fluorescence intensity increase was 200 % in *E. coli* and *B. subtilis*. Even if we did not specifically insert a specific σ factor recognition site for *C. glutamicum*, the new promoter can enhance its fluorescence expression 60 % by utilizing three σ factors from other bacteria (Fig. 2b).

This shows that we can identify the promoter activity (prokaryotic) of P_{bs} based on the addition of σ factor sequences. The newly generated P_{st} promoter sequence can serve as the skeleton structure of the new version of the cross-species promoter.

3.3. Design upstream activation sequence (UAS) to engineer eukaryotic elements of P_{bs} promoter sequence

The initiation of transcription in eukaryotes is a complex process that entails the recognition of the RNA polymerase and promoter sequence by various transcription factors (TFs). Primarily, the core promoter sequence establishes the fundamental level of transcription [38], and TF binding to the upstream activation sequence (UAS) can regulate transcriptional activity [39–41]. Techniques related to UAS engineering include serial combination, library mutation, and artificial design [42–44]. These studies indicate that UAS can enhance the efficient recognition of core promoter sequences by RNA polymerase binding sites, thereby increasing transcription efficiency.

In order to optimize the functionality of cross-species promoters in eukaryotic microorganisms, we aimed to identify effective active elements from natural yeast promoter sequences. In the construction of the eukaryotic element part of the cross-species promoter, we first conducted activity detection on a series of classic constitutive promoters. These tested promoters include the GAP promoter, GCW14 promoter, AOX1 promoter of *P. pastoris* [41,45,46], and the TDH3 promoter from *S. cerevisiae* [47]. We performed cross-speices validation of promoter activity in these two yeasts. We found that the original promoter of yeasts is more active in its own cells than in other cells. For example, the activity of the TDH3 promoter in *Saccharomyces cerevisiae* can reach

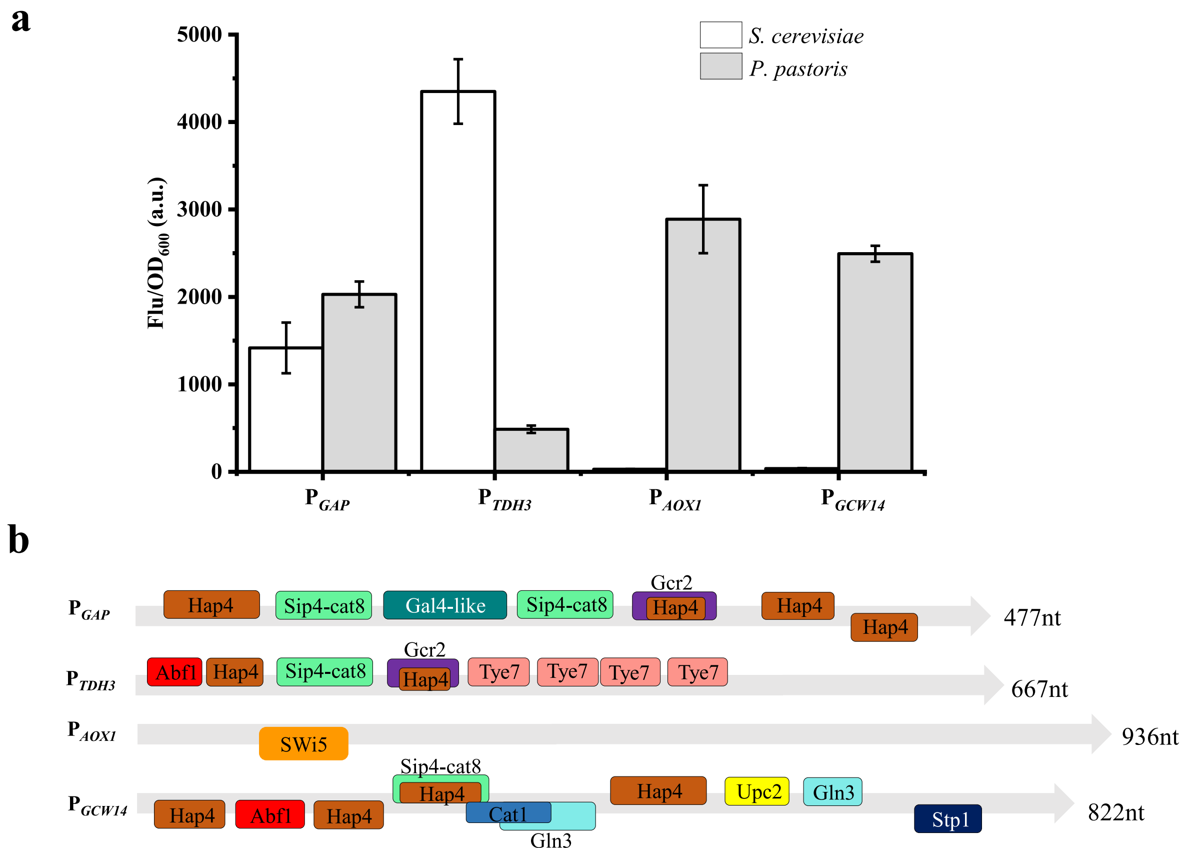

Fig. 3. Screening for TFBSs on natural promoters. (a) The broad spectrum of natural promoters from different yeast cells was verified. GAP, AOX1 and GCW14 promoters were derived from *P. pastoris*, and TDH3 promoters were derived from *S. cerevisiae*. **(b)** The predicted and selected transcription factor binding site and its relative position on the promoter sequence. Different colors represent different TFs binding positions.

about eight times that of *P pastoris*. The activity of GAP, an endogenous promoter of *P pastoris*, was reduced by about 30 % in *S. cerevisiae* (Fig. 3a). We hypothesize that transcription factor binding sites (TFBSs) on the promoter sequence are responsible for this result.

In search of potential universal recognition sites on natural promoters, we utilized the cross-species comparative genomics of transcription regulation in yeasts database YEASTRACT+ (http://www.yeastract.com/) [48] to identify conserved sequences of TFBSs, based on the promoter activity results. We selected 11 sequences with the optimized TFBSs to enhance promoter strength (Fig. 3b–Table 1). These transcription factors are thought to be transcriptional activators. There were 4 TFBSs on GAP promoter sequence and 5 TFBSs on TDH3 promoter sequence (3 repeats), 1 TFBS on AOX1 promoter sequence and 7 TFBSs on GCW14 promoter sequence (3 repeats). Relative positions of these TFBSs on promoter sequences, we used a simple sequence annexation algorithm (Supplementary file 2) to combine these TFBSs and find the best annexation sequence to improve the DNA sequence information density of TFBSs in the UAS region (Fig. 4a) (Table 2). After merging, 11 transcription factor merging sequences were generated, ranging in length from 25 to 109 nt. The combination results were fused with the promoter P_{bs} to verify the fluorescence intensity in yeast cells. The promoter that 2 TFBSs, 4 TFBSs, 5 TFBSs, 10 TFBSs and 11 TFBSs showed better performance. The relative fluorescence intensity of UAS2, UAS4, UAS5 in *P. pastoris* exceeds 800, and the relative fluorescence intensity of UAS10, UAS11 in *S. cerevisiae* exceeds 1400, ranking in the top three in each combination for the relative fluorescence intensity (Fig. 4b).

3.4. Engineering cross-species promoters by combining eukaryotic and prokaryotic elements

In this section, we integrated the results of the previous steps, which included the new P_{st} promoter skeleton, the UP-G1, UP-G2, UP-G3 sequences screened from the UP elements library, and the combination sequences of TFBSs 2 TF, 4 TF, 5 TF, 10 TF, and 11 TF. As shown in part 1,2,3 of (Fig. 5a). This led to a total of 15 combinations, according to the validation results (Fig. S2), the eight sequences 2S2, 2S3, 4S2, 5S2, 5S3, 10S2, 10S3 and 11S3 were selected for the subsequent fusion ribosome binding site (RBS) combination ligation. We attached the RBS [49] to the 3′ end of these promoter sequences of screening in 8 combinations (Fig. 5a). We examined the cross-species promoters (named $P_{sh1\text{-}8}$) on controlling GFP expression in *E. coli*, *B. subtilis*, *C. glutamicum*, *S. cerevisiae* and *P. pastoris* by fluorescence microscopy, and found that all generated cross-species promoters were active in five cell types (Fig. 5b). The promoter activity level was quantified by fluorescence intensity (Fig. 5c). And, contrast P_{st} and Fig. S2 of 2S2, 2S3, 4S2, 5S2, 5S3, 10S2, 10S3 and 11S3 sequence, we found that the fusion of RBS to add a certain extent promote the GFP expression of function, although the effect was not expected. By comparing the composition promoters that are commonly utilized in hosts, we discovered that cross-species promoters exhibited a certain degree of activity intensity in various strains. For instance, in *E. coli*, the activity range of the P_{sh} series promoter could reach 10 times that of the J23100 promoter; in *B. subtilis*, it could reach approximately 1.8 times that of the P_{43} promoter; and in *C. glutamicum*, it could attain about 80 % of the activity intensity of the P_{trc} promoter (excluding inducible regulatory sequences). In *P. pastoris*, the activity intensity of the transspecies promoter $P_{sh1/3}$ is similar to that of the GAP promoter, and the activity intensity of P_{sh7} can reach 120 % of that of the GAP promoter. In *S. cerevisiae*, the activity intensity of the cross-species promoter can amount to up to 50 % of that of the TEF1 promoter. Simultaneously, a comparison with the promoter skeleton P_{st} further validated the efficacy of these artificial UAS sequences. The activity of $P_{sh3/7/8}$ was relatively potent in both yeasts, suggesting that certain arrangements of TFBSs might enhance promoter activity. Therefore, by analyzing the transcription factor binding sites of these three promoter sequences, we found that they share the same newly generated transcription factor binding sites (Table 3). We believe that it is the permutation and combination that led to the generation of some new TFBSs, which play a promoting role in the activity. The sequence of P_{sh} in (Table S4). In addition, considering that yeast TFBSs with promoter sequences may affect their stable expression, we refer to the scheme of Zhou et al. [50]. The stable expression of P_{sh} was demonstrated in *P. pastoris* through long-run fermentation experiments (Fig. S3). We demonstrated the stability of cross-species promoter on fluorescence expression of GFP through 14 days of continuous fermentation in conventional medium.

4. Discussion

In this study, based on the P_{bs} promoter sequence, we generated new version of the cross-species promoter P_{sh} by adding σ factor recognition sites, UP elements, optimized promoter skeletons and artificial UAS sequences. We attempted to integrate conserved sequences associated with transcription initiation across diverse microorganisms, and

Table 1
TFs sequences used.

Transcription factor	Sequence of promoter	Sequence (5′-3′)	Optimized use sequence (5′-3′)	Description
Sip4-cat8	$P_{GAP/TDH3}$	CNACGGCC/CATTTGCC	CATATTCCGTTCGTCCGAAT	zinc cluster transcriptional activator, binds carbon source responsive elements [60]
Gcr2	$P_{GAP/TDH3}$	ATGGAAAA	ACTTTGCC	transcriptional activator of genes involved in glycolysis [61]
Tye7	P_{TDH3}	ATCACNCCA/GAGTGATG/CACGCATG/AACCTCAA	ATCAGCTGCT	transcriptional activator in Ty1-mediated gene expression [61]
Hap4	P_{GAP}	TTGGTT	TTGGTT	a transcriptional activator and global regulator of respiratory gene expression [61]
Abf1	$P_{TDH3/GCW14}$	TCAAAGAATACG/	GTCATGACCAGA	create a region of open chromatin near its binding site and to contribute to activated transcription [62]
Cat1	P_{GCW14}	GATAAG	GATAAG	transcriptional activator of nitrogen catabolite repression genes [63]
Gln3	P_{GCW14}	GATTAG	GATTAG	transcriptional activator in nitrogen catabolite repression system [64]
Stp1	P_{GCW14}	RYRCGGCRC	CGGCTC	activates transcription of amino acid permease genes [65]
Upc2	P_{GCW14}	TAAACGA	TCGTATA	redundant activator of filamentation with ECM22, sterol regulatory element binding protein [66]
Gal4-like	P_{GAP}	TAAAACGGAGGTCGTGTACCCGACC	TAAAACGGAGGTCGTGTACCCGACC	similar to the GAL TF in *S. cerevisiae*, Gal4-like TF is responsible for regulation of GAP promoter as an activator, yet the details of the mechanism are still unknown [67]
SWi5	P_{AOX1}	ACCAGC	ACCAGC	TF that recruits Mediator and Swi/Snf complexes [68]

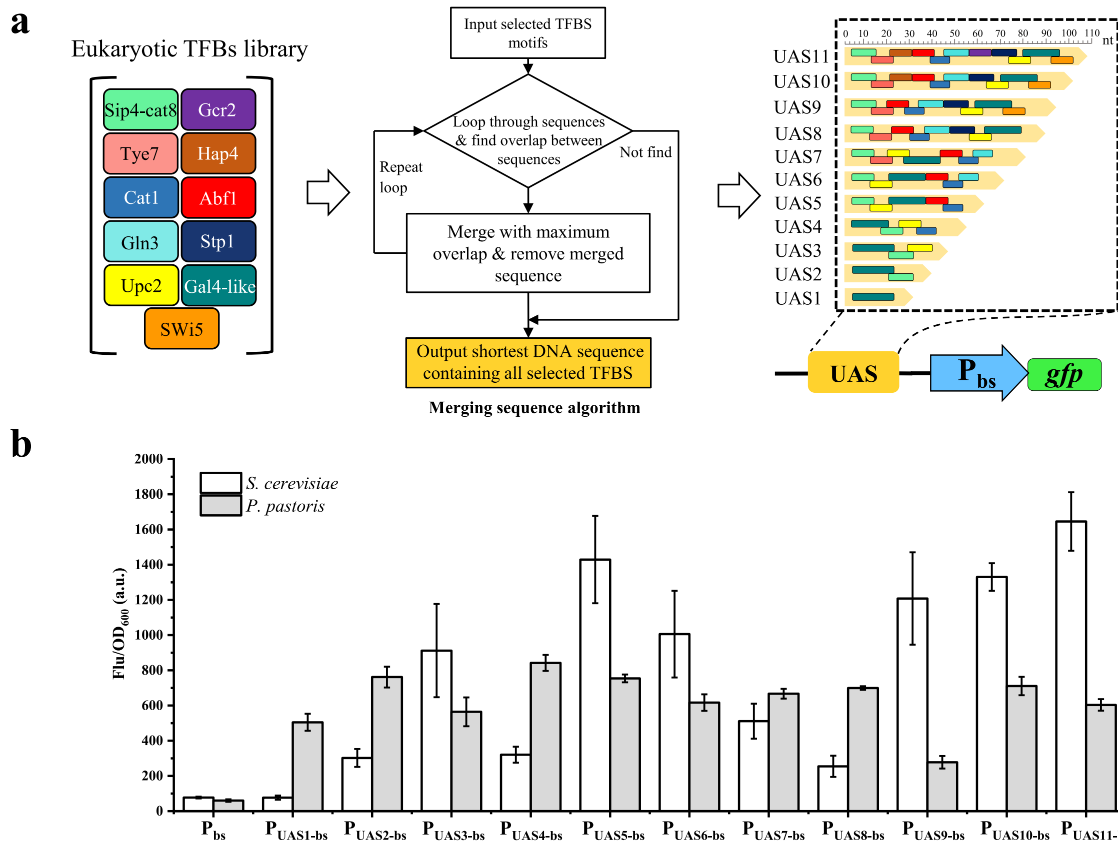

Fig. 4. Strength Identification of Yeast Endogenous Promoters and Validation of TFBSs Combination. (a) The designed minimum annexation sequence algorithm was used to align and combine from eukaryotic TFBSs library, produce artificial UAS components. **(b)** The artificial UAS components were connected in series upstream of P_{bs} promoter, and their activity was verified in *S. cerevisiae* and *P. pastoris*, respectively. The combine TFs sequence shown correspond to Table 2.

Table 2
Combination of TF conserved binding sequences.

Name	Conserved sequence assemblies of TFs (5′-3′)
UAS1	TAAAACGGAGGTCGTGTACCCGACC
UAS2	TAAAACGGAGGTCGTGTACCCGACCATATTCCGTTCGTCCGAA
UAS3	TCGTATAAAACGGAGGTCGTGTACCCGACCATATTCCGTTCGTCCGAAT
UAS4	TAAAACGGAGGTCGTGTACCCGACCATATTCCGTTCGTCCGAATCGTATAGATAAG
UAS5	CATATTCCGTTCGTCCGAATCGTATAAAACGGAGGTCGTGTACCCGACCGTCATGACCAGATAAG
UAS6	CATATTCCGTTCGTCCGAATCGTATAAAACGGAGGTCGTGTACCCGACCGTCATGACCAGATAAGATTAG
UAS7	CATATTCCGTTCGTCCGAATCAGCTGCTCGTATAAAACGGAGGTCGTGTACCCGACCGTCATGACCAGATAAGATTAG
UAS8	CATATTCCGTTCGTCCGAATCAGCTGCTGTCATGACCAGATAAGATTAGCGGCTCGTATAAAACGGAGGTCGTGTACCCGACC
UAS9	CATATTCCGTTCGTCCGAATCAGCTGCTGTCATGACCAGATAAGATTAGCGGCTCGTATAAAACGGAGGTCGTGTACCCGACCAGC
UAS10	CATATTCCGTTCGTCCGAATCAGCTGCTTGGTTGTCATGACCAGATAAGATTAGCGGCTCGTATAAAACGGAGGTCGTGTACCCGACCAGC
UAS11	CATATTCCGTTCGTCCGAATCAGCTGCTTGGTTGTCATGACCAGATAAGATTAGACTTTGCCATATGCAAAACGGCTCGTATAAAACGGAGGTCGTGTACCCGACCAGC

experimental results demonstrated the effectiveness of this approach.

Among the array of σ factors available, we selected σ^{54} due to its membership in a distinct σ factor family [51], thereby broadening the spectrum of σ factor recognition within promoter sequences. The selection of σ^{B} and σ^{D} was based on their recognition sequences aligning effectively with those of the original P_{bs} promoter. However, these combinations are not comprehensive enough due to variations in conserved sequences such as TFBSs, sigma factor recognition sequences, and core promoter sequences among different strains and microbial cells spanning biological kingdoms, particularly in eukaryotes. For instance, artificial UAS exhibit constrained activity in yeast and demonstrate varying intensities (e.g., UAS8 and UAS11). We posit that this outcome may be attributed to unidentified or recently formed transcription factor binding motifs, or disparities in the efficacy of transcription factor recognition sequences within the promoters of *S. cerevisiae* and *P. pastoris*. In particular, a focus on expanding the repertoire of eukaryotic promoter elements and detection under different culture conditions is essential for further research programs. Interestingly, this series of artificial UAS should be used in yeast promoter engineering in the future. P_{sh} had activity intensity in different strains, and we believe that this series of cross-species promoters can help solve the problem of suitability of different microbial host cells for protein activity expression, and preliminarily screen out the host suitable for protein expression. In addition, the related research or application of Internal ribosome entry sites (IRES) [52] sequence or 2A peptide sequence [53] or a short intergenic sequence (IGG1) [54] has also proved that eukaryotic cells such as yeast also have the qualification of polycistron expression. This will further expand the use of cross-species promoters and provide a powerful tool for the automation, standardization, and high-pass quantification of the gene route construction process in cell factories

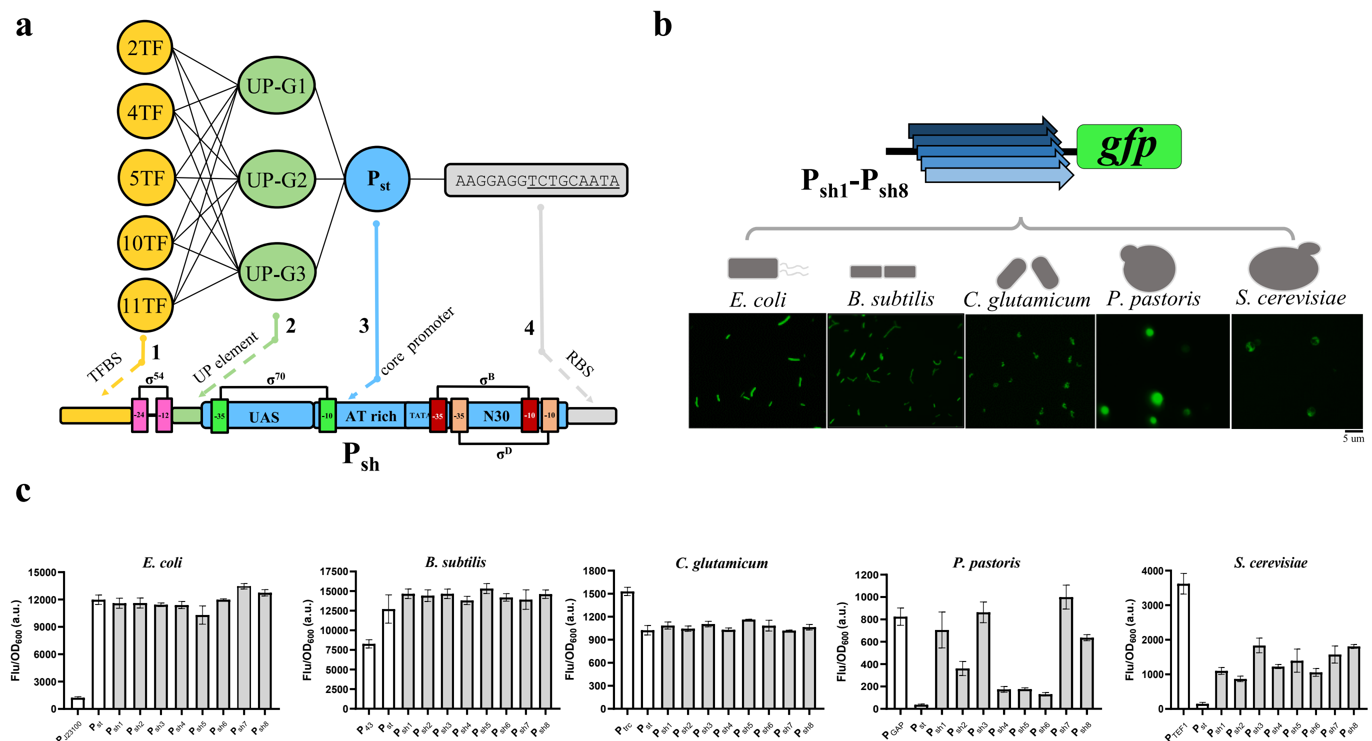

Fig. 5. Artificially assembled cross-species promoter (a) The construction of the cross-species promoter P_{sh} involved integrating five TFBSs binding sites, three UP elements, P_{st} core promoter sequences. The promoter combinations generated in steps 1, 2, and 3 were screened for eight excellent candidates to link them to the fusion RBS (fused SD-KOZAK 5′-AAGGAGGTCTGCAATA-3′) in the 4 parts. The underline indicates the yeast KOZAK sequence. **(b)** The GFP expression of P_{sh} series promoter in *E. coli*, *B. subtilis*, *C. glutamate*, *P. pastoris* and *S. cerevisiae* was observed by fluorescence microscope. **(c)** The intensity of P_{sh1-8} series promoters in different strains was verified by the fluorescence expression of GFP. Promoter J23100, P_{43}, P_{trc}, P_{GAP}, P_{TEF1}, and P_{st} were used for comparison. Data are presented as the mean ± standard deviation (n = 3).

Table 3
Common TFs and TFs generated by novel sequences.

Promoter	Common TFs	New TFs (direction is not differentiated)
P_{sh3}	Gal4-like, Sip4-cat8, Upc2	Gsm1, Pip2, Rgt1, Oaf1, Yrr1
P_{sh7}		Ino2, Ino4, Hac1, Ecm22, Oaf1, Hap4p, Oaf1, Pip2, Rgt1, Rtg3, Yap1, Yrr1
P_{sh8}		Hac1, Ecm22, Oaf1, Hap4, Oaf1, Pip2, Rgt1, Rtg3, Yap1, Yrr1

in the future.

Machine learning methods to analyze high-throughput data and establish a cross-species promoter design algorithm that takes into account parameters such as promoter activity, sequence length, suitable strain selection, etc., thereby generating a collection of artificial promoters or some more suitable RBS applicable to yeast or other microorganisms alike [55–58]. These artificially redesigned promoters hold promise for improving gene expression stability and controllability in cell factories. By integrating wet lab experiments with computational analysis using dry data from biological experiments will render the entire gene expression system more comprehensive and standardized without being confined to specific strains or cell types [56,59].

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All the authors read and agree the content of this paper and its publication.

CRediT authorship contribution statement

Wenjie Zuo: Conceptualization, Data curation, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. **Guobin Yin:** Conceptualization, Formal analysis, Supervision. **Luyao Zhang:** Data curation, Formal analysis. **Weijiao Zhang:** Investigation, Supervision. **Ruirui Xu:** Investigation, Supervision. **Yang Wang:** Investigation, Supervision, Writing – original draft, Writing – review & editing. **Jianghua Li:** Investigation, Supervision. **Zhen Kang:** Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.08.003.

References

[1] Balakrishnan R, Mori M, Segota I, Zhang Z, Aebersold R, Ludwig C, Hwa T. Principles of gene regulation quantitatively connect DNA to RNA and proteins in bacteria. Science 2022;378:eabk2066. https://doi.org/10.1126/science.abk2066.

[2] Wang Y, Liu Q, Weng H, Shi Y, Chen J, Du G, Kang Z. Construction of synthetic promoters by assembling the sigma factor binding -35 and -10 boxes. Biotechnol J 2019;14:e1800298. https://doi.org/10.1002/biot.201800298.

[3] Jodlbauer J, Rieder L, Glieder A, Wiltschi B. Bidirectional promoter libraries enable the balanced Co-expression of two target genes in *E. coli*. Methods Mol Biol 2023; 2617:75–86. https://doi.org/10.1007/978-1-0716-2930-7_5.

[4] Wang Y, Shi Y, Hu L, Du G, Chen J, Kang Z. Engineering strong and stress-responsive promoters in *Bacillus subtilis* by interlocking sigma factor binding motifs. Synthetic and Systems Biotechnology 2019;4:197–203. https://doi.org/10.1016/j.synbio.2019.10.004.

[5] Liu X, Sun M, Gao AX, Ledesma-Amaro R, Fang Q, Yang Y, Bai Z. Leaderless bicistronic design for precise and reliable control of gene expression in *Corynebacterium Glutamicum*. ACS Synth Biol 2023;12:2157–67. https://doi.org/10.1021/acssynbio.3c00246.

[6] Huang J, Chen J, Wang Y, Shi T, Ni X, Pu W, Liu J, Zhou Y, Cai N, Han S, Zheng P, Sun J. Development of a hyperosmotic stress inducible gene expression system by engineering the MtrA/MtrB-dependent NCgl1418 Promoter in *Corynebacterium glutamicum*. Front Microbiol 2021;12. https://doi.org/10.3389/fmicb.2021.718511. 718511-718511.

[7] van de Vegte YJ, Said MA, Rienstra M, van der Harst P, Verweij N. Genome-wide association studies and Mendelian randomization analyses for leisure sedentary behaviours. Nat Commun 2020;11:1770. https://doi.org/10.1038/s41467-020-15553-w.

[8] Xiong L, Zeng Y, Tang RQ, Alper HS, Bai FW, Zhao XQ. Condition-specific promoter activities in *Saccharomyces cerevisiae*. Microb Cell Factories 2018;17. https://doi.org/10.1186/s12934-018-0899-6. 58-58.

[9] Curran KA, Crook NC, Karim AS, Gupta A, Wagman AM, Alper HS. Design of synthetic yeast promoters via tuning of nucleosome architecture. Nat Commun 2014;5:4002–10. https://doi.org/10.1038/ncomms5002.

[10] Blazeck J, Garg R, Reed B, Alper HS. Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters. Biotechnol Bioeng 2012;109:2884–95. https://doi.org/10.1002/bit.24552.

[11] Redden H, Alper HS. The development and characterization of synthetic minimal yeast promoters. Nat Commun 2015;6:7810. https://doi.org/10.1038/ncomms8810.

[12] Garrigós-Martínez J, Vuoristo K, Nieto-Taype MA, Tähtiharju J, Uusitalo J, Tukiainen P, Schmid C, Tolstorukov I, Madden K, Penttilä M, Montesinos-Seguí JL, Valero F, Glieder A, Garcia-Ortega X. Bioprocess performance analysis of novel methanol-independent promoters for recombinant protein production with *Pichia pastoris*. Microb Cell Factories 2021;20. https://doi.org/10.1186/s12934-021-01564-9. 74-74.

[13] Portela RM, Vogl T, Kniely C, Fischer JE, Oliveira R, Glieder A. Synthetic core promoters as universal parts for fine-tuning expression in different yeast species. ACS Synth Biol 2017;6:471–84. https://doi.org/10.1021/acssynbio.6b00178.

[14] Lai J, Song L, Zhou Y, Zong H, Zhuge B, Lu X. Fine-tuned gene expression elements from hybrid promoter libraries in *Pichia pastoris*. ACS Synth Biol 2024;13:310–8. https://doi.org/10.1021/acssynbio.3c00534.

[15] Bernat-Camps N, Ebner K, Schusterbauer V, Fischer JE, Nieto-Taype MA, Valero F, Glieder A, Garcia-Ortega X. Enabling growth-decoupled *Komagataella phaffii* recombinant protein production based on the methanol-free P(DH) promoter. Front Bioeng Biotechnol 2023;11:1130583. https://doi.org/10.3389/fbioe.2023.1130583.

[16] Vogl T, Kickenweiz T, Pitzer J, Sturmberger L, Weninger A, Biggs BW, Kohler EM, Baumschlager A, Fischer JE, Hyden P, Wagner M, Baumann M, Borth N, Geier M, Ajikumar PK, Glieder A. Engineered bidirectional promoters enable rapid multi-gene co-expression optimization. Nat Commun 2018;9:3589. https://doi.org/10.1038/s41467-018-05915-w.

[17] Yuan J, Mo Q, Fan C. New set of yeast vectors for shuttle expression in *Escherichia coli*. ACS Omega 2021;6:7175–80. https://doi.org/10.1021/acsomega.1c00339.

[18] Guo F, Qiao Y, Xin F, Zhang W, Jiang M. Bioconversion of C1 feedstocks for chemical production using *Pichia pastoris*. Trends Biotechnol 2023;41:1066–79. https://doi.org/10.1016/j.tibtech.2023.03.006.

[19] Gao J, Jiang L, Lian J. Development of synthetic biology tools to engineer *Pichia pastoris* as a chassis for the production of natural products. Synth Syst Biotechnol 2021;6:110–9. https://doi.org/10.1016/j.synbio.2021.04.005.

[20] Patra P, Das M, Kundu P, Ghosh A. Recent advances in systems and synthetic biology approaches for developing novel cell-factories in non-conventional yeasts. Biotechnol Adv 2021;47:107695. https://doi.org/10.1016/j.biotechadv.2021.107695.

[21] Duman-Özdamar ZE, Binay B. Production of industrial enzymes via *Pichia pastoris* as a cell factory in bioreactor: current status and future aspects. Protein J 2021;40: 367–76. https://doi.org/10.1007/s10930-021-09968-7.

[22] Luckie BA, Kashyap M, Pearson AN, Chen Y, Liu Y, Valencia LE, Carrillo Romero A, Hudson GA, Tao XB, Wu B, Petzold CJ, Keasling JD. Development of *Corynebacterium glutamicum* as a monoterpene production platform. Metab Eng 2024;81:110–22. https://doi.org/10.1016/j.ymben.2023.11.009.

[23] Wang Y, Hu L, Huang H, Wang H, Zhang T, Chen J, Du G, Kang Z. Eliminating the capsule-like layer to promote glucose uptake for hyaluronan production by engineered *Corynebacterium glutamicum*. Nat Commun 2020;11:3120. https://doi.org/10.1038/s41467-020-16962-7.

[24] Cazier AP, Blazeck J. Advances in promoter engineering: novel applications and predefined transcriptional control. Biotechnol J 2021;16:e2100239. https://doi.org/10.1002/biot.202100239.

[25] Lale R, Tietze L, Fages-Lartaud M, Nesje J, Onsager I, Engelhardt K, Wong CFA, Akan M, Hummel N, Kalinowski J, Ruckert C, Hohmann-Marriott MF. A universal approach to gene expression engineering. Synth Biol (Oxf) 2022;7. https://doi.org/10.1093/synbio/ysac017. ysac017.

[26] Elgharbi F, Ben Hlima H, Ben Mabrouk S, Hmida-Sayari A. Expression of a copper activated xylanase in yeast: location of the his-tag in the protein significantly affects the enzymatic properties. Mol Biotechnol 2023;65:1109–18. https://doi.org/10.1007/s12033-022-00606-w.

[27] Chen J, Peng J, Ma C, Zhang L, Wu X, Wei H, Li J, Lü X, Gao R. Co-expression of pig IL-2 and fusion bovine cathelicidin gene by recombinant plasmids in yeast and their promotion of mouse antibacterial defense. Biology 2022;11. https://doi.org/10.3390/biology11101491.

[28] Ma J, Yan HH, Qin CQ, Liang YX, Ren DF. Accumulation of astaxanthin by Co-fermentation of *Spirulina platensis* and recombinant *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 2022;194:988–99. https://doi.org/10.1007/s12010-021-03666-x.

[29] Yang S, Liu Q, Zhang Y, Du G, Chen J, Kang Z. Construction and characterization of broad-spectrum promoters for synthetic biology. ACS Synth Biol 2018;7:287–91. https://doi.org/10.1021/acssynbio.7b00258.

[30] Rhodius VA, Mutalik VK, Gross CA. Predicting the strength of UP-elements and full-length *E. coli* sigmaE promoters. Nucleic Acids Res 2012;40:2907–24. https://doi.org/10.1093/nar/gkr1190.

[31] Mazumder A, Kapanidis AN. Recent advances in understanding sigma70-dependent transcription initiation mechanisms. J Mol Biol 2019;431:3947–59. https://doi.org/10.1016/j.jmb.2019.04.046.

[32] Ross W, Aiyar SE, Salomon J, Gourse RL. Escherichia coli promoters with UP elements of different strengths: modular structure of bacterial promoters. J Bacteriol 1998;180:5375–83. https://doi.org/10.1128/jb.180.20.5375-5383.1998.

[33] Yan Q, Fong SS. Study of in vitro transcriptional binding effects and noise using constitutive promoters combined with UP element sequences in *Escherichia coli*. J Biol Eng 2017;11. https://doi.org/10.1186/s13036-017-0075-2. 33-33.

[34] Rytter JV, Helmark S, Chen J, Lezyk MJ, Solem C, Jensen PR. Synthetic promoter libraries for *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 2014;98: 2617–23. https://doi.org/10.1007/s00253-013-5481-x.

[35] Kompaniiets D, Wang D, Yang Y, Hu Y, Liu B. Structure and molecular mechanism of bacterial transcription activation. Trends Microbiol 2023. https://doi.org/10.1016/j.tim.2023.10.001.

[36] Dostalova H, Holatko J, Busche T, Rucka L, Rapoport A, Halada P, Nesvera J, Kalinowski J, Patek M. Assignment of sigma factors of RNA polymerase to promoters in *Corynebacterium glutamicum*. Amb Express 2017;7. https://doi.org/10.1186/s13568-017-0436-8.

[37] Qiu Y, Nagarajan H, Embree M, Shieu W, Abate E, Juárez K, Cho B-K, Elkins JG, Nevin KP, Barrett CL, Lovley DR, Palsson BO, Zengler K. Characterizing the interplay between multiple levels of organization within bacterial sigma factor regulatory networks. Nat Commun 2013;4. https://doi.org/10.1038/ncomms2743.

[38] Vogl T, Ruth C, Pitzer J, Kickenweiz T, Glieder A. Synthetic core promoters for *Pichia pastoris*. ACS Synth Biol 2014;3:188–91. https://doi.org/10.1021/sb400091p.

[39] Yarrington RM, Yu Y, Yan C, Bai L, Stillman DJ. A role for mediator core in limiting coactivator recruitment in *Saccharomyces cerevisiae*. Genetics 2020;215:407–20. https://doi.org/10.1534/genetics.120.303254.

[40] Koda W, Senmatsu S, Abe T, Hoffman CS, Hirota K. Reciprocal stabilization of transcription factor binding integrates two signaling pathways to regulate fission yeast fbp1 transcription. Nucleic Acids Res 2021;49:9809–20. https://doi.org/10.1093/nar/gkab758.

[41] Ergün BG, Çalık P. Hybrid-architectured promoter design to deregulate expression in yeast. Methods Enzymol 2021;660:105–25. https://doi.org/10.1016/bs.mie.2021.05.014.

[42] Li S, Ma L, Fu W, Su R, Zhao Y, Deng Y. Programmable synthetic upstream activating sequence library for fine-tuning gene expression levels in *Saccharomyces cerevisiae*. ACS Synth Biol 2022;11:1228–39. https://doi.org/10.1021/acssynbio.1c00511.

[43] Zhao Y, Liu S, Lu Z, Zhao B, Wang S, Zhang C, Xiao D, Foo JL, Yu A. Hybrid promoter engineering strategies in *Yarrowia lipolytica*: isoamyl alcohol production as a test study. Biotechnol Biofuels 2021;14. https://doi.org/10.1186/s13068-021-02002-z. 149-149.

[44] Deng J, Wu Y, Zheng Z, Chen N, Luo X, Tang H, Keasling JD. A synthetic promoter system for well-controlled protein expression with different carbon sources in *Saccharomyces cerevisiae*. Microb Cell Factories 2021;20. https://doi.org/10.1186/s12934-021-01691-3. 202-202.

[45] Turkanoglu Ozcelik A, Yilmaz S, Inan M. *Pichia pastoris* promoters. Methods Mol Biol 2019;1923:97–112. https://doi.org/10.1007/978-1-4939-9024-5_3.

[46] Yan C, Yu W, Zhai X, Yao L, Guo X, Gao J, Zhou YJ. Characterizing and engineering promoters for metabolic engineering of *Ogataea polymorpha*. Synthetic and Systems Biotechnology 2022;7:498–505. https://doi.org/10.1016/j.synbio.2021.12.005.

[47] Duveau F, Yuan DC, Metzger BPH, Hodgins-Davis A, Wittkopp PJ. Effects of mutation and selection on plasticity of a promoter activity in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 2017;114:E11218–e11227. https://doi.org/10.1073/pnas.1713960115.

[48] Monteiro PT, Oliveira J, Pais P, Antunes M, Palma M, Cavalheiro M, Galocha M, Godinho CP, Martins LC, Bourbon N, Mota MN, Ribeiro RA, Viana R, Sá-Correia I, Teixeira MC. YEASTRACT+: a portal for cross-species comparative genomics of transcription regulation in yeasts. Nucleic Acids Res 2020;48:642–9. https://doi.org/10.1093/nar/gkz859.

[49] Xu L, Liu P, Dai Z, Fan F, Zhang X. Fine-tuning the expression of pathway gene in yeast using a regulatory library formed by fusing a synthetic minimal promoter with different Kozak variants. Microb Cell Factories 2021;20. https://doi.org/10.1186/s12934-021-01641-z. 148-148.

[50] Zhou S, Zhao L, Zuo W, Zheng Y, Zhang P, Sun Y, Wang Y, Du G, Kang Z. Minimizing endogenous cryptic plasmids to construct antibiotic-free expression systems for *Escherichia coli* Nissle 1917. Synthetic and Systems Biotechnology 2024;9:165–75. https://doi.org/10.1016/j.synbio.2024.01.006.

[51] Danson AE, Jovanovic M, Buck M, Zhang X. Mechanisms of σ54-dependent transcription initiation and regulation. J Mol Biol 2019;431:3960–74. https://doi.org/10.1016/j.jmb.2019.04.022.

[52] Huang Y, Zhang Y, Li S, Lin T, Wu J, Lin Y. Screening for functional IRESes using α-complementation system of β-galactosidase in *Pichia pastoris*. Biotechnol Biofuels 2019;12. https://doi.org/10.1186/s13068-019-1640-3.

[53] Jin X, Zhang W, Wang Y, Sheng J, Xu R, Li J, Du G, Kang Z. Biosynthesis of non-animal chondroitin sulfate from methanol using genetically engineered *Pichia pastoris*. Green Chem 2021;23:4365–74. https://doi.org/10.1039/d1gc00260k.

[54] Yue Q, Meng J, Qiu Y, Yin M, Zhang L, Zhou W, An Z, Liu Z, Yuan Q, Sun W, Li C, Zhao H, Molnár I, Xu Y, Shi S. A polycistronic system for multiplexed and precalibrated expression of multigene pathways in fungi. Nat Commun 2023;14:4267. https://doi.org/10.1038/s41467-023-40027-0.

[55] Zhang P, Wang H, Xu H, Wei L, Liu L, Hu Z, Wang X. Deep flanking sequence engineering for efficient promoter design using DeepSEED. Nat Commun 2023;14:6309. https://doi.org/10.1038/s41467-023-41899-y.

[56] Vaishnav ED, de Boer CG, Molinet J, Yassour M, Fan L, Adiconis X, Thompson DA, Levin JZ, Cubillos FA, Regev A. The evolution, evolvability and engineering of gene regulatory DNA. Nature 2022;603:455–63. https://doi.org/10.1038/s41586-022-04506-6.

[57] LaFleur TL, Hossain A, Salis HM. Automated model-predictive design of synthetic promoters to control transcriptional profiles in bacteria. Nat Commun 2022;13:5159. https://doi.org/10.1038/s41467-022-32829-5.

[58] Hossain A, Lopez E, Halper SM, Cetnar DP, Reis AC, Strickland D, Klavins E, Salis HM. Automated design of thousands of nonrepetitive parts for engineering stable genetic systems. Nat Biotechnol 2020;38:1466–75. https://doi.org/10.1038/s41587-020-0584-2.

[59] Van Brempt M, Clauwaert J, Mey F, Stock M, Maertens J, Waegeman W, De Mey M. Predictive design of sigma factor-specific promoters. Nat Commun 2020;11:5822. https://doi.org/10.1038/s41467-020-19446-w.

[60] Roth S, Kumme J, Schüller H-J. Transcriptional activators Cat8 and Sip4 discriminate between sequence variants of the carbon source-responsive promoter element in the yeast *Saccharomyces cerevisiae*. Curr Genet 2004;45:121–8. https://doi.org/10.1007/s00294-003-0476-2.

[61] Holland P, Bergenholm D, Borlin CS, Liu G, Nielsen J. Predictive models of eukaryotic transcriptional regulation reveals changes in transcription factor roles and promoter usage between metabolic conditions. Nucleic Acids Res 2019;47:4986–5000. https://doi.org/10.1093/nar/gkz253.

[62] Yarragudi A, Miyake T, Li R, Morse RH. Comparison of ABF1 and RAP1 in chromatin opening and transactivator potentiation in the budding yeast *Saccharomyces cerevisiae*. MCB (Mol Cell Biol) 2004;24:9152–64. https://doi.org/10.1128/mcb.24.20.9152-9164.2004.

[63] Stanbrough M, Magasanik B. Two transcription factors, Gln3p and Nil1p, use the same GATAAG sites to activate the expression of GAP1 of *Saccharomyces cerevisiae*. J Bacteriol 1996;178:2465–8. https://doi.org/10.1128/jb.178.8.2465-2468.1996.

[64] Blinder D, Magasanik B. Recognition of nitrogen-responsive upstream activation sequences of *Saccharomyces cerevisiae* by the product of the *GLN3* gene. J Bacteriol 1995;177:4190–3. https://doi.org/10.1128/jb.177.14.4190-4193.1995.

[65] de Boer M, Nielsen PS, Bebelman JP, Heerikhuizen H, Andersen HA, Planta RJ. Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene *BAP3* of *Saccharomyces cerevisiae*. Nucleic Acids Res 2000;28:974–81. https://doi.org/10.1093/nar/28.4.974.

[66] Gallo-Ebert C, Donigan M, Liu HY, Pascual F, Manners M, Pandya D, Swanson R, Gallagher D, Chen W, Carman GM, Nickels Jr JT. The yeast anaerobic response element AR1b regulates aerobic antifungal drug-dependent sterol gene expression. J Biol Chem 2013;288:35466–77. https://doi.org/10.1074/jbc.M113.526087.

[67] Ata O, Prielhofer R, Gasser B, Mattanovich D, Calik P. Transcriptional engineering of the glyceraldehyde-3-phosphate dehydrogenase promoter for improved heterologous protein production in *Pichia pastoris*. Biotechnol Bioeng 2017;114:2319–27. https://doi.org/10.1002/bit.26363.

[68] Dohrmann PR, Voth WP, Stillman DJ. Role of negative regulation in promoter specificity of the homologous transcriptional activators Ace2p and Swi5p. MCB (Mol Cell Biol) 1996;16:1746–58. https://doi.org/10.1128/mcb.16.4.1746.