Engineering strong and stress-responsive promoters in *Bacillus subtilis* by interlocking sigma factor binding motifs

Yang Wang\textsuperscript{a,b}, Yanan Shi\textsuperscript{a,b}, Litao Hu\textsuperscript{a,b}, Guocheng Du\textsuperscript{a,b}, Jian Chen\textsuperscript{a,b,*}, Zhen Kang\textsuperscript{a,b,*}

\textsuperscript{a} The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, 214122, China
\textsuperscript{b} The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi, 214122, China

**Abstract**

Prokaryotic gene expression is largely regulated on transcriptional levels with the involvement of promoters, RNA polymerase and sigma factors. Developing new promoters to customize gene transcriptional regulation becomes increasingly demanded in synthetic biology and biotechnology. In this study, we designed synthetic promoters in the Gram-positive model bacterium *Bacillus subtilis* by interlocking the binding motifs of σ\(^70\) for house-keeping gene expression and that of two alternative sigma factors σ\(^{38}\) and σ\(^{32}\) which are involved in responding post-exponential growth and general stress, respectively. The developed promoters are recognized by multiple sigma factors and hence generate strong transcriptional strength when host cells grow under normal or stressed conditions. With green fluorescent protein as the reporter, a set of strong promoters were identified, in which the transcriptional activities of P\(_{\text{IAA}1}\), P\(_{\text{IAB}4}\), P\(_{\text{IAB}7}\) were 18.6, 4.1, 3.3 fold of that of the commonly used promoter P\(_{\text{HSL}}\), respectively. Moreover, some of the promoters such as P\(_{\text{HSL}}\), P\(_{\text{IAB}4}\), P\(_{\text{IAB}7}\), P\(_{\text{DAG}2}\) displayed increased transcriptional activities in response to high salinity or low pH. The promoters developed in this study should enrich the biotechnological toolboxes of *B. subtilis*.

**Keywords:** Synthetic biology, Transcription, Promoter engineering, Stress-responsive, *Bacillus subtilis*

**Introduction**

Bacterial RNA polymerase indispensably requires sigma factor to initiate gene transcription \cite{1-3}. The binding motifs of sigma factors, commonly known as the canonical -35 and -10 elements are considered as the core structure of bacterial promoters. Every bacterial species has a house-keeping or principal sigma factor responsible for the transcription of essential genes and several types of alternative sigma factors governing the expression of genes required at special conditions \cite{4}. For instance, the principal sigma factor σ\(^70\) of *Escherichia coli* regulates genes that are indispensable for cell survival such as TCA cycle and protein synthesis. The alternative sigma factor σ\(^{32}\) (RpoH) governs expression of genes to deal with heat shock and the alternative sigma factor σ\(^{38}\) (RpoS) controls the expression of genes for starvation or stationary phase. *Bacillus subtilis* also has a principal sigma factor σ\(^A\) (SigA) and 10 characterized alternative sigma factors. Among them, the alternative sigma factor σ\(^{H}\) (SigH) controls gene expression at post-exponential phase and alternative sigma factor σ\(^{8}\) (SigB) controls genes for general stress response \cite{4}. Principal sigma factor is constitutively produced while alternative sigma factors are generated conditionally in response to intra- or extra-cellular stimuli \cite{3}. All the sigma factors compete for a limited number of RNA polymerase \cite{3,5}.

There are a considerable amount of natural promoters governed by more than one sigma factor. For example, in *E. coli* there are over eight hundred identified regions bound by both σ\(^70\) and σ\(^{38}\) \cite{6}. The commonly used *B. subtilis* P\(_{\text{HSL}}\) promoter is recognized by both σ\(^8\) and σ\(^{A}\) \cite{7}. Promoters governed by multiple sigma factors should have stronger transcriptional activities, as the promoter recognition chances would be raised. Developing synthetic promoters composed of both principal and alternative sigma factor binding motifs has very high importance in the field of biotechnology. Such synthetic promoters are more resistant to imperfect growing conditions, such as biotechnological processes that cause cell stress or metabolic burden and generate strong transcription of target genes \cite{8}.

As another biotechnologically versatile host strain, *B. subtilis* has excellent protein secretion capability and a high level of biosafety. *B. subtilis* has been engineered for production of proteins such as α-amylase and chemicals like vitamins and nucleotides \cite{9}. Robust transcription of target genes in various cultivation conditions is crucial for the applications of *B. subtilis* in biotechnology. To enrich the
transcriptional regulation toolboxes of \textit{B. subtilis}, we constructed strong and stress-responsive \textit{B. subtilis} promoters in this research. The engineered promoters \textit{P}_{168-1}, \textit{P}_{168-4}, \textit{P}_{168-7} showed higher activities comparing with \textit{P}_{43} \cite{7,10} and \textit{P}_{gfp} \cite{11}. More importantly, these promoters were in apparent responses to the commonly encountered growth stresses like high salinity or low pH.

2. Methods

2.1. Medium and cultivation

\textit{E. coli} and \textit{B. subtilis} strains were cultivated in Luria-Bertani (LB medium, 10 g/L Typtone, 10 g/L Sodium chloride and 5 g/L Yeast extract, pH 7.0) at 37°C. Spizizen minimal medium was used to prepare \textit{B. subtilis} competent cells as described \cite{12}. When necessary 100 µg/mL ampicillin, 10 µg/mL chloramphenicol, 20 mM sodium citrate buffer (pH 4.5) or 0.5 M NaCl were supplemented to the culture (all as the final concentration).

2.2. Strains and plasmids

The used strains and plasmids in this study were listed in Table 1 and the primers were shown in Table 2. \textit{E. coli} JM109 was used for all plasmid constructions. \textit{B. subtilis} 168 was used as the host strain to measure the activity of the synthetic promoters. The \textit{E. coli} - \textit{B. subtilis} shuttle vector pHTO1 \cite{13} was selected as the backbone for promoter library construction and screening. To build the plasmid pHTO1-gfp, \textit{gfp} gene was amplified with primers \textit{gfp-F} and \textit{gfp-R} from pMD19-gfp \cite{14}, digested with BamH I and Sma I and ligated with the equivalently cleaved pHTO1. \textit{P}_{43} promoter was amplified from pP43NMK \cite{15} with primers \textit{P}_{43-F} and \textit{P}_{43-R}. The pHTO1-gfp was linearized via PCR with primers p0-F and p0-R to remove the \textit{P}_{gfp} promoter. The linearized pHTO1-gfp and the \textit{P}_{43} PCR product were combined into pHTO1-P43-gfp by T5 exonuclease DNA assembly (TEDA) \cite{16}.

2.3. Promoter library construction

Promoter libraries borne by pHTO1 vector were constructed by replacing the \textit{P}_{gfp} promoter with the DNA comprising interlocking sigma factor binding motifs. Briefly, sigma factor binding motifs and randomized intra- and inter-σ binding motif spacers were firstly included in designated primers (\textit{P}_{gfp-F} and \textit{P}_{gfp-R} Table 2) synthesized by GENEWIZ (Suzhou, China). PCR was performed with the described primers using pHTO1-gfp as template. PCR products were phosphorlyated and end to end ligated by Blunting Kination Ligation (BKL) Kit (Takara, Beijing) to generate the final plasmids containing the promoter libraries (Table 1).

2.4. Promoter library screening and promoter characterization

For primary screening of the synthetic promoter libraries, \textit{B. subtilis} cells carrying the plasmids were cultivated in 96-well plates filled with LB medium for 24 h. Cell density (OD_{600}) and fluorescence intensity (excitation wavelength 490 nm, emission wavelength 530 nm, gain 70) were measured with BioTek Cytation Plate Reader. The culture of \textit{B. subtilis} strain carrying the pHTO1 vector was applied to subtract background fluorescence signal. During the second round of screening, 26 strong promoters that did not impair cell growth were re-assayed in shake flask cultures. Sampling was performed every 4 h, and cells were washed with 20 mM phosphate buffered saline (pH 7.0, PBS). After appropriate dilution, cell density and fluorescence intensity were measured as described above.

To test the response of synthetic promoters to stresses including low pH and high salinity, cells were pre-cultivated at normal condition (LB medium, 37°C, pH 7.0) for 6 h. Afterwards, the cultures were shifted to the designated conditions by supplementing 20 mM final concentration of sodium citrate buffer (pH 4.5) or 0.5 M final concentration of NaCl. Samples were taken every 2 h. Cells were washed with PBS, and the cell density (OD_{600}) and fluorescence intensity were measured as described above.

2.4. Promoter sequencing

Promoters with interesting properties were isolated from \textit{B. subtilis} via plasmid extraction (Sangon plasmid extraction kit). Crude plasmid samples were used to transform \textit{E. coli} JM109. Single \textit{E. coli} colonies were picked for plasmid Sanger sequencing by GENEWIZ (Suzhou, China).

3. Results and discussion

3.1. Design of synthetic promoters

The consensus sequences recognized by \textit{B. subtilis} σ^A (house-keeping sigma factor), σ^B (sigma factor for general stress response), and σ^H (sigma factor for post-exponential and sporulation gene expression) (Fig. 1A) were selectively assembled to interlock each other (Fig. 1B and 1C). The binding motifs of σ^A and σ^B have been defined as TTGACA (-35)-N14-TGNTATAAT(-10) and AGGTTT(-35)-GGGTAT(-10) in the DBTBS (a database of transcriptional regulation in \textit{B. subtilis}) \cite{17}. The binding motifs of σ^H was defined as AGGAAAT (-35)NGGAAT (-10) here according to previous report \cite{4}. RNA polymerase would accordingly be guided to the synthetic promoters with the assistance of more than one sigma factor and the frequency of promoter recognition would thus be increased, especially when alternative sigma factors were

Table 1

| Strains        | Feature                                                                 | Reference                    |
|----------------|-------------------------------------------------------------------------|------------------------------|
| \textit{E. coli} JM109 | \textit{E. coli} K-12 F' traD36 proA^+ B' lacIΔ (lacZ)ΔM15 (sas-lac-proAB) glnV44 c14 gyrA96 recA1 relA1 endA1 th i d R17 | New England Biolabs          |
| \textit{B. subtilis} 168 | \textit{B. subtilis} wild type strain                                    | Bacillus Genetic Stock Center (BGSC) |
| Plasmids        |                                                                         |                              |
| pHTO1          | \textit{E. coli} - \textit{B. subtilis} shuttle vector replicative in \textit{B. subtilis}, carrying IPTG inducible promoter \textit{P}_{gfp}, \textit{ampR}^+, \textit{carA}^+ | \cite{13}                     |
| pP43NMK        | \textit{E. coli} - \textit{B. subtilis} shuttle vector replicative in \textit{B. subtilis}, with \textit{P}_{43} promoter, \textit{ampR}^+, \textit{Km}^8                     | \cite{15}                     |
| pMD19-gfp      | The \textit{gfp} gene under the control of \textit{P}_{gfp} promoter in pHTO1. | This study |
Table 2
Primer used in this study.

| Primer | Sequence (5′–3′) |
|--------|------------------|
| gfp-F  | CGCATGGGTAAGGGAGAAGAACTTTTC |
| gfp-R  | TCCGATCCACCCGGTATATTGTATAGTTCACTCATGCC |
| PBH-F  | NTATAATNNNNNNNAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |
| PBH-R  | TCCGATCCACCCGGTATATTGTATAGTTCACTCATGCC |
| PBA-F  | NNNGAATNNNNNNNAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |
| PBA-R  | TCCGATCCACCCGGTATATTGTATAGTTCACTCATGCC |
| PHAB-F | AATNNNGGATATNNNNNNNAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |
| PHAB-R | TCCGATCCACCCGGTATATTGTATAGTTCACTCATGCC |
| PHBA-F | ATNNNNNNNNTATAATNNNNNNNAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |
| PHBA-R | TCCGATCCACCCGGTATATTGTATAGTTCACTCATGCC |
| P43-F  | TGATAGGTGGTATGTTTTCGCTTG |
| P43-R  | TGATAGGTGGTATGTTTTCGCTTG |
| p0-F   | AGCGGTACCAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |
| p0-R   | AGCGGTACCAAAGGAGGAAGGATCGAATGGGTAAAGGAGAAACTTTTC |

Fig. 1. Schematic of synthetic promoters composed of interlocking sigma factor binding motifs. (A) In many cases, promoters contain one sigma factor binding motif, including the -35 and -10 elements. The commonly used *B. subtilis* P43 comprises overlapping binding motifs of σB and σA. Diamond stands for the -35 element while rectangle indicates the -10 element. (B) Inspired by P43, there are numerous possible ways to construct synthetic promoters by interlocking the binding motifs of different sigma factors when considering variations in inter σ binding motif spacer length, motif arrangement or motif compositions. Herein, the length of the intra σ binding motif spacer was fixed as the same to the natural promoters, but the spacer of inter σ binding motif was variable. (C) Nucleotide sequences of natural promoters and synthetic promoters recognized by single or multiple sigma factors. The consensus sequences recognized by σA, σB and σH were indicated with green, violet and yellow; -35 elements and -10 elements of the sigma factor binding motifs were indicated with diamonds and rectangles, respectively. Red letters indicate the nucleotide shared by two adjacent sigma factor binding motifs. N stands for degenerate nucleotide.
enriched in response to stresses. The length of the intra σ binding motif spacer (the spacer between the -35 and -10 elements) sequence was not changed, while the nucleotides of the spacer were randomized via PCR with degenerate primers (Fig. 1B and 1C). Under this provision, there are numerous ways to combine the σ binding motifs into synthetic promoters as the length of inter σ binding motif spacer (the spacer between two -35 or two -10 elements), the arrangement of the motifs and the overall number of the assembled motifs are all variants (Fig. 1B).

In this study, we designed B. subtilis synthetic promoters by selectively combining the motifs recognized by σA, σB and σH or arbitrarily in five ways (Fig. 1C). The aim is to create synthetic promoters with strong transcriptional activities in all growth phases under different cultivation conditions. P_HA comprises interlocking binding motifs of σH and σA; P_BH comprises interlocking binding motifs of σB and σH; P_AH comprises interlocking binding motifs of σB and σA; P_AB comprises interlocking binding motifs of σA and σB, and P_HBA comprises interlocking binding motifs of σA, σB and σH. The binding motifs of σA and σB in PHBA promoters are inverted comparing to PHAB. Moreover, in PHAB Promoters the three selected -35 elements and -10 elements are adjacent to each other (Fig. 1C).

3.2. Preliminary screening of the synthetic promoters

The promoter libraries for P_HA, P_BH, P_AB, P_HAB, and P_HBA were constructed by replacing the P_grac promoter of pHT01-gfp with the designated interlocking sigma factor binding motifs. For each library, 384 promoters were constructed. Relative fluorescence intensity (Fluorescence intensity (au)/OD_600) was used to demonstrate the promoter activities. Most PBH, PHA, PHAB and P_HBA promoters displayed significantly weaker promoter activities compared to the commonly used B. subtilis inducible promoter P_grac as the upper quartiles of these promoter activities (Fig. 2, upper black dash lines) were much lower than the activity of the P_grac (Fig. 2, pink dash line). On average PHA promoter library displayed the highest activity with its median value (Fig. 2, black solid line) slightly lower than the activity of P_grac. Principally PBH promoters should only display its maximal promoter activities at post-exponential phase with growth stresses, when σA and σB were enriched.

The data here preliminarily suggests that it is possible to raise B. subtilis promoter activities by interlocking sigma factor binding motifs. However, we found more sigma factor binding motifs does not necessarily make the synthetic promoters stronger, as it was shown that P_HA library outperformed the three-sigma-factor controlled P_AH and P_AB. Elongated promoter structure may also structurally tangle promoter DNA and decrease transcriptional activity. Moreover, downstream sigma factor binding motifs might also cause the pausing of a transcriptional process initiated from upstream sigma factor binding motifs [18].

Synthetic promotes belonging to the same libraries exhibited vastly different transcriptional activities, for instance, P_HA promoters displayed the broadest activity variation (Fig. 2). The estimated activity of the strongest PHA promoter displayed more than 130 fold higher than the weakest P_HA promoter (Fig. 2, green numbers). This suggests the spacer sequences are not less important in determining promoter activities. Sigma factor binding motifs is indispensable for the promoter recognition, while the spacer sequences may play critical roles in subsequent structural modulation of RNA polymerase-DNA complex and promoter open complex formation [19]. Previous studies also showed the significance of the spacer sequences between -35 and -10 elements [19,20].

We found a few P_BH and P_HAB promoters displayed high estimated promoter activities (Fig. 2, top point of the violin plot) but impaired significantly B. subtilis cell growth. The OD_600 measured in 96-well plate was about 0.05 after 24 h of cultivation. These promoters were no longer considered. Twenty six strong promoters that did not impair cell growth were selected for further characterization in shake flask (Fig. 2).

3.3. Secondary screening of the synthetic promoters

To demonstrate the promoter activities precisely, cells cultivated in shake flasks were washed twice with 20 mM phosphate buffered saline (PBS) (pH 7.0) to remove GFP released from cell lysis. OD_600 and fluorescence intensities were measured at 6 h, 12 h, 18 h and 24 h. Relative fluorescence intensity was used again to demonstrate the promoter activities. The inducible promoter P_grac (activated with 0.1 mM isopropylthio-β-galactoside (IPTG)) and constitutive B. subtilis promoter P_A4 were used as the control promoters. We found the measured promoter activities herein (Fig. 3) were lower than that measured via 96-well plate cultivation (Fig. 2). This should be partially ascribed to cell washing, which removed GFP released into the medium (Fig. 3).

Most promoters displayed stronger promoter activities in the secondary screening than P_grac and P_A4, except P_HBA-1, P_HBA-2. We speculate that P_HBA-1 and P_HBA-2 might be more sensitive to variation in cultivation conditions. The strongest promoter P_HA-1 exhibited significantly higher strength than P_grac and P_A4 controls (Fig. 3). The relative fluorescence intensity of P_HA-1 at 12 h was 17.6 fold of P_A4. Moreover, we found the activities of these synthetic promoters formed a gradient (Fig. 3). These promoters were classified into “High activity”, “Medium activity” and “Low activity” groups. These promoters with quite different transcriptional activities should enrich the gene regulation toolbox of B. subtilis.

The sequences of these 26 synthetic promoters were also compared (Table 3). However, we did not find apparent regularity defined the correlation between promoter strength and the randomized inter- and intra-σ binding motif spacers (Fig. 3 and Table 3). Additionally, we found the sequences of P_HA-5, P_HA-6 and P_HA-4 promoters were mutated. The -35 element of σA were changed from “GGGTAT” to “GGGTAA” in P_HA-5. The -35 elements of σB and σH and the -10 element of σA were missing in P_HA-6, while the -10 element of σA and -35 element of σB were deleted in P_HA-4. There is no clue how these truncated promoters, particularly P_HA-6, executed gene transcription initiation, but our finding herein suggests the plasticity of sigma factor reorganizing B. subtilis promoters.
3.3. Characterization of synthetic promoters to different stresses

In the next step, eight synthetic promoters, PHA-1, PHA-2, PHA-4 (high activity group, Fig. 3); PHA-6, PHA-7 and PHA-5 (medium activity group, Fig. 3); PBA-2 and PBA-3 (low activity group, Fig. 3), were selected as the representatives to test their responses to low pH and high salinity pressures, which were commonly encountered during biotechnological bacteria cultivation. Cells carrying the synthetic promoters or the control promoter P_grac were cultivated under the normal condition (LB medium, pH 7.0) for 4h before being exposed to pH 4.5 or 0.5M NaCl (final concentration). The time-course of the promoter activity was recorded and compared (Fig. 4). We found transcriptions from synthetic promoters PHA-1, PBA-2, PHA-6, PHA-7 and PHA-5 were enhanced at varying degrees in response to 0.5M NaCl while PHA-2 was repressed by 0.5M NaCl (Fig. 4). In comparison, PHA-4 was in positive response to acidic pH. These results indicate that it is feasible to make a promoter stress-responsive by incorporating the binding motifs of alternative sigma factors.

*B. subtilis* σB is known for responding to general stresses. Promoters carrying the binding motif of σB, such as PHA and PHA were induced as anticipated by the supplement of NaCl or by low pH. *B. subtilis* σH assists the transcription of genes specific to post-exponential growth phase or sporulation. The incorporation of σH binding motif to synthetic promoters should be beneficial for strong gene transcription at post-exponential phase. This is in agreement with our finding that in comparing to P_grac the PHA promoter and PHA promoters displayed stronger activities after time point 8 h (Fig. 4). High salinity reduces the association of σH [21] and therefore may suppress the σH control gene expression. It explains the reduced activity of PHA-2 in presence of 0.5M NaCl (Fig. 4). However, we found the PHA-1 promoter lacking σB.

Fig. 3. Transcriptional strength of the selected strong promoters measured in the second round of screening. *B. subtilis* cells carrying the promoters were grown in LB medium in shake flasks. Cells were washed twice with PBS and fluorescence intensity and OD_{600} were measured at 6 h, 12 h, 18 h and 24 h. P_grac and P_e promers were used as the control. Transcription driven by P_grac was induced with 0.1 mM IPTG.

Table 3

Sequences of the 26 selected promoters.

| Promoters | Sequence (5’ to 3’) |
|-----------|---------------------|
| PHA       | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-2     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-4     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-6     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-7     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA       | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-2     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-3     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-4     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-5     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-6     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-7     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-8     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-9     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA       | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-2     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-4     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-6     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-7     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-8     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PHA-9     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA       | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-2     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-3     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-4     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-5     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-6     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-7     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-8     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |
| PBA-9     | AGGAATTTCACCGGTTAACTGCAGGTAATGCGGTGTATAATTGCATAGCCCAAACAGAGGAGAAGATCA |

Ribosome binding site is labeled in italic. Straight, dash and wave underlines stand for the binding motif of σ^σH, σ^σB and σ^σA, respectively.
binding motifs was induced by salt stress. The differences between PHA-2 and PHA-1 promoters were only found in the spacer region. More study is required to explain how the spacer regions could invert the response of a promoter to stresses.

4. Conclusions

Promoter is the most important regulatory element of gene expression. Developing promoters with novel properties has captured many attentions in the field of synthetic biology or biotechnology [22–25]. Stress-responding capability is one the properties that have been studied extensively. Various synthetic stress responsive promoters were created dominantly by engineering the transcriptional regulators and the cognate cis-DNA elements [26–30], which were considered as the accessory elements of promoters. In this study, we developed stress responsive promoter for application in B. subtilis by modulating the core elements of all bacterial promoters, the binding motif of sigma factor (Fig. 1). Incorporating the binding motifs of different sigma factors would assimilating the endogenous regulation of sigma factors to synthetic promoters and confer promoters stress-responsive capability and strong activity, especially when cells encountering growth transition or environmental changes.

The strongest promoter necessarily containing the binding motif of σ^A, which is the principal sigma factor of B. subtilis. B. subtilis promoters unrecognizable to σ^A are unlikely to generate strong transcriptional strength, as indicated by the low activity of PBH (Fig. 2). Comparing to PHA, the PBA showed much weaker activities, which may be a preliminary indication of the σ^H’s advantage over the σ^B in competing for RNA polymerase. Promoter library construction and large/medium-throughput screening seem inevitable when developing customized promoters as synthetic promoters composed of same organization of sigma factor binding motifs may have strikingly different transcriptional strengths (Fig. 2 and Fig. 3) or different responses towards the same stressor (Fig. 4 and Table 3). In many sophisticated promoter engineering strategies, synthetic promoters designed based on promoter modularity require some irrational optimizations as many factors such as DNA topological structure or post-transcriptional regulation may unpredictably affect the promoter activities.

Future work may expand this promoter engineering method to the other industrial microorganisms such as Corynebacterium glutamicum, Clostridium acetobutylicum, Synechococcus sp. and Streptomyces coelicolor.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (31970085and 31670092), the National Key R&D program of China (2018YFA0901401), the Fundamental Research Funds for the Central Universities (JUSRP51707A), a grant from the Key Technologies R&D Program of Jiangsu Province (BE2019630) and
the 111 Project. Y. W. was sponsored by the China Postdoctoral Science Foundation (2019M651702).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2019.10.004.

References

[1] Feklistov A, Sharon BD, Darst SA, et al. Bacterial sigma factors: a historical, structural, and genomic perspective. Annu Rev Microbiol 2014;68:357–76.
[2] Helmann JD. Where to begin? Sigma factors and the selectivity of transcription initiation in bacteria. Mol Microbiol 2019;112:335–47.
[3] Browning DF, Busby SJ. Local and global regulation of transcription initiation in bacteria. Nat Rev Microbiol 2016;14:638–50.
[4] Haldenwang WG. The sigma factors of Bacillus subtilis. Microbiol Rev 1995;59:1–30.
[5] Mauri M, Klumpp S. A model for sigma factor competition in bacterial cells. PLoS Comput Biol 2014;10:e1003845.
[6] Cho BK, Kim D, Knight EM, et al. Genome-scale reconstruction of the sigma factor network in Escherichia coli: topology and functional states. BMC Biol 2014;12:4.
[7] Wang PZ, Doi RH. Overlapping promoters transcribed by Bacillus subtilis sigma 55 and sigma37 RNA polymerase holoenzymes during growth and stationary phases. J Biol Chem 1984;259:8619–25.
[8] Wang Y, Liu Q, Weng H, et al. Construction of synthetic promoters by assembling the sigma factor binding -35 and -10 boxes. Biotechnol J 2018:e1800298.
[9] Hohmann HP, van Dijl JM, Krishnappa L, et al. Host organisms: Bacillus subtilis. Ind Biotechnol: Microorganisms 2017;1:221–97.
[10] Wang LF, Doi RH. Promoterswitchingduringdevelopmentandtheterminationsiteofthesigma 43 operon of Bacillus subtilis. Mol Gen Genet 1987;207:114–9.
[11] Phan TT, Nguyen HD, Schumann W. Development of a strong intracellular expression system for Bacillus subtilis by optimizing promoter elements. J Biotechnol 2012;157:167–72.
[12] Sosada Y, Kada T. Formation of competent Bacillus subtilis cells. J Bacteriol 1983;153:813–21.
[13] Nguyen HD, Phan TT, Schumann W. Expression vectors for the rapid purification of recombinant proteins in Bacillus subtilis. Curr Microbiol 2007;55:89–93.
[14] Zeng L, Wang Z, Vainstein A, et al. Cloning, localization, and expression analysis of a new tonoplast monosaccharide transporter from Vitis vinifera L. J Plant Growth Regul 2011;30:199–212.
[15] Zhang XZ, Cui ZL, Hong Q, et al. High-level expression and secretion of methyl parathion hydrolase in Bacillus subtilis WB800. Appl Environ Microbiol 2005;71:4101–3.
[16] Xia Y, Li K, Li J, et al. T5 exonuclease-dependent assembly offers a low-cost method for efficient cloning and site-directed mutagenesis. Nucleic Acids Res 2019;47:e15.
[17] Sierro N, Makina Y, de Hoon M, et al. DFBRS: a database of transcriptional regulation in Bacillus subtilis containing upstream intergenic conservation information. Nucleic Acids Res 2008;36:D93–6.
[18] Perdue SA, Roberts JW. 2(70) dependent transcription pausing in Escherichia coli. J Mol Biol 2011;412:782–92.
[19] Hook-Barnard IG, Hinton DM. The promoter spacer influences transcription initiation via sigma70 region 1.1 of Escherichia coli RNA polymerase. Proc Natl Acad Sci U S A 2009;106:737–42.
[20] Singh SS, Typan A, Hengge R, et al. Escherichia coli Sigma(70) senses sequence and conformation of the promoter spacer region. Nucleic Acids Res 2011;39:5109–18.
[21] Widdierich N, Rodrigues CD, Commichau FM, et al. Salt-sensitivity of o3 and SpoOA prevents sporulation of Bacillus subtilis at high osmolality avoiding death during cellular differentiation. Mol Microbiol 2016;100:108–24.
[22] Blazek J, Alper HS. Promoter engineering: recent advances in controlling transcription at the most fundamental level. Biotechnol J 2013;9:46–58.
[23] Wang HH, Kim H, Cong L, et al. Genome-scale promoter engineering by coselection MAGE. Nat Methods 2012;9:591–3.
[24] Engstrom MD, Pfleger BF. Transcription control engineering and applications in synthetic biology. Synth Syst Biotechnol 2017;2:176–91.
[25] Segall-Shapiro TH, Sontag ED, Voigt CA. Engineered promoters enable constant gene expression at any copy number in bacteria. Nat Biotechnol 2018;36:352–8.
[26] Rajkumar AS, Liu G, Bergenholm D, et al. Engineering of synthetic, stress-responsive yeast promoters. Nucleic Acids Res 2016;44:e136.
[27] Yang S, Liu Q, Zhang Y, et al. Construction and characterization of broad-spectrum promoters for synthetic biology. ACS Synth Biol 2018;7:287–91.
[28] Phelan RM, Sachs D, Petkiewicz SJ, et al. Development of next generation synthetic biology tools for use in Streptomyces venezuelae. ACS Synth Biol 2017;6:e159–66.
[29] Monteiro LMO, Arruda LM, Silva-Rocha R. Emergent properties in complex synthetic bacterial promoters. ACS Synth Biol 2018;7:602–12.
[30] Presnell KV, Flexer-Harrison M, Alper HS. Design and synthesis of synthetic UP elements for modulation of gene expression in Escherichia coli. Synth Syst Biotechnol 2019;4:99–106.