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Novel *sinIR* promoter for *Bacillus subtilis* DB104 recombinant protein expression system

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Abstract Transcriptome analysis revealed that the *sinR* gene encoding a transition-state regulator of *Bacillus pumilus*, genetically close to *B. subtilis*, was expressed at high levels during growth. The *sinR* gene is the second gene of the *sinIR* operon consisting of three promoters and two structural genes in *B. subtilis*. This study used the *sinIR* promoter of *B. subtilis* DB104 to construct a recombinant protein expression system. First, the expression ability depending on the number of *sinIR* promoter was investigated using enhanced green fluorescent protein (eGFP). The expression level of eGFP was slightly higher when using two promoters (P*sin*2) than using original promoters. The P*sin*2 promoter was further engineered by modifying the repressor binding site and −35 and −10 regions. Shine-Dalgarno (SD) sequence of the *sinI* gene was modified to the consensus sequence. Finally, combining the engineered P*sin*2 promoter with the modified SD sequence increased the expression level of eGFP by about 13.4-fold over the original promoter. Our results suggest that the optimized *sinIR* promoter could be used as a novel tool for recombinant protein expression in *B. subtilis*.

Keywords *Bacillus subtilis* DB104 · Expression system · Promoter engineering · *sinIR* promoter

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Introduction

Bacillus subtilis, a rod-shaped Gram-positive aerobic bacterium, has been widely used as a host of recombinant proteins because of its well-known genetic background and superior protein secretion capability [1-3]. It also has easy handling, fast and cost-effective high-density culture, and absence of a significant codon bias [2,4,5]. In recent decades, *B. subtilis* has been used for producing industrial enzymes, antibiotics, medicinal proteins, and so on [6-8]. However, it has the disadvantage of producing many extracellular proteases that recognize and degrade heterologous proteins [9]. *B. subtilis* DB104 derived from *B. subtilis* 168 is a strain deficient in two extracellular proteases with less than 4% extracellular protease activity compared to its parental strain [10]. Thus, this strain is one of the most used strains to produce industrially extracellular enzymes [11].

A promoter is an essential factor in a protein expression system that can directly affect gene expression. Promoter engineering has been developed as a strategy to construct a strong promoter. Most commonly, promoter engineering involves changing core regions of the promoter, such as upstream (UP) element and, −35, −16, −10, +1 regions [12]. For instance, alteration of −10 and −35 regions and UP sequence in *ylbP* promoter can enhance the activity of recombinant β-galactosidase about 26-fold compared with its wild-type promoter [2]. Other researchers have optimized *groESL* promoter core region including UP elements and −35, −15, −10 regions to the consensus sequence and found that the amount of recombinant protein is enhanced up to about 30% of the total cellular protein in *B. subtilis* [4]. As another strategy, repressor binding site of promoter can be changed. For example, Sun et al. have engineered the *cbh1* promoter by replacing repressor binding sites with activators in *Trichoderma reesei*, resulting in 5.0-fold higher protein expression [13]. The expression of a protein is also affected by the strength of Shine-Dalgarno (SD) sequence. Optimized sequences can successfully increase the production of recombinant proteins in many bacterial strains [14,15].

Recently, a novel and effective strategy for screening strong promoters has been introduced by transcriptome analysis [16,17]. Transcriptome analysis is a technology that enables detection of expression levels and quantification of transcripts in a greater range. However, transcriptome studies of *B. subtilis* have been limited to transcriptional regulation in certain environments, such as specific media or growth conditions, rather than the expression level pattern [18-25]. For this reason, several studies have been conducted by borrowing transcriptome analysis results from other species such as *B. licheniformis* [16] and *B. megaterium* [26] to screen promoters in *B. subtilis*. Transcriptome analysis has also been performed during the growth of *B. pumilus* BA06, which is genetically closer to *B. subtilis* based on genomic alignments [27]. In that study, *sinR* gene showed constant high expression levels from the early stage to the late stage of bacterial growth [27].

The *sinR* gene is the second gene of the *sinIR* operon consisting of three distinct promoters (P1, P2, and P3) and two structural genes (*sinI* and *sinR*). P1 and P2 are located upstream of *sinI*. P3 is located between *sinI* and *sinR* [28]. SinI, the first gene product of this operon, is also known as an antagonist of SinR [29]. SinR is a DNA-binding protein that regulates the transcription of several post-exponential genes [30,31]. The transcription of *sinIR* promoters is regulated by three transition-state regulators (Spo0A, Hpr, and AbrB) [32]. When Spo0A is phosphorylated, it is bound to the front of the P1 region as an activator protein to regulate transcription [33]. Hpr and AbrB are bound near the P1 region as repressors to inhibit transcription [33]. During vegetative growth, P3 is constantly expressed [34]. P1 and P2 start to transcribe during the onset of the stationary phase and after two hours of stationary phase, respectively [34]. Thus, genes under the control of *sinIR* promoters could be expressed throughout the growth.

In this study, a novel gene expression system in *B. subtilis* DB104 was developed using *sinIR* promoter referring to results of transcriptome analysis of *B. pumilus* BA06. Transcriptome levels of engineered promoters were evaluated using enhanced green fluorescent protein (eGFP). Modifying the number of promoters, repressor binding sites, −35 and −10 elements, and the SD sequence in the *sinIR* operon significantly improved the transcriptional level of the *sinIR* promoter.

Materials and Methods

Bacterial strains, growth condition, and transformation

Bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* DH5α was used as a recipient in cloning experiments. *B. subtilis* 168 was used as a donor of genomic DNA. *B. subtilis* DB104 was used for the expression ability of promoters through the reporter protein, enhanced green fluorescent protein (eGFP). Recombinant *B. subtilis* DB104 cells were grown in Luria-Bertani (LB) medium at 30 ºC under aeration with shaking at 250 rpm. The following concentrations and antibiotics were used for selection: 50 μg/mL ampicillin (for *E. coli*) and 10 μg/mL kanamycin (for *B. subtilis*). Transformation of *E. coli* DH5α was carried out using the heat shock method [35]. Transformation of *B. subtilis* DB104 was performed as previously described [11].

Construction of recombinant plasmids

Primers used in this study are listed in Table 2. The *sinIR* operon is transcribed by three discrete promoters: promoters P1 and P2 preceding the *sinI* gene, and promoter P3 abutting to the *sinR* gene. The DNA fragment containing each promoter region was amplified from the genomic DNA of *B. subtilis* 168. Promoters P*sin*2 (including promoters P1 and P2) and P*sin*3 (including promoters P1, P2, and P3) were amplified using the common forward primer Psin-F with reverse primer Psin2-OR and Psin3-OR, respectively. In addition, the *egfp* gene was amplified from pUB19-*ΔcotB-gfp* [36]. It originated from the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). The *egfp-2* gene fused with promoter P*sin*2 was amplified using primer pair Psin2-OF/GFP-R. The *egfp-3* gene linked with promoter P*sin*3 was amplified using primer pair Psin3-OF/GFP-R. Because structural gene sequences of *egfp-2* and *egfp-3* are identical, they are referred to as *egfp* in the rest of the text. The promoter and *egfp* genes were then recombined by overlap extension PCR. PCR-amplified fragments were digested with a pair of *Mlu*I and *Hind*III restriction enzymes (Takara, Tokyo, Japan) to construct amplicons P*sin*2-*egfp* and P*sin*3-*egfp*. The pUB19 vector plasmid constructed in a previous study [36] was used as an *E. coli–Bacillus* shuttle vector. To construct a vector, pUB19 was digested with a pair of *Hind*III and *Mlu*I restriction enzymes. P*sin*2-*egfp* and P*sin*3-*egfp* fragments were ligated into the pUB19 vector to construct pUB19-P*sin*2-*egfp* (7.5 kb) and pUB19-P*sin*3-*egfp* (7.7 kb), respectively. This process is briefly shown in Fig. 1.

Proper primer sets were used to construct recombinant plasmids carrying P*sin*2 mutants as shown in Table 2. Mutant promoters P*sin*2-H, P*sin*2-A, P*sin*2-SD, P*sin*2-P1, and P*sin*2-P2 were amplified from plasmid pUB19-P*sin*2-*egfp* using primer sets of pUB19-F/PH-OR, pUB19-F/PA-OR, pUB19-F/PSD-OR, pUB19-F/PP1-OR, and pUB19-F/PP2-OR, respectively. Each *egfp* gene for recombination with each promoter was amplified using primer sets PH-OF/GFP-R, PA-OF/GFP-R, PSD-OF/GFP-R, PP1-OF/GFP-R, and PP2-OF/GFP-R and recombined by overlap PCR, respectively. As a result, DNA fragments P*sin*2-H-*egfp*, P*sin*2-A-*egfp*, P*sin*2-SD-*egfp*, P*sin*2-P1-*egfp*, and P*sin*2-P2-*egfp* were obtained. Other mutant promoters were amplified using the same methods with appropriate plasmid templates and primer sets. P*sin*2-HA-*egfp* was amplified from pUB19-P*sin*2-H-*egfp*. P*sin*2-HASD-*egfp* was amplified from pUB19-P*sin*2-HA-*egfp*. P*sin*2-P-*egfp*, P*sin*2-PSD-*egfp*, and P*sin*2-HAPSD-*egfp* were amplified from pUB19-P*sin*2-P1-*egfp*, pUB19-P*sin*2-P-*egfp*, and pUB19-P*sin*2-PSD-*egfp*, respectively. Each DNA fragment was digested with *Mlu*I and *Hind*III and subcloned into the pUB19 vector to

Table 1 List of bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source of reference
Strains		
Escherichia coli DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	
Bacillus subtilis 168	*trp*C2	
Bacillus subtilis DB104	*his npr*R2 *npr*E18 Δ*apr*A3	
Plasmids		
pEGFP-N1	Eukaryotic expression vector carrying *egfp*	Clontech
pUB19	*E. coli-Bacillus* shuttle vector, Ap^r, Km^r	[36]
pUB19-P*sin*3-*egfp*	eGFP under *sinIR* promoter control, Ap^r, Km^r	This study
pUB19-P*sin*2-*egfp*	eGFP under *sinI* promoter control, Ap^r, Km^r	This study
pUB19-P*sin*2-H-*egfp*	P*sin*2 with Hpr binding site removed, Ap^r, Km^r	This study
pUB19-P*sin*2-A-*egfp*	P*sin*2 with AbrB binding site removed, Ap^r, Km^r	This study
pUB19-P*sin*2-HA-*egfp*	P*sin*2 with Hpr and AbrB binding sites removed, Ap^r, Km^r	This study
pUB19-P*sin*2-SD-*egfp*	P*sin*2 with strong Shine-Dalgarno (SD) sequence, Ap^r, Km^r	This study
pUB19-P*sin*2-P1-*egfp*	P*sin*2 with modified P1 that replaced by the consensus sequence of σ^A-dependent promoters, Ap^r, Km^r	This study
pUB19-P*sin*2-P2-*egfp*	P*sin*2 with modified P2 that replaced by the consensus sequence of σ^A -dependent promoters, Ap^r, Km^r	This study
pUB19-P*sin*2-P-*egfp*	P*sin*2 with modified P1 and P2 that replaced by the consensus sequence of σ^A -dependent promoters, Ap^r, Km^r	This study
pUB19-P*sin*2-HASD-*egfp*	P*sin*2 with Hpr and AbrB binding sites removed and strong SD sequence, Ap^r, Km^r	This study
pUB19-P*sin*2-PSD-*egfp*	P*sin*2 with modified P1 and P2, and strong SD sequence, Ap^r, Km^r	This study
pUB19-P*sin*2-HAPSD-*egfp*	P*sin*2 with Hpr and AbrB binding sites removed, modified P1 and P2, and strong SD sequence, Ap^r, Km^r	This study

Ap: Ampicillin, Km: Kanamycin, ^{r}: resistance

Table 2 List of oligonucleotides used in this study

Oligonucleotide	Sequences 5' → 3'	Used for
Psin-F	NNNNNCG**ACGCGT**CGACCATTCGACATCATTCTCG	P*sin*3, P*sin*2
pUB19-F	CGAGGAAAGATGCTGTTCTTGT	All of modification
GFP-R	TATGTTGTGTGGAATTGTGAGCG	
Psin2-OF	ATTTTAGGAGGAGAAACTGCATGGTGAGCAAGGGCGAG	P*sin*2
Psin2-OR	TCCTCGCCCTTGCTCACCATGCAGTTTCTCCTCCTAAAAT	
Psin3-OF	TATAATATCACAAGGAAGGTGATGACAAATGGTGAGCAAGGGCGA	P*sin*3
Psin3-OR	CTCGCCCTTGCTCACCATTGTCATCACCTTCCTTGTGATATTAT	
PH-OF	GATTATAAAGGTATATTGGAAAAAAATTCTGG	P*sin*2-H
PH-OR	CCAATATACCTTTATAATCGTATTTTCTCAAAAAAAACG	
PA-OF	GGTATATTGGAAAAAAATTCTGGTGATTTAAAATGACTTCC	P*sin*2-A, P*sin*2-HA
PA-OR	GCTTCATTAGTCTCTGGAAGTCATTTTAAATCACCAG	
PSD-OF	GAAATACATAAACAAGTATTTAAGGAGGAGAAAC	P*sin*2-SD, P*sin*2-HASD, P*sin*2-PSD
PSD-OR	GCAGTTTCTCCTCCTTAAATACTTGTTTATG	
PP1-OF	CTCGTTTTTTTTGACAAAATACGATTATAATAAAGGTATAATGG	P*sin*2-P1
PP1-OR	GAATTTTTTTCCATTATACCTTTATTATAATCGTATTTTGTCAAA	
PP2-OF	GGCAATTGACATCCAGAGACTAATGAAGCATATAATAAG	P*sin*2-P2, P*sin*2-P
PP2-OR	GTCTCTGGATGTCAATTGCCATTAAATCACC	
PHA-OF	ATGGAAAAAAATTCTGGTGATTTAAATTGACATCCAGAGA	P*sin*2-HAPSD
PHA-OR	CACCAGAATTTTTTTCCATTATACCTTTATAATCGTATTTTGTC	

3' and 5'- overlap sequences were underlined, and inserted *Mlu*I site was shown in bold

construct the recombinant plasmid listed in Table 1.

Measurement of eGFP fluorescence in *B. subtilis* DB104

A single colony of an appropriate *B. subtilis* DB104 strain picked on LB agar plates was inoculated into 10 mL of LB broth containing antibiotics and cultured overnight. The preculture was transferred into 50 mL of LB broth containing antibiotics in 500 mL baffled flask to be cultured for 48 h. During culture, the culture medium was sampled every 12 h. One hundred μL of the sample was transferred to a black flat 96-well plate (SPL Life Sciences, Pocheon, Korea) and the fluorescence value of eGFP was measured with a Victor^{TM} X4 multiplate reader (PerkinElmer, Waltham, MA, USA). Data were averaged from three independent experiments. Raw data of eGFP fluorescence measurement was presented in Additional file 1: Table S1.

Statistical analysis

All data are presented as average ± standard deviation from independent triplicate experiments. The statistical significance was conducted with two-way analysis of variance (ANOVA) using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Differences with p <0.05 were considered statistically significant. Tables S2 to S6 show analyzed results of Figs. 2 to 6, respectively (Additional file 1).

Results

Effect of the number of promoters on eGFP expression level

First, a pUB19-P*sin*3-*egfp* plasmid (P*sin*3) with all three promoters constituting the *sinIR* operon and a pUB19-P*sin*2-*egfp* plasmid (P*sin*2) with the third promoter removed were constructed (Fig. 1 and Fig. 2A). The expression level of eGFP according to the incubation time was compared in *B. subtilis* DB104 transformants having these recombinant plasmids. Fluorescence was not expressed in *B. subtilis* DB104 containing the pUB19 plasmid as a negative control (Additional file 1: Fig. S1). Expression levels of eGFP in P*sin*2 were greater by 18% and 33% at 36 and 48 hours, respectively, compared to eGFP level in P*sin*3 (Fig. 2B). Since the eGFP expression in P*sin*2 was higher than that in P*sin*3, we performed additional promoter engineering based on P*sin*2. Cell growths of these recombinant strains were determined by measuring optical density at 600 nm. Their growths were increased for 24h, but no longer increased at 36 or 48 h (Fig. 2C).

Modifications of repressor binding sites, –35 and –10 region, and Shine-Dalgarno sequence

To investigate the effect of the removal of repressor binding site on transcription, the two repressor binding sites (Hpr and AbrB)

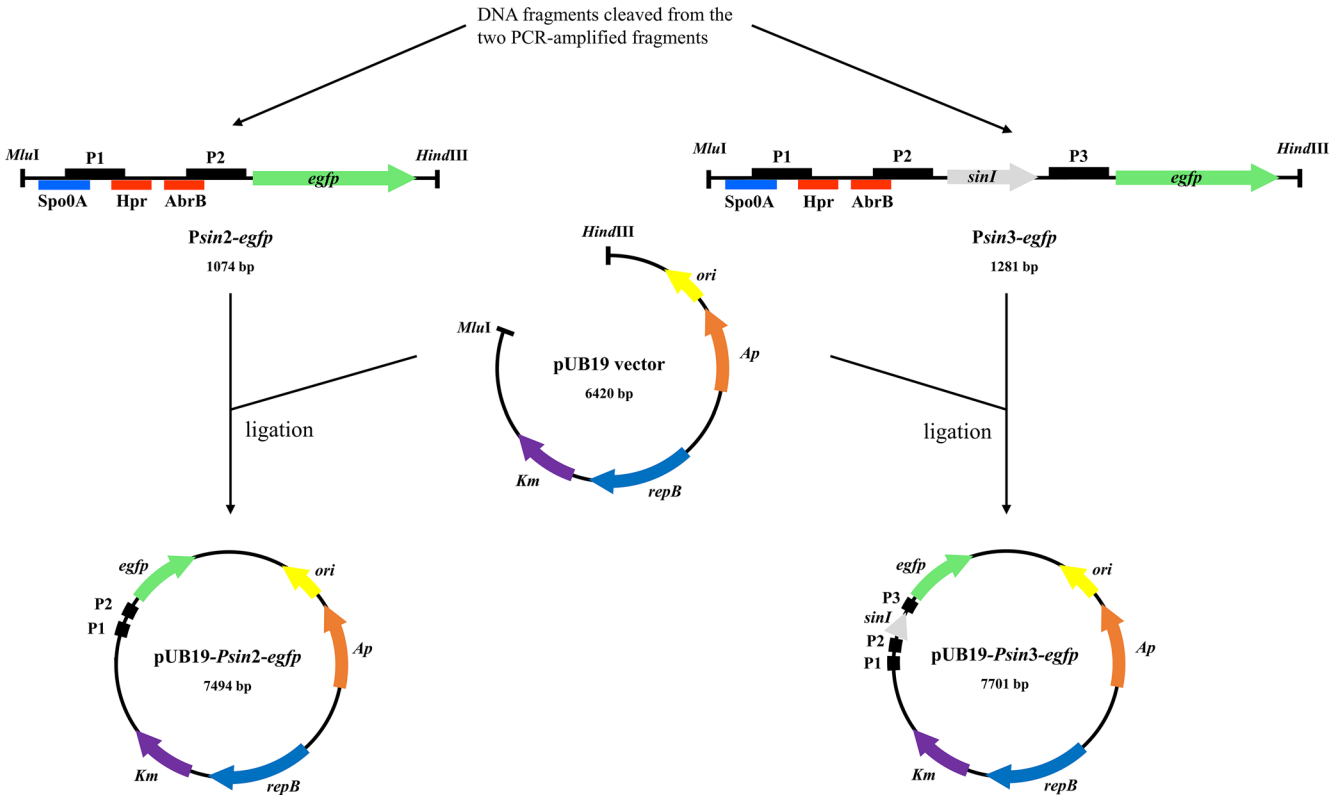

Fig. 1 Construction scheme for recombinant plasmids according to the number of promoters. *Ap* and *Km* represent ampicillin and kanamycin resistance markers, respectively. *ori* and *repB* represent replication origin and replication protein B, respectively. P1, P2, and P3 represent the three distinct promoter sequence of *sinIR* gene

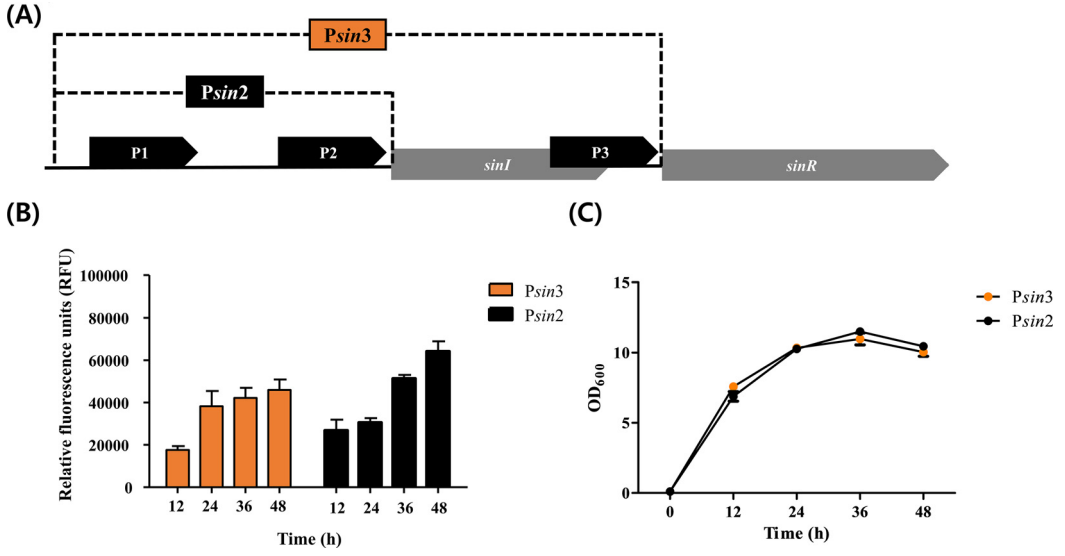

Fig. 2 Comparison of promoter strength according to the number of *sinIR* promoters. (A) Composition of *sinIR* operon. Regions of P*sin*2 and P*sin*3 are indicated by dotted lines. (B) Comparison of relative fluorescence units (RFU) according to the number of promoters. RFU driving expression of *egfp* was measured in triplicate and averaged with standard deviation. Two-way ANOVA with Bonferroni's post hoc correction test was performed (Additional file 1: Table S2). (C) Optical density (OD_{600}) values of P*sin*3 and P*sin*2 during growth

(A)

	Hpr binding site	AbrB binding site
Consensus sequence	….ATANTAT….	….TGGNA….
P*sin*2	tataATAAAGGtata	ttaaTGGCAaatg
P*sin*2-H	tata____AAGGtata	
P*sin*2-A		ttaa_____aatg
P*sin*2-HA	tata____AAGGtata	ttaa_____aatg

(B)

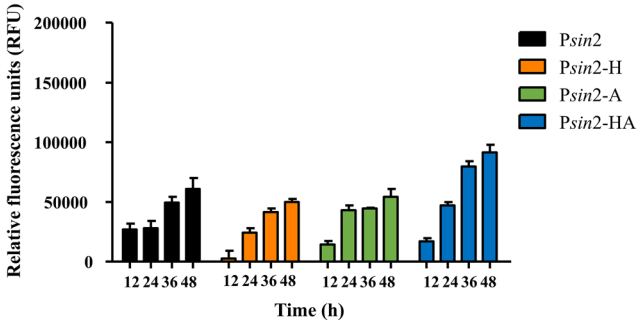

Fig. 3 Comparison of mutant promoter strength after removing binding sites of repressor proteins. (A) Changed sequences of P*sin*2-H, P*sin*2-A, and P*sin*2-HA. Removed sequence is indicated by underline. The lowercase letters indicate the unmodified sequences. (B) Relative fluorescence level under the control of P*sin*2 derivatives. The fluorescence driving expression of *egfp* was measured in triplicate and averaged with standard deviation. Two-way ANOVA with Bonferroni's post hoc correction test was performed (Additional file 1: Table S3)

(A)

	P1 (–35……–10)	P2 (–35……–10)
Consensus sequence	…. TTGACA…….....TATAAT ….	…… TTGACA ……..…….....TATAAT …..
P*sin*2	tttt TTGAGAaaatacgatTATAAA ggta	gcaa ATGACTtccagagacttaatgaagcaTACAAT aagt
P*sin*2-P1	tttt TAGACAaaatacgatTATAAT ggta	
P*sin*2-P2		gcaa TTGACAtccagagacttaatgaagcaTATAAT aagt
P*sin*2-P	tttt TAGACAaaatacgatTATAAT ggta	gcaa TTGACAtccagagacttaatgaagcaTATAAT aagt

(B)

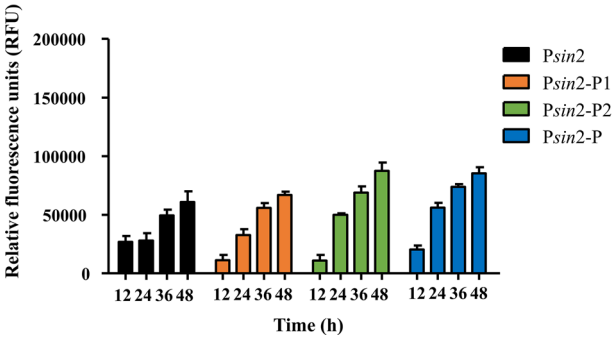

Fig. 4 Comparison of mutant promoter strength that changed –35 and –10 region sequence. (A) Changed sequences of P*sin*2-P1, P*sin*2-P2, and P*sin*2-P. Underlined nucleotides indicate mutated sequence to consensus sequence of σ^A-dependent promoter in *B. subtilis*. The lowercase letters indicate the unmodified sequences. (B) Relative fluorescence level under the control of P*sin*2 derivatives. The fluorescence driving expression of *egfp* was measured in triplicate and averaged with standard deviation. Two-way ANOVA with Bonferroni's post hoc correction test was performed (Additional file 1: Table S4)

were removed individually or together. Therefore, three recombinant plasmids were constructed for this experiment. They are listed in Table 1. The pUB19-P*sin*2-H-*egfp* plasmid (P*sin*2-H) had a three-base (ATA) deletion at the Hpr binding site. The pUB19-P*sin*2-A-*egfp* plasmid (P*sin*2-A) had a five-base (TGGCA) deletion at the AbrB binding site. The pUB19-P*sin*2-HA-*egfp* (P*sin*2-HA) plasmid had a total of eight-base deletion at both binding sites (Fig. 3A). Compared to P*sin*2 as a control, P*sin*2-H and P*sin*2-A showed decreased expression levels of eGFP by 18% and 11%, respectively, after 48 h incubation. However, eGFP expression was increased by 50% in P*sin*2-HA in which both repressor binding sites were removed (Fig. 3B). The results after 36 h incubation were similar to those after 48 h incubation.

Nucleotide sequences of –35 and –10 regions in a promoter are critical factors determining promoter strength. In *B. subtilis*, the *sinIR* promoter is one of σ^A-dependent promoters. However, nucleotide sequences of –35 and –10 regions do not precisely match consensus sequences of the σ^A-dependent promoters (TTGACA and TATAAT, respectively) [34,37]. To investigate whether converting existing sequences of –35 and –10 regions into consensus sequences of the σ^A-dependent promoter could affect promoter activity, corresponding sequences of P1 and P2 promoters were modified (Table 1 and Fig. 4A). A pUB19-P*sin*2-P1-*egfp* plasmid (P*sin*2-P1) with two base substitutions in –35 (G to C) and –10 (A to T) regions of the P1 promoter alone was constructed. A pUB19-P*sin*2-P2-*egfp* plasmid (P*sin*2-P2) with three base substitutions in the -35 (A to T, T to A) and -10 (C to T) regions of the P2 promoter alone was also constructed. In the case of the pUB19-P*sin*2-P-*egfp* plasmid (P*sin*2-P), identical base substitutions were made in the –35 and –10 regions of both P1 and P2 promoters. Expression levels of eGFP in P*sin*2-P1, P*sin*2-P2, and P*sin*2-P were increased by 10, 43, and 40%, respectively,

(A)

	Shine-Dalgarno seqence
Consensus sequence	….TAAGGAGG….
P*sin*2	tattTTAGGAGGagaa
P*sin*2-SD	tattTAAGGAGGagaa

(B)

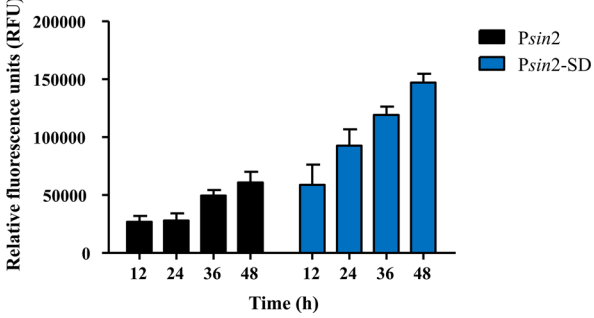

Fig. 5 Comparison of mutant promoter strength that changed Shine-Dalgarno sequence. (A) Changed sequence of P*sin*2-SD. Underlined nucleotide indicates mutated sequence. The lowercase letters indicate the unmodified sequences. (B) Relative fluorescence level under the control of P*sin*2 derivative. The fluorescence driving expression of *egfp* was measured in triplicate and averaged with standard deviation. Two-way ANOVA with Bonferroni's post hoc correction test was performed (Additional file 1: Table S5)

(A)

	Hpr binding site	AbrB binding site	P1 (–35……–10)	P2 (–35……–10)
Consensus sequence	….ATANTAT….	….TGGNA….	…. TTGACA…….TATAAT ….	…… TTGACA…….TATAAT …..
P*sin*2	tataATAAAGGtata	ttaaTGGCAaatg	tttt TTGAGAaaatacgatTATAAA ggta	gcaa ATGACTccagagacttaatgaagcaTACAAT aagt
P*sin*2-HASD	tata____AAGGtata	ttaa_____aatg		
P*sin*2-PSD			tttt TAGACAaaatacgatTATAAT ggta	gcaa TTGACAccagagacttaatgaagcaTATAAT aagt
P*sin*2-HAPSD	tata____AAGGtata	ttaa_____aatg	tttt TAGACAaaatacgatTATAAT ggta	gcaa TTGACAccagagacttaatgaagcaTATAAT aagt

(B)

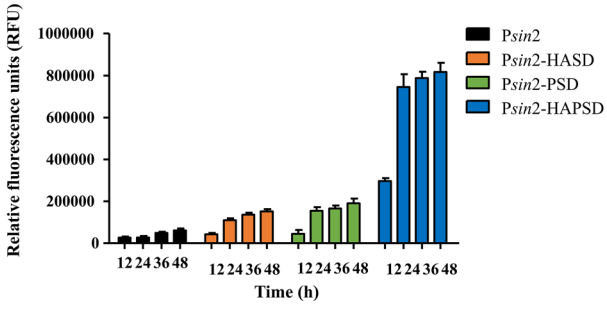

Fig. 6 Comparison of mutant promoter strength with several promoter elements modified. (A) Changed sequences of P*sin*2-HASD, P*sin*2-PSD, and P*sin*2-HAPSD. Underlined nucleotides indicate mutated sequence. The lowercase letters indicate the unmodified sequences. Underlines without letter indicate deleted sequence. (B) Relative fluorescence level under the control of P*sin*2 derivative. The fluorescence driving expression of *egfp* was measured in triplicate and averaged with standard deviation. Two-way ANOVA with Bonferroni's post hoc correction test was performed (Additional file 1: Table S6)

after 48 h of incubation compared to with level in P*sin*2 control (Fig. 4B). Results after 36 h of incubation were similar to those after 48 h of incubation.

The SD sequence (TTAGGAGG) of the *sinI* gene differs by only one base compared to the SD sequence (TAAGGAGG) known to be strong in *B. subtilis*. To investigate effect in the change of the SD sequence on the expression of eGFP, the pUB19-P*sin*2-SD-*egfp* plasmid (P*sin*2-SD) with modified SD sequence of the *sinI* gene was constructed (Table 1 and Fig. 5A). After 36 h incubation and 48 h incubation, expression levels of eGFP in P*sin*2-SD were both increased 2.4-fold compared to that in the P*sin*2 control group (Fig. 5B).

Optimizing a strong promoter for high-level expression of GFP

All modifications in the *sinIR* operon, including removal of the repressor binding sites, alteration of the −35 and −10 regions, and alteration of the SD sequence, increased expression levels of eGFP compared to the P*sin*2 control. Three recombinant plasmids were additionally constructed based on the P*sin*2-SD plasmid to optimize a strong promoter by combining each element that contributed to the enhancement of promoter activity (Table 1 and Fig. 6A). The pUB19-P*sin*2-HASD-*egfp* (P*sin*2-HASD) plasmid with two repressor binding sites deleted and the pUB19-P*sin*2-PSD-*egfp* (P*sin*2-PSD) plasmid −35 and −10 regions modified were constructed, respectively. Finally, the pUB19-P*sin*2-HAPSD-*egfp* (P*sin*2-HAPSD) plasmid was constructed by removing the two repressor binding sites from P*sin*2-PSD. Expression levels of eGFP in P*sin*2-HASD and P*sin*2-PSD were increased by 2.75-fold and 3.35-fold after 36 h culture or 2.5-fold and 3.16-fold after 48 h culture, respectively, compared to the eGFP level in P*sin*2 (Fig. 6B). In particular, eGFP expression in P*sin*2-HAPSD was increased by 15.94-fold and 13.41-fold, respectively, after 36 and 48 h incubation compared to that in P*sin*2.

Discussion

The promoter is a fundamental element that can influence protein production. Strategy such as screening from microbial genomes [38,39] and synthesizing synthetic promoters [40-42] have been used to obtain powerful promoters to construct a protein expression system. However, screening and generating promoter libraries is a labor- and time- consuming task. Thus, several recent studies have used more efficient RNA-seq to determine strong promoters [26,43]. Han L, et al. have investigated the transcriptome of *B*. *pumilus* BA06 [27]. Referring to this study, we selected the *sinIR* promoter, which could constantly express throughout growth, and attempted promoter engineering to apply it to the promoter expression system in *B*. *subtills* DB104.

P1, P2, and P3 as the three promoters in the *sinIR* operon have different expression time and regulatory mechanisms. Therefore, we first compared protein expression ability according to the number of *sinIR* promoters. As a result, P*sin*2 containing two promoters (P1 and P2) with *sinI* gene removed exhibited slightly higher expression than Psin3, which contained three promoters (P1, P2, and P3) and a *sinI* structure gene. The three *sinIR* promoters are controlled differently during bacterial growth. P3 is expressed from early growth to two hours after the stationary phase starts [33]. After that, the expression by P3 stops and the transcription by P2 commences [33]. On the other hand, the expression of genes controlled by P1 is repressed during exponential growth but upregulated rapidly upon entering the stationary phase [34]. The amount of SinI after P1 activation is about 10-fold more than SinR despite P1 transcribing mRNA that encodes both *sinI* and *sinR* [28,34]. For these reasons, fluorescence expression in P*sin*3 was higher than that in P*sin*2 up to 24 h before entering the stationary phase (Fig. 2). Although it contained additional promoter P3, eGFP expression in P*sin*3 was less than expected, which could be attributed to overexpression of the *sinI* gene. SinI and SinR are master regulators in *B*. *subtilis* biofilm formation [44]. SinI is an antagonist of SinR. It binds to SinR to prevent it from inhibiting biofilm formation [45]. The *sinI* gene was overexpressed in P*sin*3. Thus, biofilm was formed more than that in P*sin*2 (Additional file 1: Fig. S2). The expression of *sinI* gene in P*sin*3 seems to affect the expression of eGFP.

Transition state regulators included in the regulation of the *sinIR* promoter operate complicatedly to prevent unwanted responses during the exponential phase. The *sinIR* operon is controlled by three transcription factors; Spo0A, Hpr, and AbrB [33]. Phosphorylated Spo0A attaches to the −35 region of P1 and acts as an activator [33]. Two other proteins, Hpr and AbrB, are attached to repress transcription at the −10 region of P1 and −35 region of P2 regions, respectively (Fig. 1) [28,33,46]. The binding site of each transcription factor does not overlap with each other. We intended to improve the activity of the promoter by removing transcription inhibition by these two repressors [46,47]. Contrary to our expectation, eGFP expression was not affected when each repressor binding site was removed individually. However, when the two repressor binding sites were both removed, the expression of GFP was increased, indicating that each repressor alone could not sufficiently inhibit transcription of the *sinIR* promoter.

Many related studies have shown that −35 and −10 regions are the important sequences for determining promoter strength [48-50]. We modified −35 and −10 sequences of the P1 and P2 promoters to the consensus sequence respectively or both simultaneously. Among P1 and P2 promoters, only sequence change of the P2 promoter increased the expression of eGFP regardless of the change of the P1 promoter sequence. Similar studies have been conducted for other promoters. It was found that activities of P_{aprE} and P_{srfA} were improved by changing −35 sequence to match the consensus sequence [12,51]. In addition, studies that substituted −35 and −10 sequences of P_{aprN}, P_{ylb}, P_{groES} and P_{cry3Aa} found that when more sequences were changed to match the consensus sequence, more protein expression was affected [2,52-54].

The SD sequence is present in the 5' untranslated mRNA region. It complementarily binds with anti-SD sequences present in the 16S rRNA 3' terminus of the small ribosomal subunit to initiate translation [55]. This sequence also plays an important role in recombinant protein production [56]. In this study, when the SD sequence of the *sinIR* operon was changed to the strong consensus sequence of *B*. *subtilis* [57,58], the expression level of the eGFP was significantly increased. The modified SD sequence in mRNA might affect mRNA stability and translation efficiency [59], which appears to positively impact the expression level of eGFP. Modifications in the SD sequence in *Corynebacterium glutamicum* can also affect expression levels of target proteins [60].

The combination of modified elements in this experiment positively affected eGFP expression. Removal of repressor binding sites, sequence changes in the −35 and −10 regions, and introduction of a strong SD sequence contributed to a 13.4-fold increase in eGFP expression compared to the wild-type *sinIR* promoter. Many studies on promoter engineering have shown increased protein expression by increasing transcription levels, mainly through modification of the UP element and −35 and −10 regions in the promoter region [2,4,53,61,62]. We also confirmed that protein expression could be positively affected through modification of repressor binding sites and SD sequence. In the following study, the P*sin*2-HAPSD promoter will be compared to commercial promoters and applied to produce recombinant proteins in *B*. *subtilis* DB104.

In conclusion, this study attempted to develop a novel protein expression system in *B. subtilis* DB104 using a *sinIR* promoter, which was chosen based on transcriptome analysis results of *B. pumilus* BA06. We found that removing the repressor protein binding site from the *sinIR* promoter region or changing the −35 and −10 sequences and the SD sequence with their respective consensus sequences enhanced the transcription level, respectively. In addition, integration of these elements in the *sinIR* promoter increased the transcription level by 13.4-fold compared to the

wild-type promoter. Promoter selection based on transcriptomic analysis and promoter engineering is expected to be utilized to develop a recombinant protein expression system in *B. subtilis* DB104.

Competing interests The authors declare that they have no competing interests.

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