Improvement surfactin production by substitution of promoters in *Bacillus subtilis* TD7

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### Abstract:
Surfactin is one of the most widely used biosurfactants, which exhibits excellent surface activity plus other biological effects. It has potential applications in microbially enhanced oil recovery, environmental bioremediation, agricultural bio-control, pharmacy, cosmetics and food industries. The low yield of surfactant in wild strains is the key factor restricting its industrial applications. Since promoters are the key element in gene expression, constructing genetically engineered bacteria by promoter substitution is an effective method to enhance surfactin production. This study focused on constructing strains with efficient surfactin production by replacing the native srfA promoter with a better one. Two different promoter patterns with different homologous arm positions were used for srfA promoter substitution. The most efficient installation way was determined to replacing the sequence between the transcriptions start site and the ribosome binding site of srfA. In addition, eight endogenous strong auto-inducible phase-dependent promoters were chosen and used to substitute the native promoter srfA using the CRISPR-Cas9 system. As a result, high surfactin yielding strains with potential application in industry were obtained. According to the results, the yield of three strains with promoters P43, Pₚₑᵥₑ, and Pᵥₑₚ was 3.5, 2.8, and 2.3 times higher than that of the wild strain *Bacillus subtilis* TD7.

### Keywords:
Surfactin, CRISPR-Cas9, promoter substitution, *Bacillus subtilis*, phase-dependent promoter

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### 1 Introduction

Biosurfactants produced by a wide variety of diverse microorganisms are of low ecotoxicity, good biodegradability, and environmental biocompatibility, and are regarded as a new field in green technology (Muthusamy et al., 2008; Rebello et al., 2018; Jimoh and Lin, 2019). The global market for biosurfactants reached 1.6 billion USD in 2018 (https://www.gminsights.com/industry-analysis/biosurfactants-market-report). Surfactin is one of the most potent biosurfactants with the best surface activity and excellent biological properties (Peypoux et al., 1999; Seydlova and Svobodova, 2008). Therefore, surfactin has potential applications in cosmetics, food processing, microbially enhanced oil recovery (MEOR), and environmental bioremediation (Yoneda et al., 2001; Pereira et al., 2013; Bezza and Chirwa, 2017a, b; Nitschke and Silva, 2018). Furthermore, it may serve as biocontrol agent, tumor growth inhibitor, disinfectant and industrial cleaner (Rodrigues et al., 2006; Sen, 2010; Gudina et al., 2013; Fernandes et al., 2014; Liu et al., 2019; Penha et al., 2020). A large-scale industrial application of surfactin, however, is impeded by the low productivity of production strains causing high production costs (Chen et al., 2015). Therefore, constructing overproducing strains to improve productivity is a promising solution to fulfill the requirements of industrial-scale production (Hu et al., 2019).

Surfactin is a secondary metabolite assembled by a non-ribosomal peptide synthetase, which is encoded by the srfA gene cluster (Roongsawang et al., 2011). The transcription
of srfA is controlled by the promoter P_{srfA} (Nakano et al., 1988). Based on the synthesis mechanism, various surfactin-producing strains have been engineered by modifying the transcriptional regulatory factors for enhancing surfactin transmembrane efflux, strengthening metabolic pathways of precursors, as well as systematic genetic manipulation of multiple modules (Jung et al., 2012; Coutte et al., 2015; Li et al., 2015; Yang et al., 2015; Dhali et al., 2017; Gao et al., 2017; Wang et al., 2019; Wu et al., 2019). Previous promoter modifications (Table S1) include the replacement of the P_{srfA} promoter by isopropyl -d-thiogalactoside (IPTG)-mediated inducible promoters such as P_{vac} and P_{32} and constitutive promoters such as P_{repU} and P_{repL} (Sun et al., 2009; Coutte et al., 2010; Willenbacher et al., 2016; Jiao et al., 2017). IPTG is an expensive inducing agent, which is non-degradable and may therefore cause environmental pollution. All these flaws prohibit its utilization in large-scale fermentation. On the other hand, phase-dependent auto-inducible promoters enabling a high-level expression of the target gene with relatively low cost are highly desirable for industrial application (Guan et al., 2015; Yu et al., 2015; Song et al., 2016; Liu et al., 2018; Kang et al., 2020). Such promoters were investigated and are classified into four classes: the class I (exponential phase) promoters show transcriptional activity at exponential phase but no activity at stationary phase, the class II (middle-log and early stationary-phase) promoters mainly transcribe at mid-exponential phase to early stationary phase, the class III (lag-log and stationary-phase) promoters are effective after middle-log phase, and the class IV (stationary phase) promoters are mainly active at stationary phase (Yang et al., 2017). Based on these, eight phase-dependent auto-inducible promoters of classes II (P43, P_{poyG}, and P_{psac}), III (P_{psr}, P_{ylbP}, and P_{pyqD}), and IV (P_{pmmgA} and P_{pyqD}) were applied for replacing the native srfA promoter.

Moreover, previous studies deleted the sequences between the transcription start site (TSS) and the ribosome binding site (RBS), while replacing the native srfA promoter. However, the sequence between TSS and the RBS may play an important role in ribosome binding and protein translation. In order to enhance surfactin production, we designed two different installation modes with the different sequences upstream or downstream of P_{srfA} and compared these two modes using CRISPR-Cas9 tools. Furthermore, eight different classes of highly efficient promoters were selected to replace the srfA promoter for increasing the surfactin yield of the wild strain B. subtilis TD7.

### 2 Materials and Methods

#### 2.1 Bacterial strains and culture conditions

_Bacillus subtilis_ TD7, isolated from Daqing oil field and conserved in our laboratory (Liu et al., 2012), was used as host strain in this study; _Bacillus subtilis_ 168 was used as the promoter template. The sequences of various promoters derived from the genome of _B. subtilis_ 168 are listed in Table S2. _Escherichia coli_ JM83 was used for plasmid construction and replication. More details for these strains are listed in Table 1.

Strain cultivation and fermentation were performed in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, or 1.8 % Agar for LB solid medium) and fermentation medium with the following composition: 70 g/L sucrose, 1 g/L yeast extract, 25 g/L NaNO3, 0.333 g/L KH2PO4, 1 g/L Na2HPO4 • 12 H2O, 0.15 g/L MgSO4 • 7 H2O, 7.5 mg/L CaCl2, 6 mg/L MnSO4 • H2O, and 6 mg/L FeSO4 • 7 H2O (pH 7.0) (Jiao et al., 2017). Spizizen medium was used to prepare _B. subtilis_ competent cells (Anagnostopoulus and Spizizen, 1961). Kanamycin was used for positive colony selection at a concentration of 30 mg/mL for _E. coli_ and 10 mg/mL for _B. subtilis_.

#### 2.2 DNA manipulation and plasmid construction

_E. coli_ transformation, DNA and plasmid extraction and purification were performed following the standard methods and manual instructions of kits (Shanghai Generay Biotech Co., Ltd). CRISPR-Cas9 plasmid pJOE8999 (Hangzhou Disai Biotech Co., Ltd). The single-guide RNA (sgRNA) sequence was obtained from the website (https://crispy.secondarymetabolites.org). All primers and oligonucleotides were designed with Primer Premier 5 and synthesized by GenScript (Nanjing) Co., Ltd. (Table S3). DNA sequencing verification was performed by Beijing Liuhe Huada Gene Technology Co., Ltd. Enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

As shown in Figure 1, the recombinant plasmids were constructed in several steps. Firstly, sgRNA was obtained by phosphorylation and annealing the oligos pN20-F/R in a thermocycler with the following parameters: 97 °C for 10
Figure 1. Plasmid construction. (a) Four fragments including sgRNA, the upstream and downstream homologous arms of the P\textit{psrfA} and new promoters were inserted into the CRISPR-Cas9 plasmid. (b) gRNA guides Cas9 to cleave the target region in genome by generating base-pairing. (c) Gene replacement by homologous recombination.

min, 90 °C for 4 min, 70 °C for 10 min, 55 °C for 10 min, 40 °C for 10 min, ramp down to 25 °C. The sgRNA was inserted to pJOE8999 at S\textit{fi}I sites to construct pJ-sgP\textit{psrfA}. In the second step, we designed primers pU-F/R, and pD-F/R. Then use \textit{B. subtilis} TD7 chromosomal DNA as template, the upstream and downstream homologous arm fragments were obtained by PCR. The PCR parameters were as follows: pre-denaturation at 97 °C for 5 min, denaturation at 97 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 2 min, 32 cycles for step 2 to step 4, 72 °C for 10 min, 25 °C. The arms were then linked together by overlap-extension PCR (Heckman and Pease, 2007). After purification, we obtained the fragment U-XbaI-D and then inserted it into the X\textit{baI} site of the plasmid pJ-sgP\textit{psrfA}. In the next step, those target promoters were amplified from \textit{B. subtilis} 168 genomes and cloned into the pJ-UD\textit{psrfA} at X\textit{baI} site. Finally, we obtained a set of recombinant plasmids.

2.3 Strain construction

Genetic recombination was performed as described by Altenbuchner (Altenbuchner, 2016). Competent \textit{B. subtilis} TD7 cells were prepared with Spizizen medium (Anagnostopoulos and Spizizen, 1961). Fresh \textit{B. subtilis} TD7 colonies were obtained from overnight cultivation on LB plates at 37 °C and were picked up and inoculated into shake flasks with 5 mL GM1 medium. Cultivation was carried out for 20 h at 37 °C and 190 rpm. Then, 500 mL broth were transferred into another shake flask with 5 mL GM1 medium and cultured for 5 h at 37 °C and 190 rpm. Mid-late logarithmic bacterial cells were then given (1.5 mL broth) into 5 mL GM2 medium and cultivated at 37 °C and 190 rpm for 1.5 h to obtain competent cells. Recombinant vectors were transformed into the competent cells. Then the cells were resuscitated by cultivation at 37 °C for 1 h without shaking and another hour at 37 °C and 190 rpm. Afterwards the strains were spread on LB plates containing kanamycin and incubated overnight at 37 °C. According to colony PCR screening results, one positive colony was streaked on LB plate with kanamycin and 0.2% mannose and cultivated at 28 °C for 2 days to induce the cas9 expression under the control of P\textit{man}. The genome was cleaved at target localization by the sgRNA, then the promoter replacing was completed by double-crossover homologous recombination. We screened the stains that finished the promoter substitu-
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2.4 Cultivation of recombinant strains and surfactin analysis

The strains stored at -80 °C were firstly reactivated by spreading on LB plates and incubation at 37 °C for 12 h. Then single colonies were picked and used to inoculate in 30 mL LB liquid medium (250 ml bottle). The cultures were inoculated at 37 °C and 200 rpm for 14 h. Then, 2 mL (2 %) seed broth were inoculated into the fermentation medium. Fermentation was carried out at 37 °C and 200 rpm for 120 h. The absorbance at 600 nm wavelength (OD600) was measured, and 2 mL fermentation broth were given into EP tubes for surfactin detection. The pH was adjusted to 2.0 with 6 mol/L HCl, and the product was extracted thrice using ethyl acetate. The crude product was obtained by drying at 70 °C. Surfactin was dissolved in 1 mL methanol and filtered through a 0.22 m pore-size filter membrane. The concentration of surfactin was measured with reverse-phase high pressure liquid chromatography (RP-HPLC) with an ODS-BP C18 column (5 mm, Φ4.6 mm 250 mm). All strains were fermented in three batches with three parallel samples in each batch.

For HPLC-analysis, acetonitrile and water (containing 0.1 % acetic acid) were used as mobile phase at a gradient of 80 % acetonitrile for 0-2 min, 80 % acetonitrile for 2-22 min, 100 % acetonitrile for 22-35 min, 100 % to 80 % acetonitrile for 35-40 min, and 80 % acetonitrile for 40-45 min. The chromatograms were recorded at 205 nm at a column temperature of 30 °C and injection volumes of 25 mL. The surfactin variants were also analyzed by electrospray ionization mass spectrometry (ESI-MS).

3 Results

3.1 Substitution of the native srfA promoter in different patterns

Two pairs of primers were designed for different upstream and downstream homologous arms of srfA promoters. Finally, two plasmids pJ-P431 and pJ-P432 with the same promoter but different homologous arms for genome editing were obtained. Using these plasmids, we obtained two recombinant strains whose original promoter PsrfA was replaced by P43 at two different sites. As shown in Figure 2, B. subtilis TP1 retains the sequences between TSS and RBS in srfA, but the sequences between the upstream gene terminator and PsrfA were knocked out. In contrary, B. subtilis TP1-2 retained the sequences between the upstream terminator and PsrfA, while the sequences between TSS and RBS in srfA was removed.

3.2 Surfactin production by two differently installed promoters

The surfactin yield of these engineered strains was assessed by ESI-MS and HPLC after purification. After the fermentation with B. subtilis TD7, the surfactin isoforms were detected. The m/z values of these surfactin isoforms of hydroxy fatty acids with carbon chain lengths from C11 to C16 were 978.70, 992.74, 1006.75, 1020.74, 1034.74, 1035.78, 1036.80, 1048.81, 1050.02 (Figure...
Figure 3. HPLC detection of Surfactin produced by the wild strain *B. subtilis* TD7 and the recombined strains *B. subtilis* TP1 after cultured at 37 °C for 72 h. A: surfactin C13, B: surfactin iso-C14, C: surfactin C14, D: surfactin C15, E: surfactin C16.

Table 2. The proportion of surfactin isoforms produced by wild strain and mutations with different promoter installation ways

| Component      | Relative MW (M/Z) | TD7     | TP1     | TP1-2   |
|----------------|-------------------|---------|---------|---------|
| C13-surfactin  | 1006.75           | 6.6±0.1 | 18.0±0.4| 16.6±0.3|
| isoC14-surfactin| 1020.74           | 10.9±6.4| 25.7±0.7| 26.6±0.2|
| C14-surfactin  | 1020.74           | 28.6±3.4| 10.6±0.5| 10.5±0.4|
| C15-surfactin  | 1034.76           | 48.5±0.5| 39.0±0.7| 40.7±0.2|
| C16-surfactin  | 1048.81           | 5.4±0.0  | 6.8±0.8  | 5.7±0.4 |

Since surfactin isoforms of C13 up to C16 constituted the major fraction of products for all strains (Figure 3), the total amount of these compounds was used as a measure of surfactin productivity (Figure 4). In Table 2, the proportion of different isoforms given. The engineered strains produced more surfactin C13 and iso-C14, but less surfactin C14 and C15 in comparison with the original one. Ultimately, the surfactin yield of the original strain *B. subtilis* TD7 was 0.65±0.03 g/L, whereas the engineered strains *B. subtilis* TP1 and *B. subtilis* TP1-2 yielded 2.28±0.08 g/L and 1.50±0.11 g/L, respectively. Based on these data, the newly designed promoter installation site (Figure 2(b)) was used for subsequent experiments.

3.3 Substitution of the original P*_{srfA}* promoter by eight strong phase-dependent promoters

In the present study, eight efficient promoters of the classes II (P43, P_{spoVG}, and P_{yvyD}), III (P_{lytR}, P_{ylbP}, and P_{sigX}), and IV (P_{mmgA} and P_{yqfD}) were selected to replace the native srfA promoter in *B. subtilis* (Table 1). As shown in Figure 5, surfactin was produced rapidly and reached the maximum amount at 72 h. Based on this result, 72 h incubation time was used for all tests.

Surfactin yield of different engineered strains were measured at 72 h (Figure 6). Surfactin yields of strains with class II (middle-log and early stationary phases) promoters were higher those with other classes promoters. Surfactin yields of *B. subtilis* TP1, TP2 and TP3 were 2.14±0.17 g/L, 1.75±0.27 g/L and 1.40±0.22 g/L, which were 3.5, 2.8 and 2.3 times higher than that of the parent strain *B. subtilis* TD7 (0.62±0.05 g/L). Thus, these strains have potential for further studies and industrial application. The class III (lag-log and stationary phases) promoters had no obvious effect on product yield resulting the mutants *B. subtilis* TP4, TP5, and TP6 yielded 0.58±0.18 g/L, 0.40±0.06 g/L, and 0.28±0.07 g/L, respectively. Strains with class III or IV promoters had low yields, probably because they lost the high-activity expression window for highly active expression of assistant genes and cofactors. Therefore, the promoters for middle-log and early stationary phases are more suitable for increasing surfactin yield.

Cell growth was monitored to see the effect of promoter substitution on cell growth. The growth curve (Figure S2) indicates that the strains with class II promoters grew retarded and produced less biomass, indicating that surfactin production had a negative effect on cell growth. Strains with class IV promoters began to decline the earliest. These results suggest that promoters may have adverse effects on cell growth and surfactin yield. The change in yield per OD_{600} ratio (Figure S3) also confirmed the influence of the promoters on surfactin production capacity.

4 Discussion

Surfactin, an important lipopeptide-type biosurfactant, has good surface activity and some specific biological activities. However, the low yield limits its application (Peypoux et al., 1999; Geetha et al., 2018). Since the promoter is a key element of gene expression system and directly affects the
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**Figure 5.** Surfactin production curve of the wild strain and promoter modified strains during fermentation for 5 days at 37 °C and 200 rpm.

Gene expression level, its an efficient strategy to replace the promoter with a stronger one than the wild type to increase the yield. There are many phase-dependent auto-inducible promoters that can be expressed at a high level, but using phase-dependent promoters for surfactin is a new attempt. Using phase-dependent promoters instead of chemically inducible promoters will reduced cost, toxicity and is easier of operation, because such promoters require chemical induction compounds like IPTG. IPTG is toxic and persistent in the environment and requires additional operation to add it during fermentation processes (Sun et al., 2009; Jiao et al., 2017). The addition of chemical inducers will also limit the use of the product in pharmaceutical and food industries. This study was focused on testing the substitution of a phase-dependent promoters to for the native *srfA* promoter to increase surfactin yield.

In order to obtain better results, we first designed a new promoter installation mode, different from the previous research, which put the target promoter P43 at a different site. The results of improved effect indicate that the installation method does affect the efficiency of the *srfA* promoter. We conclude from our results that the sequence between TSS and RBS of *srfA* is important for ribosome recruitment and mRNA translation, while the upstream sequence affects the new promoter. However, more work needs to be done to clarify this effect.

Using the new promoter installation site, eight strains of the native *srfA* promoter been replaced by various phase-dependent promoters P43, P*spoVG*, P*yvyD*, P*lytR*, P*ylp*, P*sigX*, P*mmgA* and P*yqfD* were obtained for the first time. The surfactin yield of those strains with different promoters follows the order: class II, class III and class IV, which implies that the strains with class II promoters had the highest surfactin yield. Since surfactin is a secondary metabolite and is synthesized by NRPSs (Nonribosomal peptide synthetase) (Marahiel, 2016). Thus, the promoter work to produce many NRPSs very early in order to accumulate enough NRPSs for surfactin production in early and fully stationary phases.

**Figure 6.** Surfactin yields of the wild strain and the modified strains after 72h culture.

There is still potential to increase the surfactin yield, if compared to the current highest surfactin yield which obtained by modifying multi-module 83 genes (Wu et al., 2019). To further improve surfactin production, different combinations of the three class II promoters P43, P*spoVG*, and P*yvyD* may be used to obtain an optimal combination for *srfA* gene translation.

**5 Conclusion**

Compared with the previous studies, we designed a more efficient site for replacing the native *srfA* promoter in *B. subtilis* by keeping the sequence between TSS and RBS of *srfA*. This study tested the 3 class II (middle-log and early stationary phases) phase-dependent promoters P43, P*spoVG*, and P*yvyD*. These promoters enhanced the yield of surfactin 3.5, 2.8, and 2.3 times. Since these inducer independent strains are able to produce surfactin without IPTG or other inducing agents, they are environmentally friendly, economical and suitable for various industrial production. We firstly found the fact that the promoters of middle-log and early stationary phases play an important role in surfactin production, while promoters of the class II and class IV were not efficient.

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**Conflict of Interest**

The authors have no conflicts of interest to declare.
Ethical approval

Ethics approval was not required since no human subjects or animals were performed for this study.

Author Contributions

Bo-Zhong Mu, Wolfgang Sand, Hui-Zhan Zhang and Jiang Ye contributed to the design of the work and the analysis of the data. Fang-Fang Liu, Yi-Wei Qiao, Yu-Zhe Guo, Fang-Yue Kuang, Xi-Qing Lin performed the research. Fang-Fang Liu, Yi-Fan Liu, Jin-Feng Liu, and Shi-Zhong Yang drafted and modified the paper. All authors gave final approval of the version to be published.

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Supplymentary Material

**Figure S1.** ESI-MS of surfactin production from Bacillus subtilis TD7.

**Figure S2.** Growth curve of the wild-type strain and different mutants during cultivated for 5 days at 37 °C and 200 rpm.

**Figure S3.** Surfactin yield per OD$_{600}$ ratio of the wild-type strain and different mutants.
Table S1. Mutant surfactin producers by promoter exchange

| Strains          | Promoters | Production in shake flask | Reference               |
|------------------|-----------|---------------------------|-------------------------|
| B. subtilis      | Pspac     | 3.86 g/L (IPTG)           | Sun et al., 2009        |
| B. subtilis      | P_repU    | 1.47 g/g biomass          | Coutte et al., 2010     |
| B. subtilis      | P_reg     | 0.26 g/L                  | Willenbacher et al., 2016|
| B. subtilis      | Pg3       | 8.61 g/L (IPTG)           | Jiao et al., 2017       |
| THY15/Pg3        |           |                           |                         |

Table S2. Promoter sequence

| Promoters | Sequence (5’-3’) |
|-----------|------------------|
| P43       | attgaatgcggaagatgatatatatccctttgtagtaccggcgtgtaattctttttctatttcagaacaacgcatgagtttttctatttcttttttttttttttttttttggacattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
**Table S3. Primers sequence**

| Primer     | Sequence (5’ - 3’)                                          | Description                                      |
|------------|-------------------------------------------------------------|--------------------------------------------------|
| pN20-F     | tgcTTCTCTGTAATAATGTTTAG                                     |                                                   |
| pN20-R     | aaacCTAAAGATTTTACAGAAA                                      | Manufacture sgRNA                                 |
| pU-1F      | gcaataagaagcctttACGCTTTACATAATTTGATG                       | Amplify upstream arm for pattern 1                |
| pU-1R      | gctctagagcAGACACCCCTTGCGAAGAG                              | Amplify upstream arm for pattern 2                |
| pD-1F      | ttcgcaagggtgccgtctcgtagacGAAAAACATGAATAATAGCCA             | Amplify upstream arm for pattern 1                |
| pD-1R      | agattatttcttaattTCGATAAAATGATGCAAGAT                      | Amplify downstream arm for pattern 2              |
| pU-2F      | cccgggcaataagcctttCTTTAATCGGTGTCGTTT                    | Amplify promoter P43 for pattern 2                |
| pU-2R      | gctctagagcTCATTTCCACTAAACATTTTAC                          | Amplify promoter P43 for pattern 2                |
| pD-2F      | gtttagtggaaagatcgtagacGATTGAGATATTATTTCC                 | Amplify promoter P43 for pattern 2                |
| pD-2R      | gatgagattttcataatTTTCCAGTATCCCTATCG                       | Amplify promoter P43 for pattern 2                |
| P43-1F     | ttgcaagggtgccgtctCGAGATGATTGATTATTTCC                    | Amplify promoter P43 for pattern 2                |
| P43-1R     | ttatctgttttcGGAACCTACCTACTATTTAT                         | Amplify promoter P43 for pattern 2                |
| P43-2F     | gttgtagtggaaagatcgtagacGATTGAGATATTATTTCC                | Amplify promoter P43 for pattern 2                |
| P43-2R     | aaatttatttccacatttgacccatatcctatcctatcgtacGCGAAGAT      | Amplify promoter P43 for pattern 2                |
| spoVG-F    | ttcgcaagggtgcTTGGGAAGTAAACGGAAGT                         | Amplify promoter P_{spoVG}                       |
| spoVG-R    | ttatctgtttttCTATATAAACAATTAGTGTATCAA                     | Amplify promoter P_{spoVG}                       |
| yvyD-F     | ttgcagaagggtgcTTGATCAATTGCTCCTTCTCT                       | Amplify promoter P_{yvyD}                        |
| yvyD-R     | ttatctgtttttCTAAATGATGTTCTATTCTCC                       | Amplify promoter P_{yvyD}                        |
| sigX-F     | ttgcagaagggtgcTTGAAAGCCCAACAGGAT                        | Amplify promoter P_{sigX}                        |
| sigX-R     | ttatctgtttttCTGATGATGATCTATGTTAGTTGAAAGGT               | Amplify promoter P_{sigX}                        |
| lytR-F     | ttgcagaagggtgcTTGACCCCTACATAAGTACCTTCC                   | Amplify promoter P_{lytR}                        |
| lytR-R     | ttatctgtttttCTAATGATGTTACATTACCCAGTGTAA                  | Amplify promoter P_{lytR}                        |
| ylbP-F     | ttgcagaagggtgcCTAGCAAGGTGAAAGTCC                         | Amplify promoter P_{ylbP}                        |
| ylbP-R     | ttatctgtttttCTTTATAGGATGTTAACCATT                         | Amplify promoter P_{ylbP}                        |
| yqfD-F     | ttgcagaagggtgcTTGACCCCTAGCATGACCTAT                      | Amplify promoter P_{yqfD}                        |
| yqfD-R     | ttatctgtttttCTTATAGGATGATTGATGATTG                      | Amplify promoter P_{yqfD}                        |
| mmg-F      | ttgcagaagggtgcTTGACCCCTAGCATGACCTAT                      | Amplify promoter P_{mmgF}                        |
| mmg-R      | ttatctgtttttCTTTATAGGATGATTGATG                         | Amplify promoter P_{mmgR}                        |