RESEARCH ARTICLE

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 focuses 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, P_{spoVG}, and P_{yvyD} was 3.5, 2.8, and 2.3 times higher than that of the wild stain *Bacillus subtilis* TD7.

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

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Received: May 19, 2021; **Accepted:** July 22, 2021; **Published Online:** August 27, 2021

Citation: Liu, F.-F., Liu, Y.-F., Qiao, Y.-W., Guo, Y.-Z., Kuang, F.-Y., Lin, X.-Q., Ye, J., Liu, J.-F., Yang, S.-Z., Zhang, H.-Z., Sand, W., Mu, B.-Z., 2021. Improvement surfactin production by substitution of promoters in *Bacillus subtilis* TD7. *Applied Environmental Biotechnology*, 6(1): 31-41. http://doi.org/10.26789/AEB.2021.01.004

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 excessed 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 fulfil 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_{spac} and Pg3 and constitutive promoters such as P_{veg} and P_{repU}. (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_{spoVG}, and P_{yvyD}), III (P_{lytR}, P_{ylbP}, and P_{sigX}), and IV (P_{mmgA} and P_{yqfD}) 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 seleted 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.

Table 1. Strains with various types of promoters used in this study

Strains	Promoter	Promoter type	Sources
E. coli JM83	Used for plasmid construction		Stored in lab
B. subtilis TD7	Patent strain		Stored in lab
B. subtilis TP1	P43 (designed pattern)	Classes II	This study
B. subtilis TP1-2	P43 (contrastive pattern)	Classes II	This study
B. subtilis TP2	P_{spoVG}	Classes II	This study
B. subtilis TP3	P_{yvyD}	Classes II	This study
B. subtilis TP4	P_{sigX}	Classes III	This study
B. subtilis TP5	P_{lytR}	Classes III	This study
B. subtilis TP6	P_{ylbP}	Classes III	This study
B. subtilis TP7	P_{yqfD}	Classes IV	This study
B. subtilis TP8	P_{mmgA}	Classes IV	This study

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 $NaNO_3$, 0.333 g/L KH_2PO_4, 1 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 0.15 g/L $MgSO_4 \cdot 7\ H_2O$, 7.5 mg/L $CaCl_2$, 6 mg/L $MnSO_4 \cdot H_2O$, and 6 mg/L $FeSO_4 \cdot 7\ H_2O$ (pH 7.0) (Jiao et al., 2017). Spizizen medium was used to prepare *B. subtilis* competent cells (Anagnostopoulos 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 Disiai 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

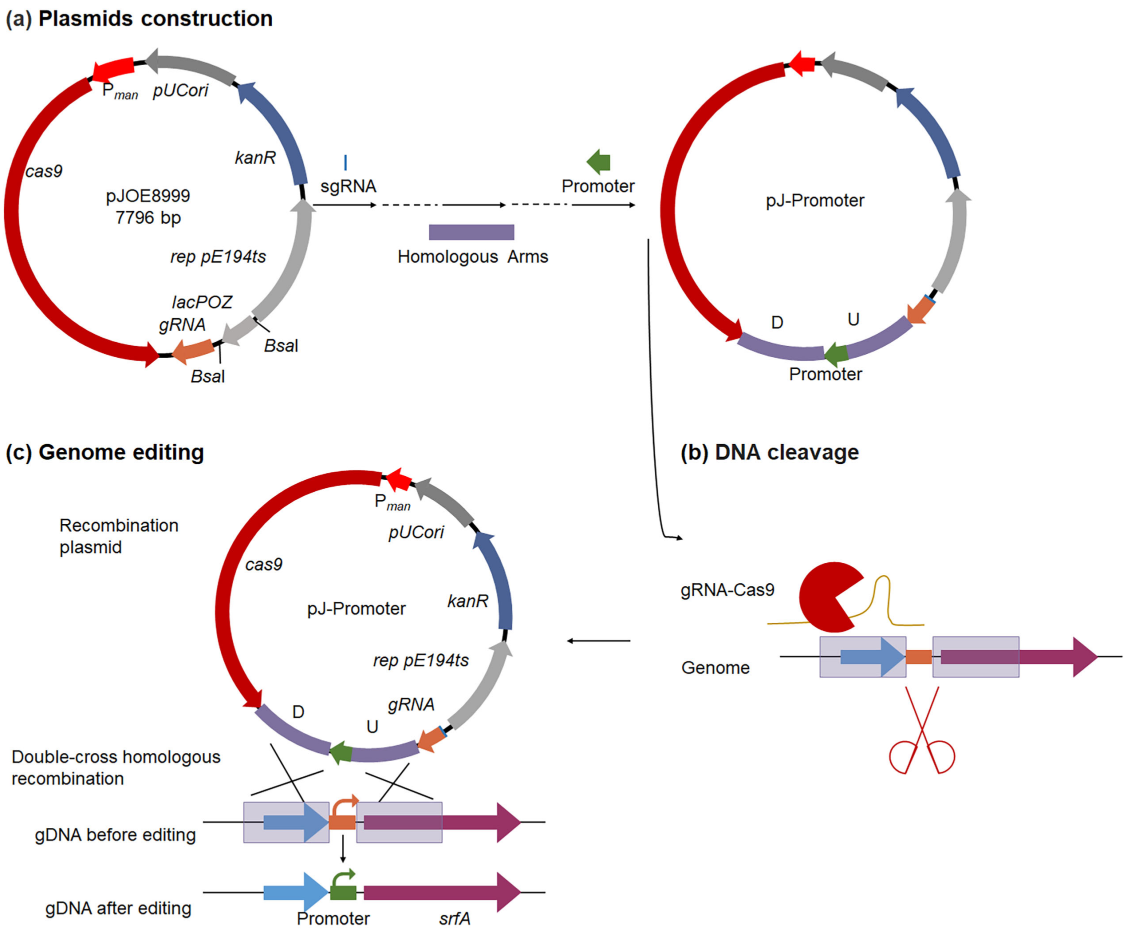

Figure 1. Plasmid construction. (a) Four fragments including sgRNA, the upstream and downstream homologous arms of the P_{srfA} and new promoters were inserted into the CRISPR-Cas9 plasmid. (b) gRNA guids Cas9 to cleave the target regain in genome by generating base-pairing. (c) Gene replacement by homologous 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 *Sfi*I sites to construct pJ-sgP$_{srfA}$. In the second step, we designed primers pU-F/R, and pD-F/R. Then use *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 *XbaI* site of the plasmid pJ-sgP$_{srfA}$to produce the vector pJ-UD$_{srfA}$. In the next step, those target promoters were amplified from *B. subtilis* 168 genomes and cloned into the pJ-UD$_{srfA}$at *XbaI* site. Finally, we obtained a set of recombinant plasmids.

2.3 Strain construction

Genetic recombination was performed as described by Altenbuchner (Altenbuchner, 2016). Competent *B. subtilis* TD7 cells were prepared with Spizizen medium (Anagnostopoulos and Spizizen, 1961). Fresh *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 *cas*9 expression under the control of P_{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-

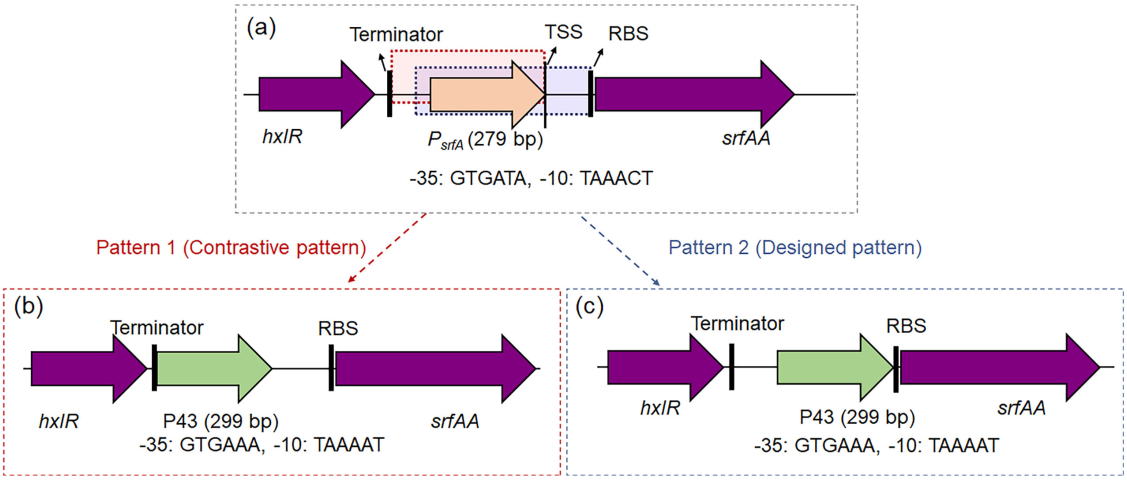

Figure 2. Schematic diagram of the upstream and downstream of the P_{srfA} in *B. subtilis* wild-type and recombined strains. (a) Original composition of the native *srfA* operon. (b) Promoter region composition of the strain, whose P_{srfA} was replaced by P43 with conserving the bases between RBS and TSS. (c) The promoter region composition of the strain of P_{srfA} replaced by P43 with the bases between RBS and TSS deleted and bases between the promoter and the *hxlR* terminator conserved.

tion by colony PCR and sequencing. These positive strains were then placed on LB plates without antibiotics using a toothpick and cultivated for overnight at 50 °C. One positive colony with successful promoter exchange was streaked on a LB plate and incubated for 12 h at 42 °C. All colonies were cultivated for 12 h on two LB plates (with and without kanamycin) at 42 °C. The colonies, which thrived only on LB plates without kanamycin, were evaluated for plasmid deletion using colony PCR.

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 (OD_{600}) 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 C_{18} 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-$P43_1$ and pJ-$P43_2$ with the same promoter but different homologous arms for genome editing were obtained. Using these plasmids, we obtained two recombinant strains whose original promoter P_{srfA} 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 P_{srfA} were knocked out. In contrary, *B. subtilis* TP1-2 retained the sequences between the upstream terminator and P_{srfA}, 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, 1.49.81, and 1050.02 (Figure

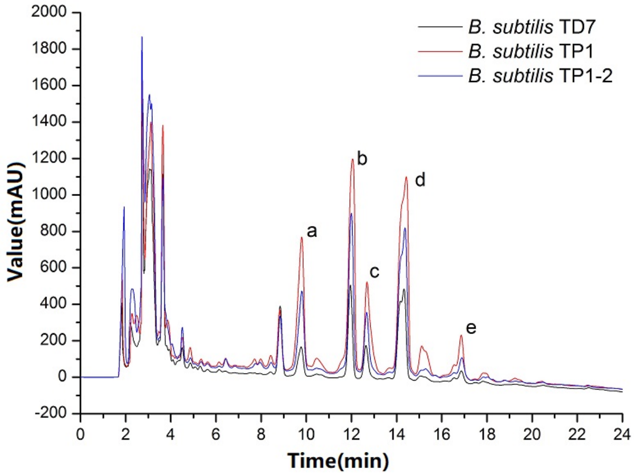

Figure 3. HPLC detection of Surfactin produced by the wild strain *B. subtilis* TD7 and the recombined strains *B. subtilis* TP1 after cultured at 37 °C for 72 h. A: surfactin C13, B: surfactin iso-C14, C: surfactin C14, D: surfactin C15, E: surfactin C16.

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

Component	Relative MW (M/Z)	Relation proportion (%)		
		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

S1). Since surfactin isoforms of C13 up to C16 consituted the major fraction of products for all strains (Figure 3), the total amount of these compounds was used as a measure of surfaction productivity (Figure 4). In Table 2, the proportion of different isoforms given. The engineered starins 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 date, the newly designed promoter installation site (Figure 2(b)) was used for subsquent 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.

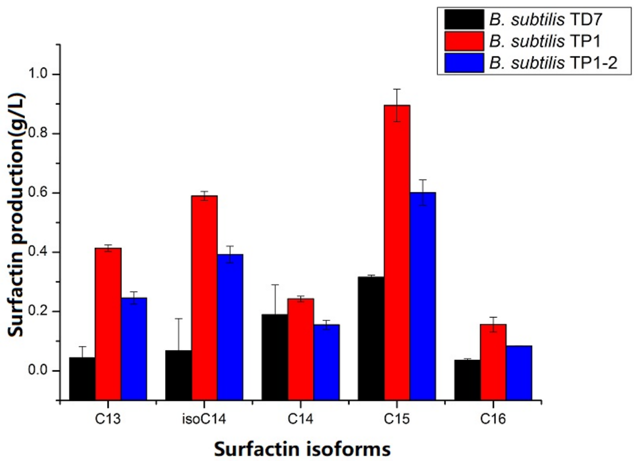

Figure 4. Surfactin isoforms ratio of the wild strain and recombined strains *B. subtilis* TP1 and *B. subtilis* TP1-2.

4 Discussion

Surfactin, an important lipopeptide-type biosurfactant, has good surface activity and some specific biological activities. However, the 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

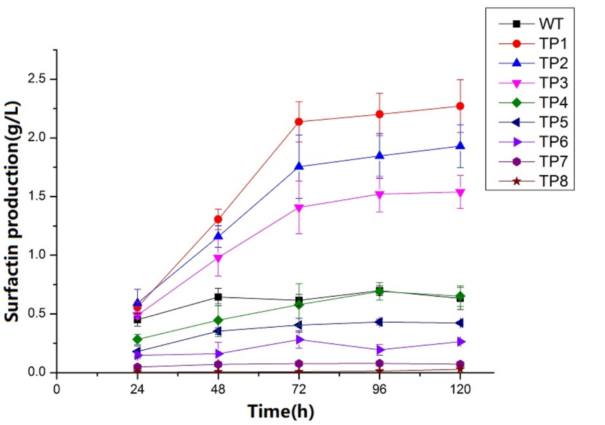

Figure 5. Surfactin production curve of the wild strain and promoter modified strains during fermentation for 5 days at 37 °C and 200 rpm.

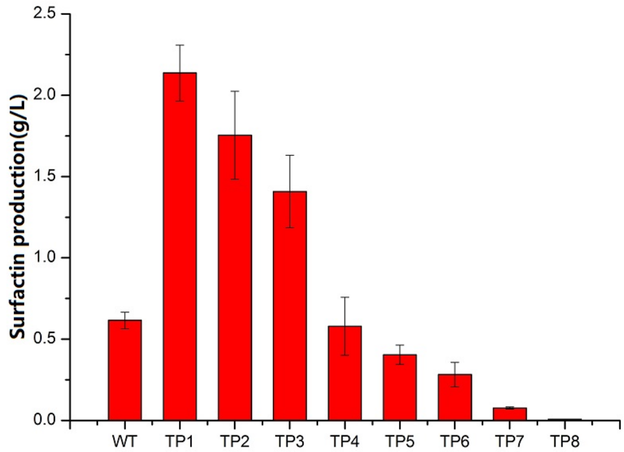

Figure 6. Surfactin yields of the wild strain and the modified strains after 72h culture.

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 inducation 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_{ylbP}, 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.

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.

Funding Sources

This work was supported by the Shanghai International Collaboration Program (18230743300), and “the Fundamental Research Funds for the Central Universitie” (No. JKJ012016028 and 50321102017017).

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 peoformed 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, Xiu-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.

References

Altenbuchner, J., 2016. Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 System. *Applied and Environmental Microbiology*, 82(17): 5421-5427.
https://doi.org/10.1128/AEM.01453-16

Anagnostopoulos, C. and Spizizen, J., 1961. Requirements for transformation in *Bacillus subtilis*. *Journal of bacteriology*, 81(5): 741-746.

Bezza, F.A. and Chirwa, E.M.N., 2017a. Pyrene biodegradation enhancement potential of lipopeptide biosurfactant produced by Paenibacillus dendritiformis CN5 strain. *Journal of Hazardous Materials*, 321: 218-227.
https://doi.org/10.1016/j.jhazmat.2016.08.035

Bezza, F.A. and Chirwa, E.M.N., 2017b. The role of lipopeptide biosurfactant on microbial remediation of aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil. *Chemical Engineering Journal*, 309: 563-576.
https://doi.org/10.1016/j.cej.2016.10.055

Chen, W.C., Juang, R.S. and Wei, Y.H., 2015. Applications of a lipopeptide biosurfactant, surfactin, produced by microorganisms. *Biochemical Engineering Journal*, 103: 158-169.
https://doi.org/10.1016/j.bej.2015.07.009

Coutte, F., Leclère, V., Béchet, M., Guez, J.-S., Lecouturier, D., Chollet-Imbert, M., Dhulster, P., Jacques, P., 2010. Effect of pps disruption and constitutive expression of *srfA* on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives. *Journal of Applied Microbiology*, 109(2): 480-491.
https://doi.org/10.1111/j.1365-2672.2010.04683.x

Coutte, F., Niehren, J., Dhali, D., John, M. and Jacques, P., 2015. Modeling leucine's metabolic pathway and knockout prediction improving the production of surfactin, a biosurfactant from *Bacillus subtilis*. *Biotechnology Journal*, 10(8): 1216-1234.
https://doi.org/10.1002/biot.201400541

Dhali, D., Coutte, F., Arias, A.A., Auger, S., Bidnenko, V., Chataigné, G., Lalk, M., Niehren, J., DeSousa, J., Versari, C., 2017. Genetic engineering of the branched fatty acid metabolic pathway of *Bacillus subtilis* for the overproduction of surfactin C-14 isoform. *Biotechnology Journal*, 12(7).
https://doi.org/10.1002/biot.201600574

Fernandes, P.E., Sao, J., Zerdas ERMA, Andrade, N.J., Fernandes, C.M., Silva, L.D., 2014. Influence of the hydrophobicity and surface roughness of mangoes and tomatoes on the adhesion of Salmonella enterica serovar Typhimurium and evaluation of cleaning procedures using surfactin. *Food Control*, 41: 21-26.
https://doi.org/10.1016/j.foodcont.2013.12.024

Gao, L., Han, J., Liu, H., Qu, X., Lu, Z., Bie, X., 2017. Plipastatin and surfactin coproduction by *Bacillus subtilis* pB2-L and their effects on microorganisms. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 110(8): 1007-1018.
https://doi.org/10.1007/s10482-017-0874-y

Geetha, S.J., Banat, I.M. and Joshi, S.J., 2018. Biosurfactants: Production and potential applications in microbial enhanced oil recovery (MEOR). *Biocatalysis and Agricultural Biotechnology*, 14: 23-32.
https://doi.org/10.1016/j.bcab.2018.01.010

Guan, C., Cui W., Cheng, J., Zhou, L., Guo, J., Hu, X., Xiao, G., Zhou, Z., 2015. Construction and development of an auto-regulatory gene expression system in *Bacillus subtilis*. *Microbial Cell Factories*, 14.
https://doi.org/10.1186/s12934-015-0341-2

Gudina, E.J., Rangarajan, V., Sen, R., Rodrigues, L.R., 2013. Potential therapeutic applications of biosurfactants. *Trends in Pharmacological Sciences*, 34(12): 667-675.
https://doi.org/10.1016/j.tips.2013.10.002

Heckman, K.L. and Pease, L.R., 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nature Protocols*, 2(4): 924-932.
https://doi.org/10.1038/nprot.2007.132

Hu, F., Liu, Y. and Li, S., 2019. Rational strain improvement for surfactin production: enhancing the yield and generating novel structures. *Microbial Cell Factories*, 18(1).
https://doi.org/10.1186/s12934-019-1089-x

Jiao, S., Li, X., Yu, H., Yang, H., Li, X., Shen, Z., 2017. In situ enhancement of surfactin biosynthesis in *Bacillus subtilis* using novel artificial inducible promoters. *Biotechnology and Bioengineering*, 114(4): 832-842.
https://doi.org/10.1002/bit.26197

Jimoh, A.A. and Lin, J., 2019. Biosurfactant: A new frontier for greener technology and environmental sustainability. *Ecotox Environ Safe*, 184.
https://doi.org/10.1016/j.ecoenv.2019.109607

Jung, J., Yu, K.O., Ramzi, A.B., Choe, S.H., Kim, S.W., Han, S.O., 2012. Improvement of surfactin production in *Bacillus subtilis* using synthetic wastewater by overexpression of specific extracellular signaling peptides, comX and phrC. *Biotechnology and Bioengineering*, 109(9): 2349-2356.
https://doi.org/10.1002/bit.24524

Kang, X.M., Cai, X., Huang, Z.H., Liu, Z.Q., Zheng, Y.G., 2020. Construction of a highly active secretory expression system in *Bacillus subtilis* of a recombinant amidase by promoter and signal peptide engineering. *International Journal of Biological Macromolecules*, 143: 833-841.
https://doi.org/10.1016/j.ijbiomac.2019.09.144

Li, X., Yang, H., Zhang, D., Xue, L., Shen, Z., 2015. Overexpression of specific proton motive force-dependent transporters facilitate the export of surfactin in *Bacillus subtilis*. *Journal of Industrial Microbiology & Biotechnology*, 42(1): 93-103.
https://doi.org/10.1007/s10295-014-1527-z

Liu, D., Mao, Z., Guo, J., Wei, L., Ma, H., Tang, Y.J., Tao, C., Wang, Z., Zhao, X., 2018. Construction, model-based analysis, and characterization of a promoter library for fine-tuned gene expression in *Bacillus subtilis*. *Acs Synthetic Biology*, 7(7): 1785-1797.
https://doi.org/10.1021/acssynbio.8b00115

Liu, J., Li, W., Zhu, X., Zhao, H., Lu, Y., Zhang, C., Lu, C., 2019. Surfactin effectively inhibits Staphylococcus aureus adhesion and biofilm formation on surfaces. *Applied Microbiology and Biotechnology*, 103(11): 4565-4574.
https://doi.org/10.1007/s00253-019-09808-w

Liu, J.F., Yang, J., Yang, S.Z., Ye, R.Q., Mu, B.Z., 2012. Effects of different amino acids in culture media on surfactin variants produced by *Bacillus subtilis* TD7. *Applied Biochemistry and Biotechnology*, 166(8): 2091-2100.
https://doi.org/10.1007/s12010-012-9636-5

Marahiel, M.A., 2016. A structural model for multimodular NRPS assembly lines. *Natural Product Reports*, 33(2): 136-140.
https://doi.org/10.1039/c5np00082c

Muthusamy, K., Gopalakrishnan, S., Ravi, T.K., Sivachidambaram, P., 2008. Biosurfactants: properties, commercial production and application. *Current Science*, 94(6): 736-747.

Nakano, M.M., Marahiel, M.A. and Zuber, P., 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *Journal of bacteriology*, 170(12): 5662-5668.
https://doi.org/10.1128/jb.170.12.5662-5668.1988

Nitschke, M. and Silva, S.S.E., 2018. Recent food applications of microbial surfactants. *Critical Reviews in Food Science and Nutrition*, 58(4): 631-638.
https://doi.org/10.1080/10408398.2016.1208635

Penha, R.O., Vandenberghe, L.P.S., Faulds, C., Soccol, V.T., Soccol, C.R., 2020. Bacillus lipopeptides as powerful pest control agents for a more sustainable and healthy agriculture: recent studies and innovations. *Planta*, 251(3).
https://doi.org/10.1007/s00425-020-03357-7

Pereira, J.F.B., Gudina, E.J., Costa, R., Vitorino, R., Teixeira, J.A., Coutinho, J.A.P., Rodrigues, L.R., 2013. Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. *Fuel*, 111: 259-268.
https://doi.org/10.1016/j.fuel.2013.04.040

Peypoux, F., Bonmatin, J.M. and Wallach, J., 1999. Recent trends in the biochemistry of surfactin. *Applied Microbiology and Biotechnology*, 51(5): 553-563.
https://doi.org/10.1007/s002530051432

Rebello, S., Aneesh, E.M., Sindhu, R., Binod, P., Pandey, A., 2018. Biosynthesis and technological advancements of biosurfactants. *Energy Environment and Sustainability*, 167-183.
https://doi.org/10.1007/978-981-10-7434-9_10

Rodrigues, L., Banat, I.M., Teixeira, J., Oliveira, R., 2006. Biosurfactants: potential applications in medicine. *Journal of Antimicrobial Chemotherapy*, 57(4): 609-618.
https://doi.org/10.1093/jac/dkl024

Roongsawang, N., Washio, K. and Morikawa, M., 2011. Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. *International Journal of Molecular Sciences*, 12(1): 141-172.
https://doi.org/10.3390/ijms12010141

Sen, R., 2010. Surfactin: biosynthesis, genetics and potential applications. *Advances in Experimental Medicine and Biology*, 672: 316-323.

Seydlova, G. and Svobodova, J., 2008. Review of surfactin chemical properties and the potential biomedical applications. *Central European Journal of Medicine*, 3(2):123-133.
https://doi.org/10.2478/s11536-008-0002-5

Song, Y., Nikoloff, J.M., Fu, G., Chen, J., Li, Q., Xie, N., Ping, Z., Sun, J., Zhang, D., Mark, I., 2016. Promoter screening from *Bacillus subtilis* in various conditions hunting for synthetic biology and industrial applications. *Plos One*, 11(7).
https://doi.org/10.1371/journal.pone.0158447

Sun, H., Bie, X., Lu, F., Lu, Y., Wu, Y., Lu, Z., 2009. Enhancement of surfactin production of *Bacillus subtilis* fmbR by replacement of the native promoter with the P*spac* promoter. *Canadian Journal of Microbiology*, 55(8): 1003-1006.
https://doi.org/10.1139/W09-044

Wang, C., Cao, Y., Wang, Y., Sun, L., Song, H., 2019. Enhancing surfactin production by using systematic CRISPRi repression to screen amino acid biosynthesis genes in *Bacillus subtilis*. *Microbial Cell Factories*, 18(1):90.
https://doi.org/10.1186/s12934-019-1139-4

Willenbacher, J., Mohr, T., Henkel, M., Gebhard, S., Mascher, T., Syldatk, C., Hausmann, R., 2016. Substitution of the native *srfA* promoter by constitutive P-veg in two *B. subtilis* strains and evaluation of the effect on surfactin production. *Journal of Biotechnology*, 224: 14-17.
https://doi.org/10.1016/j.jbiotec.2016.03.002

Wu, Q., Zhi, Y., Xu, Y., 2019. Systematically engineering the biosynthesis of a green biosurfactant surfactin by *Bacillus subtilis* 168. *Metabolic Engineering*, 52: 87-97.
https://doi.org/10.1016/j.ymben.2018.11.004

Yang, S., Du, G.C., Chen, J., Zhen, K,, 2017. Characterization and application of endogenous phase-dependent promoters in *Bacillus subtilis*. *Applied Microbiology and Biotechnology*, 101(10): 4151-4161.
https://doi.org/10.1007/s00253-017-8142-7

Yang, Y., Wu, H.J., Lin, L., Zhu, Q.-Q., Borriss, R., 2015. A plasmid-born Rap-Phr system regulates surfactin production, sporulation and genetic competence in the heterologous host, *Bacillus subtilis* OKB105. *Applied Microbiology and Biotechnology*, 99(17): 7241-7252.
https://doi.org/10.1007/s00253-015-6604-3

Yoneda, T., Tsuzuki, T., Ogata, E., Fusyo, Y., 2001. Surfactin sodium salt: an excellent bio-surfactant for cosmetics. *Journal of Cosmetic Science*, 52(2): 153-154.

Yu X., Xu J., Liu X., Chu, X., Wang, P., Tian, J., Wu, N., Fan, Y., 2015. Identification of a highly efficient stationary phase promoter in *Bacillus subtilis*. *Scientific Reports*, 5.
https://doi.org/10.1038/srep18405

Supplymentary Material

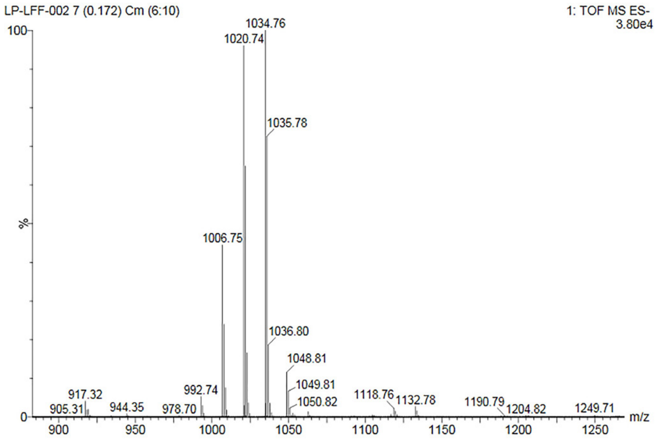

Figure S1. ESI-MS of surfactin production from Bacillus subtilis TD7.

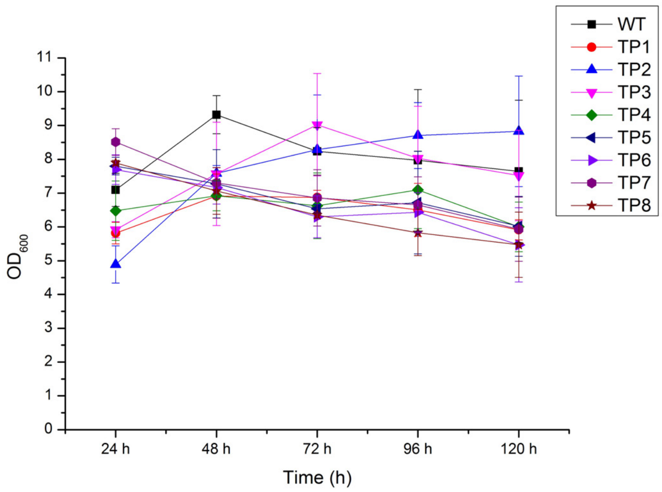

Figure S2. Growth curve of the wild-type strin and different mutants during cultivated for 5 days at 37 °C and 200 rpm.

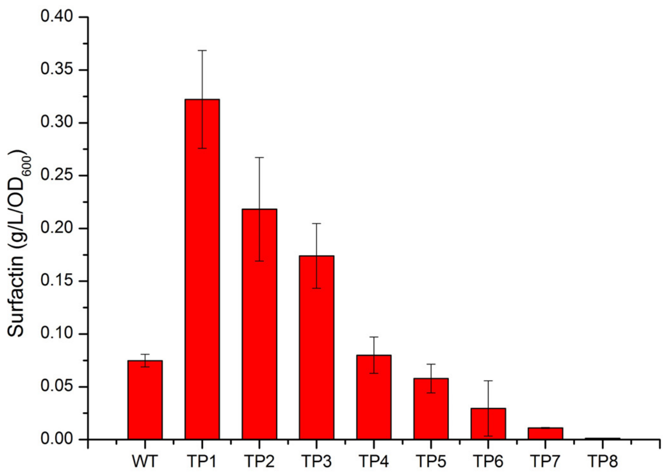

Figure S3. Surfactin yield per OD_{600} ratio of the wild-type strin and different mutants.

Table S1. Mutant surfactin producers by promoter exchange

Strains	Promoters	Production in shake flask	Reference
B. subtilis fmbR-1	P_{spac}	3.86 g/L (IPTG)	Sun et al., 2009
B. subtilis BBG113	P_{repU}	1.47 g/g biomass	Coutte et al., 2010
B. subtilis 3A38	P_{veg}	0.26 g/L	Willenbacher et al., 2016
B. subtilis THY15/Pg3 THY15/Pg3	Pg3	8.61 g/L (IPTG)	Jiao et al., 2017

Table S2. Promoter sequence

Promoters	Sequence (5'-3')
P43	attgagtggatgattatattcctttgataggtggtatgttttcgcttgaacttttaaatacagccattgaacatacggttgatttaataactgacaaacatcaccctcttgctaaagcggccaaggacgctgccgccggggctgtttgcgtttttgccgtgatttcgtgtatcattggtttacttatttttttgccaaagctgtaatggctgaaaattcttacatttattttacatttttagaaatgggcgtgaaaaaaagcgcgcgattatgtaaaatataaagtgatagcggtacc
P_{spoVG}	tgcggaagtaaacgaagtgtacggacaatattttgacactcacaaaccggcgagatcttgtgttgaagtcgcgagactcccgaaggatgcgttagtcgagatcgaagttattgcactggtgaaataataagaaaagtgattctgggagagccgggatcactttttttatttaccttatgcccgaaatgaaagctttatgacctaattgtgtaactatatcctatttttttcaaaaaatattttaaaaacgagcaggatttcagaaaaaatcgtggaattgatacactaatgcttttatatag
P_{yvyD}	gatcaattggtctctttctcttttccctctcatgagttctgtgagtatttaaaggaacattttctgattcattatagaaaatggatgctgtctattcatcaatgtatggaacccttttttaatcaattaggcgtgtgtgaggtatttgtttcgttcaatcagcatatacatatacctccgaaccgccaataacagagcaaatacaaacaaaattcgacaaagttcactgaattttcacaaaagatttatgtttcagcaggaattgtaaagggtaaaagagaaatagatacatatccttaa
P_{sigX}	ggaagcccacaacggatcaattactgtgcacagccgaatagataaaggaacaacattttctttttatattccgacaaaacggtaaaatcgagtctgaatttgccgaagaatcttgttccataagaaacacccgctgactgagcgggtgtttttttaatagccaacattaataaaatttaaggatatgttaatataaattcccttccaaattccagttactcgtaatatagttgtaatgtaacttttcaagctattcatacgac
P_{lytR}	gctaaccctacataagtaccttcttttgtttcaatgttactgtctggcgatacatcttcaccttgactcttttgactattaaccccgcaacccgaaagaagcaatataaagaacagtaaagcaataaatttttttcatttttttcacctcattatattttatcgtcaacctattttatattttaaagaaaaattaagaaacaatgaaactttttttttataaaaaacgactattttaggatttcattcttgtattaaatagagttgtatttattggaaatttaactcataatgaaagtaattt
P_{ylbP}	caagcaggtcaaagtccgaattgatactcgtgtccgcactgcaatcagccctgaattcctccccgcctttataaagccggattccttcagactgaatggccgcagcctgttcttgccgcctcgtcacaacagtcacgtcgtgataaagtgacaaataataggcgcataaaagaccaacggagcctccgccgataattccaattttcatgatgtcacacccaatttagcatttacgtattatcatagcagaagtaagaagaaattacttctcaaagatcccatgtgcttaaaattaaagtttaaatatttggatttttttaaataaagcgtttacaatatatgtagaaac
P_{yqfD}	gatgcccctgcacctatccctaaccgtatggaacaggcaagacgggaagcggaagaaagacgcagggaaacagcaagaaacctgaaagggctggaacgagatcttgctgctgccaaacaaaaaacagtatacacaaaacaaaaaatgcttcaggtgaataaagacaccgtcgtacaggggatcgttctaggagaggtgttcggacctccacgggcgaaaaaacctcaccgtacgatgcgcccggcccgtaaaaattaaagtgttagaacctcctttcaaatcatacatatgagatgaaag
P_{mmgA}	tgcaccgcatatcgaacgggcagtattaacttcagatgtgctttatcaggcagatcgatatatcgcgtctattccggcttccggctatcacccgaagataaacagcccaggggtcacagatgaagtactgaagaaaatgaggaacggtttgattaaggtaaggccgtatacagtcaatcgtccggaagatatgaagcgtctcattgaagcgggagcagacggcatgtttaccgactttccagaaaaggcttcggcattgctgaaaaatgaatagttgttagaaggaggctgtttgacgcagccttctttttcattcattcatgcccgtttcaaagcatacattcatagaagac

Table S3. Primers sequence

Primer	Sequence (5' - 3')	Descriptation
pN20-F	tacgTTTCTGTAAATAATGTTTAG	Manufacture sgRNA
pN20-R	aaacCTAAACATTATTTACAGAAA	
pU-1F	gccaataaggcctttACGCTTTCATAATTTCGTAG	Amplify upsteam arm for pattern 1
pU-1R	gctctagagcAGACACCCTTGCGAAGAG	
pD-1F	ttcgcaagggtgtctgctctagagcGAAAACAATGAATAAATAGCCA	Amplify upsteam arm for pattern 1
pD- 1R	agattatttcttaatTCGATAAATGAATGCGAGAT	
pU-2F	cccgggccaataaggcctttCTTTAATCGTTGCGTCGTCT	Amplify upsteam arm for pattern 2
pU-2R	gctctagagcTCATTTCCACTAAACATTATTTAC	
pD-2F	gtttagtggaaatgagctctagagcTATGGAAATAACTTTTTACCCT	Amplify downstream arm for pattern 2
pD- 2R	gatgaagattatttcttaatTTTCCCAGTATCCCATCG	
P43-1F	ttcgcaagggtgtctATTGAGTGGATGATTATATTCC	Amplify promoter P43 for pattern 2
P43-1R	ttattcattgttttcGGTACCGCTATCACTTTATAT	
P43-2F	gtttagtggaaatgaATTGAGTGGATGATTATATTCC	Amplify promoter P43 for pattern 2
P43-2R	aaagttatttccatattgtcatacctcccctaatGGTACCGCTATCACTTTATAT	
spoVG-F	ttcgcaagggtgtctTGCGGAAGTAAACGAAGT	Amplify promoter P_{spoVG}
spoVG-R	ttattcattgttttcCTATATAAAAGCATTAGTGTATCAA	
yvyD-F	ttcgcaagggtgtctTGATCAATTGGTCTCTTTCTC	Amplify promoter P_{yvyD}
yvyD-R	ttattcattgttttcTTAAGGATATGTATCTATTTCTCTTT	
sigX-F	ttcgcaagggtgtctGGAAGCCCACAACGGATC	Amplify promoter P_{sigX}
sigX-R	ttattcattgttttcGTCGTATGAATAGCTTGAAAAGTT	
lytR-F	ttcgcaagggtgtctGCTAACCCTACATAAGTACCTTC	Amplify promoter P_{lytR}
lytR-R	ttattcattgttttcAAATTACTTTCATTATGAGTTAA	
ylbP-F	ttcgcaagggtgtctCAAGCAGGTCAAAGTCCG	Amplify promoter P_{ylbP}
ylbP-R	ttattcattgttttcGTTTCTACATATATTGTAAACGCTT	
yqfD-F	ttcgcaagggtgtctGATGCCCCTGCACCTAT	Amplify promoter P_{yqfD}
yqfD-R	ttattcattgttttcCTTTCATCTCATATGTATGATTTG	
mmg-F	ttcgcaagggtgtctTTGCACCGCATATCGAAC	Amplify promoter P_{mmgA}
mmg-R	ttattcattgttttcGTCTTCTATGAATGTATGCTTTG	