PNAS
www.pnas.org

Supplementary Information for

An operator-based expression toolkit for *Bacillus subtilis* enables fine-tuning of gene expression and biosynthetic pathway regulation

Gang Fu[a], Jie Yue[a], Dandan Li[a], Yixin Li[a], Sang Yup Lee[c] *, Dawei Zhang[a,b] *

[a]Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, 300308, P.R. China
[b]University of Chinese Academy of Sciences, Beijing, 100049, P.R. China.
[c]Metabolic and Biomolecular Engineering National Research Laboratory, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea.

Sang Yup Lee, Dawei Zhang.
Email: leesy@kaist.ac.kr, zhang_dw@tib.cas.cn

This PDF file includes:

Other supplementary materials for this manuscript include the following:

Datasets S1 to S28

SI Appendix Materials and Methods

Strains and growth conditions

Strains used or constructed in this study are listed in Table S1. The *E. coli* DH5α (Invitrogen Life Technologies, U.S.A.) strain was used as a cloning host for plasmid construction. *B. subtilis* 1A751 (BGSC, U.S.A.) was used for library construction, protein production and other tests. All characterization experiments were carried out using Luria-Bertani (LB) broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) at 37°C with constant shaking at 200 rpm. To obtain protein expression data, *B. subtilis* 1A751 was grown in super-rich (SR) medium (25 g/L yeast extract, 15 g/L tryptone, 3 g/L K_2HPO_4; pH 7.2) at 37°C with shaking at 220 rpm. As appropriate, antibiotics (50 μg/mL kanamycin and 5 μg/mL chloramphenicol) were added in the medium. To transform plasmids into *B. subtilis*, cells were prepared by a two-step transformation method described by Spizizen(1). For the protein expression test, a single colony from a freshly transformed plate was used to inoculate 5 mL of LB medium as a seed culture supplemented with appropriate antibiotics and grown at 37°C with constant shaking at 220 rpm. The overnight cultures were diluted to 1% in a 250-mL flask containing 25 mL of fresh prewarmed SR medium with the appropriate antibiotics. When required, 1% (w/v) maltose was added to the medium immediately after inoculation. The flasks were incubated at 37°C with shaking (220 rpm) in a shaker incubator (NB-205, N-BIOTEK).

Construction of plasmids

Recombinant plasmids constructed in this study are listed in Table S2. Heterologous DNA elements or genes that used for optimization and validation of MATE system are listed in Table S3. Primers used for construction of plasmids were synthesized by GENEWIZ (Suzhou, China) and are listed in Table S4. The promoter-probe plasmid pDG was designed to fuse a promoter sequence of choice to the green fluorescence reporter gene *gfpmut2* and to integrate the fusion into the *B. subtilis amyE* locus(2). To identify the *malO* operator sequence of the *mal* operon promoter, the P_{malA} region (-226 to +25 relative to the transcriptional initiation site) was amplified with primers P_{malA}-F and P_{malA}-R and then cloned between the *BamHI* and *EcoRI* sites of the pDG plasmid to generate pDG-P_{malA}. Truncated mutants of the P_{malA} promoter were constructed based on pDG-P_{malA} by inverted PCR, where consecutive 18-bp sequence fragments between the -226 and -35 regions of the promoter were gradually excluded one at a time with each newly generated mutant. The linearized plasmid was amplified and then phosphorylated and self-ligated, generating 15 plasmids named pDG-P_{malA}-M1 to pDG-P_{malA}-M15. These plasmids were further used to determine the potential sequence of the *malO* operon in P_{malA}. To further explore the importance of different regions of the *malO* operator, each region of the operator was replaced by its complementary sequence by inverted PCR with the primer pairs *malO*-up-F/*malO*-up-R, *malO*-IR1-F/*malO*-IR1-R, *malO*-IR2-F/*malO*-IR2-R, *malO*-Spacer-F/*malO*-Spacer-R, and *malO*-F/*malO*-R, resulting in the plasmids pDG-P_{malA}-Δup, pDG-P_{malA}-ΔIR1, pDG-P_{malA}-ΔIR2, pDG-P_{malA}-ΔSpacer, and pDG-P_{malA}-Δ*malO*, respectively. For promoter variants with tandem *malO* operators, inverted PCR was performed using the plasmid pDG-P_{malA} as a template with the primer pairs *malO*2-F/*malO*2-R, *malO*4-F/*malO*4-R, *malO*6-F/*malO*6-R, *malO*2d11-F/*malO*2d11-R, *malO*2d16-F/*malO*2d16-R, *malO*2d21-F/*malO*2d21-R, *malO*2d26-F/*malO*2d26-R, *malO*2d31-F/*malO*2d31-R and *malO*2d36-F/*malO*2d36-R to generate the plasmids pDG- -O2, pDG- O4, pDG-O6, pDG-D11, pDG-D16, pDG-D21, pDG-D26, pDG-D31 and pDG-D36, respectively. To test the maltose-switchable activation and repression of cell division in *B. subtilis*, the *divIVA* gene was amplified with the primers divIVA-F/divIVA-R and used to replace the *gfpmut2* gene of the pDG-P_{malA} plasmid under the control of P_{malA}-Δcre3, generating the plasmids pDivON *and* pDivOff. For metabolite valve regulation tests in riboflavin or violacein production, the *rib* operon (*ribDEAHT*) was amplified from genome DNA and cloned into pMATE15-Δcre3 by primers Rib-F/Rib-R/ pRib-VF/pRib-VR, generating pMATE- RibOn. The original promoter of the *ribC* gene was replaced by P_{malA}-g1M2 via an AraR-based genome-editing method(3). The violacein biosynthesis pathway genes *vioA*, *vioB*, *vioC*, *vioD* and *vioE,* from *Chromobacterium violaceum* were codon optimized and synthesized as an artificial operon *vioABCDE* by GENEWIZ (Suzhou, China). To construct the violacein expression vector pVio, the vio operon was amplified by primers Vio-F/Vio-R/and cloned into pMATE15-Δcre3 by primers pVio-VF/pVio-VR.

Promoter variants with an auxiliary *malO* operator were constructed based on the plasmid pDG-P_{malA} that carried the native P_{malA} promoter. The variants contained an auxiliary distal *malO*

operator upstream of the native *malO* with different spacing distances. For variants containing different numbers of auxiliary *malO* operators, the distance between each operator (center to center) was the same as the distance between the native *malO* and -35 region within the promoter. The auxiliary *malO* operator sequence was introduced by inverted PCR at various upstream positions. The plasmid pDG-P_{malA} from which variants were derived was used as a control in a GFP fluorescence test.

To achieve a higher expression level from the maltose-inducible expression system (MATE-ON), the genomic DNA of six strains that harbored the strongest P_{malA} promoter mutants was isolated and used as a PCR template. The expression cassette consisting of the P_{malA} promoter, *gfpmut2* reporter gene and *rrn* T1 terminator was amplified and cloned into the pUB110-derived, high-copy-number plasmid pMA5, generating the plasmids pMATE01, pMATE02, pMATE06, pMATE07, pMATE11, pMATE12, pMATE13, pMATE14 and pMATE15. To further improve the stringency of the MATE-ON system, a theophylline-inducible riboswitch was fused to the 3' terminus of the P_{malA} promoter with or without the original RBS of the P_{malA} promoter. The DNA fragment of the theophylline-inducible riboswitch was synthesized by GENEWIZ (Suzhou, China) and cloned into the plasmid pMATE06 via the multimer PCR cloning method, generating the plasmids pMATE15-TheoRS1 (with RBS from P_{malA}) and pMATE15-TheoRS2 (w/o RBS from P_{malA}).

Construction of operator mutant library

Site-directed mutagenesis was employed to generate a mutant library containing a large diversity of *malO* operator sequences in P_{malA} promoter mutants. To achieve fast construction of a high-capacity library from *B. subtilis*, a sequence-independent cloning method called "multimeric PCR cloning" was used(4). The plasmid pDG-P_{malA}, which contains the sequences of wild-type *malO* and reporter gene *gfpmut2*, was used as a template, and primers (*malO*-mut-F1/ *malO*-mut-F2/ *malO*-mut-R) harboring degenerated oligonucleotides at the *malO* site and flanked with 20-bp overhangs homologous to vector sequences were used to amplify the insert fragment. Additionally, the vector fragment was amplified with the primers vector-mut-F/vector-mut-R, which contained 3' and 5' 20-bp overhangs homologous to the insert sequence. The desired library was generated by fusing these DNA fragments at equal molar ratios, and the constructs were amplified via prolonged overlap extension PCR with Q5 polymerase (NEB, USA) for 40 cycles, consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 3 min. The products were used to transform competent *B. subtilis* cells directly.

High-throughput screening

For the efficient selection of P_{malA} promoter mutants with high levels of transcription, a fluorescence-activated cell sorting (FACS) method was employed to screen the GFP signals of different cells. After transforming the multimeric plasmid product into competent cells, over 1800 colonies were obtained in each LB agar plate containing 5 μg/mL chloramphenicol. The colonies from LB agar plates were collected and inoculated into 5 mL of fresh LB medium with 1% (w/v) maltose as an inducer and 5 μg/mL chloramphenicol. After cultivating at 37°C with constant shaking at 200 rpm, the overnight culture was collected by centrifuging at 4°C and 4000 rpm for 10 min, and then, the cells were diluted 200-fold and resuspended in 0.1 M (pH 6.0) phosphate-buffered saline (PBS). Aliquots (1 mL) of cells were injected into a Beckman Coulter flow cytometer (Beckman, USA) to analyze the fluorescence signal. Cells were excited using a 488 nm wavelength laser, and the forward scatter (FSC), side scatter (SSC) and green fluorescence (530/30 filter) were measured. Cells were gated from noise using an FSC versus SSC plot. *B. subtilis* cells transformed with the plasmids pDG and pDG-P_{malA} were used as negative and positive controls, respectively. A total of over 30000 cells were analyzed, and the top 1% of cells with the greatest GFP fluorescence signals were sorted and individually collected in separate wells of 96-well microtiter plates (Corning, USA) with fresh LB medium with 5 μg/mL chloramphenicol. Three rounds of screening were performed, and over 900 strains were collected and cultivated in a Microtron microplate shaker (INFOSE HT) at 37°C and 800 rpm for 16 h. These sorted cells were then evaluated by using them to inoculate 96-deep-well microplates containing 0.5 mL of fresh LB medium with or without 1% (w/v) maltose as an inducer. After cultivating for 20 h, the fluorescence signal (E_x 483 nm/E_m 507 nm) and biomass (OD_{600}) of cultures in microplates were determined by an MD M3 microplate

reader (Molecular Devices, USA). The fluorescence strength of different strains was normalized to cell growth (OD_{600}). The plasmid DNA from each strain that demonstrated the strongest levels of fluorescence was isolated and sequenced, and the sequences of the *malO* operator were then aligned with the wild-type operator through MEGA 5 software.

Genome manipulation

Primers used for markerless gene deletion and overexpression were synthesized by GENEWIZ (Suzhou, China) and are listed in Table S4. For markerless gene deletion, an AraR-based gene knockout method was employed(3). The 0.9-kb antibiotic (C) fragment, which contained the entire cassette of a chloramphenicol resistance gene, was amplified from the plasmid pDL. The 1.2-kb araR (R) fragment was amplified from *B. subtilis* 168 genome DNA. The upstream fragment (U) and downstream fragment (D) of the target genes (*malA*, *malA-Ter*, and *ptsG*) (G) for deletion were amplified from genomic DNA with the primers UP-F/UP-R and DN-F/DN-R, respectively. Then, the five fragments, in the order U-D-CR-G, were fused by overlap extension PCR(5) and transformed into *B. subtilis* competent cells. After screening with 5 μg/mL chloramphenicol, the cells were cultivated in LB medium without any antibiotic to stimulate genome self-recombination, followed by screening with 100 μg/mL spectinomycin to obtain the gene markerless deletion strains 1A751ΔmalA, 1A751ΔmalA-Ter, and 1A751ΔptsG. For the gene overexpression analysis, pDL. which integrated target genes into the *amyE* locus of *B. subtilis*, was used as a vector. The target genes (*malR, malP,* and *malR-malP*) were amplified from genomic DNA and cloned into a plasmid driven by the inducible promoter P_{malA}. The constructs were then linearized with PstI and integrated into the genome by double-crossover integration. Positive selection of integration was performed with 5 μg/mL chloramphenicol. Colony PCR was employed to verify partial integration, and the PCR products were then sequenced by GENEWIZ (Suzhou, China).

Homogeneity and reproducibility analysis

B. subtilis 1A751, 1A751ΔmalA-Ter and 1A751ΔmalA-Ter-RP, harboring the pMATE15-Δcre3 plasmid, were grown in LB medium until the early logarithmic phase for use as seed cultures. For our homogeneity analysis, cultures were inoculated into fresh LB medium and induced with 1% (w/v) maltose for 1 h, 2 h and 4 h. For the repeatability analysis, three randomly selected colonies from different strains were inoculated into fresh LB medium, and each was grown to the early logarithmic phase. Then, cultures were induced with 0.5%, 1% and 2% (w/v) maltose for 8 h. The induced cells were collected, washed and diluted 1/1000 in PBS. Flow cytometry was performed using a Beckman Coulter Cell Sorter (Beckman). GFP fluorescence was excited with a 488 nm laser, and emission was measured through an FL2 channel (525/50 nm). Data were recorded for 100000 cells per sample and analyzed using FlowJo V10 (FlowJo, LLC). Histograms of events versus GFP fluorescence were plotted and used to determine the mean fluorescence and coefficients of variation for the total population and gated subpopulations consisting of nonfluorescent and fluorescent cells. In the case of overlaid histograms, the smoothing function was applied at a factor of 2 to allow easy visualization of individual lines.

PAGE analysis

The expression level of different target proteins in host cell were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Cultured samples were harvested after 24 h of induction, and equivalent numbers of cells were harvested after centrifugation and resuspension in lysis buffer (150 mM NaCl, 50 mM Tris-HCl; pH 7.6). After adding an equal volume of 2x SDS-PAGE loading buffer (Solarbio, China), the fractions were incubated in a boiling water bath for 10 min and separated on a 12% NuPAGE Bis-Tris Gel (Invitrogen Life Technologies, USA) with MOPS SDS Running Buffer (Invitrogen Life Technologies, USA). The PageRuler Prestained Protein Ladder (Invitrogen Life Technologies) was used as a molecular mass marker covering the 10- to 180-kDa range. Following electrophoresis, proteins were visualized using Coomassie Brilliant Blue R-250 (Solarbio, China).

The oligomeric state of transcription MalR was analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE). The soluble protein fractions in the cytoplasm were solubilized in 5× non-reducing sample buffer, and the samples were loaded onto acrylamide native gels stained with

Coomassie blue. The running buffer and native PAGE gel were prepared as described previously (6). NativeMarker™ Unstained Protein Standard (Invitrogen Life Technologies, USA) was used as a molecular weight marker.

Analytical methods

Cell growth was monitored by measuring optical density at 600 nm (OD_{600}) with UV-Vis spectrophotometer to monitor the growth of *B. subtilis* strains. Detection of L-tryptophan in fermentation culture was carried by the approach as described previously (7). The correlation of Absorbance at 600 nm and concentration of L-tryptophan was determined as illustrated in (Fig. S20B), which was the standard for calculation. For the detection of crude violacein, 1 mL of the fermentation culture was centrifuged at 16,000 × g for 2 min and the crude violacein in the pellet was extracted with ethanol. The titer of crude violacein was determined by measuring the absorbance of the crude violacein solution at 570 nm. The correlation of Absorbance at 570 nm and concentration of crude violacein was determined (Fig. S20A) as reported previously(7, 8). For riboflavin measurement, samples were first diluted with 0.05 M NaOH and centrifuged at 16,000 × g for 2 min to remove the cells, the supernatant was then diluted by acetic acid sodium-acetate buffer solution (pH 5.0) to the linear range of the spectrophotometer and the absorbance at 444 nm was recorded and calculated as reported previously(9, 10).

SI Appendix Text S1

High-throughput screening of *malO* mutants

Mutagenesis library of the *malO* operator was constructed based on the sequence characteristics of the *malO* operator described above (Fig. S3). The degenerated nucleotides were designed with a focus on the UP and spacer regions of the operator, while two inverted-repeated regions were mutated to the consensus sequence of *malO* operators in different *Bacillus* species. More than 10^6 transformants were obtained, and approximately 98% of the colonies on LB-agar plates supplemented with maltose exhibited green fluorescence, meeting the desired quality requirements for using the mutagenesis library for further high-throughput screening. Cells displaying the top 1% green fluorescence in terms of signal strength were windowed and collected by a cytometer in each round, followed by secondary validation of the fluorescence in a deep-well plate. Finally, fifteen mutants, denoted operator mutants (OM_n), were isolated (Fig. S4A). The IR1 region of the *malO* operator was the hotspot of mutation; the cytosine was prone to mutation to thymine (Fig. S4B). Moreover, a 4-bp (TTAW) mutation (5 out of 9 mutants) was found frequently in the UP region of *malO*. Interestingly, as a result of the mutation of IR1 (TTTCCC), the symmetric inverted-repeat sequence in *malO* changed from TTTCCC-N_7-GGGAAA to TTTTTC-N_9-GAAAAA, a sequence similar to that in the predicted *malO* operator found in *Staphylococcaceae* sp*., B. pumilus* and *B. clausii* (Fig. S1C). Thus, we hypothesized that this mutation plays an important role in enabling the observed increase in P_{malA} promoter strength.

SI Appendix Text S2

Construction of MATE-ON and MATE-OFF system

Construction of MATE-ON system:

To exclude the influence of host cells on gene expression and to evaluate the expression capacity of different promoter mutants in *B. subtilis,* GFP expression cassettes harboring different *malO* mutants were cloned into the high-copy-number plasmid pMA5, generating the pMATE plasmid series. Among them, six plasmids harboring the OM01, OM02, OM06, OM12, OM13 and OM15 mutations on the *malO* operator resulted in much higher levels of GFP than those produced using the wild-type promoter (Fig. S5). The plasmid with OM15 exhibited the highest level of GFP and was thus selected as the expression vector candidate for further experiments.

To develop a long-term, continuous, and robust host cell for the expression vector, the maltose utilization pathway was engineered. For this, the *malA* gene encoding 6-phospho-alpha-glucosidase, which hydrolyzes the inducer maltose-6-phosphate, was deleted to generate the 1A751ΔmalA strain. Additionally, a reported terminator-like sequence (ΔG=-30.1 kJ/mol) downstream of the *malA* gene(11) was removed from the 1A751ΔmalA strain to generate the 1A751ΔmalA-Ter strain. Moreover, the transcription activator gene *malR* and maltose transporter gene *malP* were overexpressed separately or jointly using P_{malA}. The eight engineered host strains were evaluated by a fluorescence assay (Fig. S6A).

Construction of MATE-OFF system:

There was an interesting study showing that LuxR, a transcriptional activator, can be converted to a transcriptional repressor by positioning the *lux* box between the -35 and -10 regions with partial overlap of the consensus region(12). Mimicking this strategy, a maltose-repressible promoter was designed by repositioning the *malO* operator between the -35 and -10 regions of P_{malA} (Fig. S13A). Then, the -35 and -10 regions were mutated to the consensus sequence of the σ^A promoters to improve the basal activity of P_{malA} in the absence of an inducer. To construct maltose-repressible promoters with different repression levels in *B. subtilis*, four repressible promoters harboring different *malO* operators (WT, OM2, OM6 and OM15) were generated and evaluated by GFP fluorescence assay. Considering the stability of the GFP reporter protein, a ssrA degradation tag was fused to the C-terminus of the reporter protein to prevent the accumulation of the GFP protein in the host cells. A marked fluorescence reduction was observed for all constructs upon induction with maltose in a dose-dependent manner. Of these four maltose-repressible promoters, pMATE-g1 exhibited a maximal 2-fold repression of GFP fluorescence (Fig. S13B). To study the temporal responses of fluorescence repression by the best maltose-repressible promoter, a strain harboring pMATE-g1 was characterized over a 10-h period after adding maltose at 4 h of cultivation (Fig. S13C). Prior to maltose-mediated induction, the production of the GFP reporter protein driven by the maltose-repressible promoter increased over time. Once the inducer was added, the fluorescence signal began to decline and finally stabilized at one-third of the signal measured from the control without the addition of maltose.

SI Appendix Text S3

Alleviation of the Carbon Catabolite Repression (CCR) effect of the MATE-ON system

For most bacterial expression systems using sugar or sugar alcohols as an inducer, the CCR effect is one of the big barriers for scaling up protein production at an industrial level. The inducibility of these sugar-induced expression systems is strongly dependent on the composition of the cell culture medium. When glucose is added to the culture medium, the expression of these systems is fully repressed until the glucose is exhausted. Likewise, the activity of the maltose-inducible promoter P_{malA} in *B. subtilis* is fully repressed by the catabolite control protein CcpA in the presence of glucose. A preferred strategy in most sugar-inducible systems is to construct a CcpA-deficient mutant host strain. However, as a general transcriptional regulator in carbon metabolism, CcpA and its cofactors play a major role in the coordinated regulation of catabolism and anabolism to ensure optimum cell propagation in the presence and absence of a preferred PTS carbohydrate. Several studies have reported that the CcpA-defective mutant of *B. subtilis* grows at a low rate in a minimal medium with glucose and ammonium as carbon and nitrogen sources compared to the wild-type strain(13). In addition, the glutamate pool is low in a *ccpA* mutant due to the loss of CCR of the *rocG* gene, encoding catabolic glutamate dehydrogenase which is subject to direct CcpA-dependent glucose repression(14). Thus, to alleviate the CCR effect without affecting the growth of the host cell, the binding site of CcpA in the promoter P_{malA} was chosen as a target instead of CcpA itself. To achieve this, the catabolite response element (*cre*) of $P_{malA,}$ which recruits the CcpA protein, was deleted from the promoter P_{malA} while carrying OM15 mutation on the *malO* operator (Fig. S7A). Subsequently, the effect of catabolite repression on these *cre* deletion promoter constructs was investigated in *B. subtilis* 1A751 in the presence of glucose at different concentrations. In contrast to our expectations, the *cre* deletions in P_{malA} (Δcre-1, Δcre-2 and Δcre-3) led to only a slight alleviation of the CCR effect at a low concentration (0.25% w/v) of glucose (Fig. S7B). Thus, another strategy was employed to alleviate the CCR effect by decreasing the glucose uptake efficiency in *B. subtilis*. Although glucose uptake mainly relies on the glucose-specific PTS permease PtsG ($IIC\text{-}BA^{Glc}$), a hexose/H^+ symporter GlcP contributes approximately 30% of the glucose transport in the presence of glucose(15). In addition, the glucose uptake protein GlcU, which was first described in *Staphylococcus xylosus*, is also responsible for glucose uptake(16, 17). Thus, the deletion of the glucose-specific PTS permease would decrease but not eliminate the uptake of glucose in *B. subtilis*. Additionally, a PTS permease-deficient mutant of *B. subtilis* was found to possess a much higher maltose transport rate than the wild-type strain(18). Thus, the component of the main transporter system of glucose encoded by the *ptsG gene* was deleted, expecting that the glucose uptake rate would be decreased. The *ptsG* deletion strain was used in combination with the *cre* deletion promoters. This combination mostly alleviated the CCR effect on the MATE-ON system in the presence of glucose at higher concentrations. Unexpectedly, a marked decrease in the leaky expression level was observed in strains with *cre* deletion promoters without compromising promoter strength (Fig. S7B), especially for the construct Δcre-3, which showed a great improvement in its induction fold-change (50-fold) compared to the control (10-fold) in a high-copy-number plasmid. Based on these results, two promoter constructs with left- or right-half complementary mutations in the *cre* box were generated and validated for stringency. The fluorescence assay showed that the stringency of the promoter was markedly improved when the left or right half of the *cre* box was mutated (Fig. S7C). However, the promoter activity differed among the three *cre* box mutants, suggesting that a potential transcriptional activation element or crosstalk regulation element might overlap with the *cre* box in these constructs.

SI Appendix Text S4

Improvement of stringency of the MATE-ON system

The robust maltose-activated expression system, MATE-ON, was established by combinatorial optimization of expression elements, comprising an optimized *malO* operator, a modified *cre* box, and an engineered *mal* operon. Even though the strength of the system was sufficient for protein production, the leaky expression level and the induction fold-change of this version of the MATE-ON system were not ideal, especially for proteins causing a big translational burden or toxicity to host cells. Further effort to lower the leaky expression of the MATE-ON system, including modification of the -35/-10 core region and alteration of the ribosome-binding site (RBS) in the P_{malA} promoter, has proven infeasible due to the negative effect on system robustness. However, it was found that the *cre* deletion mutants showed a much lower level of leaky expression while maintaining comparable promoter strength. To further improve the stringency of the MATE-ON system without affecting transcriptional strength, ON-type riboswitches were employed with the *cre* deletion promoter mutant, achieving dual transcriptional and translational control over target protein expression.

To this end, lysine-ON riboswitch mutants (LysRS) from *Corynebacterium glutamicum*(19) and a synthetic theophylline-activated riboswitch E (TheoRS) from *B. subtilis*(20) were inserted downstream of the *cre* deletion promoter mutant (Fig. S8A). As expected, the inducible strength of the MATE-ON system was barely influenced by these translational elements, while the leaky expression level was drastically decreased by introducing these riboswitches in the absence of inducers (Fig. S8B and S8C), especially when the construct was fused with theophylline-activated riboswitch E. The improvement of stringency of the LysRS16 and LysRS59 constructs was not as good as the theophylline-activated riboswitch because the inducer of these two Lys-ON riboswitches, lysine, is commonly present in most rich culture media. Considering the orthogonality of the inducer, theophylline, a natural alkaloid derivative of xanthine isolated from plants that is not easily degraded by bacteria, is also a better choice for our system. In addition, both TheoRS constructs exhibited a dose-dependent response to the inducer theophylline in the presence of maltose (Fig. S8D), while the construct Δcre3-TheoRS2, which contained only one RBS from TheoRS, showed approximately 2-fold higher induction strength (31602 A.U. vs. 14185 A.U.) than that with two ribosome binding sites. Furthermore, an orthogonal induction experiment was performed to determine the optimal concentrations for the inducers, maltose and theophylline. Induction with 1.25% (w/v) maltose and 4 mM theophylline resulted in the highest expression level of GFP (Fig. S8E). Taken together, an enhanced version of the MATE-ON system exhibiting both high stringency and robustness was established, allowing the stringent expression of the target reporter protein GFPmut2 without compromising the robustness of the system (Fig. S9).

SI Appendix Text S5

Homogeneity, reproducibility, and robustness analysis of the MATE system

For inducer-based bacterial expression systems, an all-or-none induction phenomenon is always found due to the transport efficiency of inducers, which leads to great heterogeneity among the cell populations(21, 22). At the subsaturated levels of an inducer, expression levels vary greatly within a population of genetically identical cells, resulting in two subpopulations: one fraction that is fully induced and the other fraction that is not induced at all(23, 24). To investigate the homogeneity and gene expression stability of the MATE system, flow cytometry analysis was performed to measure the expression level of maltose-induced reporter protein GFPmut2. The results (Fig. S11 and S12) showed that in comparison to the parent host strain, the strain with the engineered *mal* operon showed markedly improved homogeneity and reproducibility with or without the inducer throughout the experiment. A narrower distribution of fluorescence within each population was observed, as well as closer alignment of the means for triplicates of two chassis strains with engineered *mal* operons.

Next, we examined the effectiveness and robustness of the MATE-ON system in recombinant protein production. Production of nine heterologous proteins (D-psicose 3-epimerase from *Ruminococcus* sp., DPE; chitinase from *Bacillus circulans*, ChiA1; phytase from *E. coli*, AppA; luciferase from firefly, Luc; β-galactosidase from *Bacillus circulans*, BgaD; fibroblast growth factor 21 from *homo sapiens*, hFGF21; β-mannanase from *B. licheniformis*, ManA; alkaline protease from *B. licheniformis*, SubC and green fluorescence protein from *Aequorea victoria*, GFPmut2) derived from prokaryotic or eukaryotic organisms was tested (Fig. S10). These heterologous proteins could be successfully overproduced using the MATE-ON system, and over 60% of the total cellular proteins were produced as the target proteins. In some cases, the target cytoplasmic proteins (ChiA1, BgaD and GFPmut2) were excreted into the medium without any signal peptide sequence. A reasonable explanation for this phenomenon is the intracellular turgor pressure caused by the hyperexpression of the target protein within cells, which was also observed in a previous study using *E. coli* BL21 (DE3) as the host cell(25). These results highlight the applicability of our MATE system as a promising toolkit for the high-yield production of desired proteins.

SI Appendix Text S6

Exploitation of the *malO* operator as a promoter enhancer

To determine the effect of duplicated operators on the promoter activity, different numbers of auxiliary *malO* operators were appended upstream of the native P_{malA} promoter. Three synthetic promoters with adjacent multi-operators (two operators, O2; four operators, O4; and six operators, O6) were constructed, and their activities were analyzed by fluorescence assay. Compared with the native promoter (O1), synthetic promoters with duplicated operators exhibited weakening of inducible activities (Fig. S15A). The promoter activity exhibited an obvious negative correlation with the number of auxiliary operators. A reasonable explanation for this phenomenon can be made by the interaction between MalR and multiple operators. Native PAGE analysis showed that the transcriptional activator MalR was functional in an oligomeric form (Fig. S16). When multiple operators were presented in the promoter region, the oligomeric transcription factor typically binds to the regulated promoters with multiple operators, forming a DNA loop structure(26). This loop structure could interfere with the interaction between RNAP and the promoter, resulting in decreased strength of these synthetic promoters with auxiliary operators. Conversely, this effect also provides us with a convenient strategy to rapidly build a series of promoters with a broad range of activities in bacteria.

Second, whether the distance between two adjacent operators could alter the activity of the promoter was investigated. To achieve this, an auxiliary *malO* was positioned at different distances (11–36 nt) upstream of the native operator. Without exception, synthetic promoters with auxiliary operators all exhibited an approximately 50% reduction in promoter strength compared with that containing only one native operator, while no obvious difference was found among these constructs (Fig. S15B). On the other hand, an oscillating pattern emerged in the fluorescence assay when the distance between the operators was expanded to hundreds of nucleotides (Fig. S15C). The promoter activity gradually decreased as the operator spacing reached 150 nt, and then, the activity started to oscillate for operator spacing between 150 and 400 nt. Surprisingly, the activity of the promoter drastically increased by 2.84-fold when the spacing between operators was 450 nt. The sites neighboring the 450 nt position (between 439 and 467 nt) were also analyzed, but no improvement in promoter strength was observed. This phenomenon indicates that an auxiliary *malO* operator upstream of target promoter could be exploited as an enhancer element for increasing the activity of promoter, even though the activation window of the auxiliary operator was narrow and needs to be further explored.

Third, the *malO* operator was also applied in other promoters to verify the universality of the activation effect in *B. subtilis*. The promoter Pr, a constitutive promoter controlling the first gene of the *rib* operon in *B. subtilis*, was used as an example. The *malO* operator was positioned upstream of the -35 box at different spacing distances (center to center), generating six synthetic promoters (Fig. S15D). An approximate 35% decrease in mCherry fluorescence was observed in five constructs upon maltose induction. However, no oscillation of promoter activity was found for these synthetic promoters probably due to the absence of the second operator in the Pr promoter. Finally, when the operator spacing was 99 nt, the promoter activity was increased by 1.58-fold upon maltose induction. These results suggest that the *malO* operator could inducibly activate other promoters in *B. subtilis*.

SI Appendix Text S7

Fine-tuning of the violacein biosynthetic pathway using the MATE system

Genetic elements from the MATE-ON or MATE-OFF system that induce activation or repression of target genes, respectively, were used to coordinately regulate the violacein biosynthetic pathway. Five genes, namely *vioA*, *vioB*, *vioC*, *vioD* and *vioE,* from *C. violaceum* that are responsible for the biosynthesis of violacein from L-tryptophan were codon optimized and assembled as an artificial *vioABCDE* operon controlled by the P_{malA}-Δcre3 promoter, generating the violacein-producing 1A751-pVio strain (Fig. 7F). In the presence of maltose, the color of the 1A751-pVio culture medium turned into light purple, indicating that the biosynthesis of violacein was successfully activated (Fig. S18). To monitor the inhibitory effect of violacein on the host cell, the cell concentrations of 1A751-pVio cultured with and without maltose were measured. In comparison with the negative control harboring the empty plasmid pMATE15, the violacein-producing 1A751-pVio strain showed a similar growth curve in the absence of maltose induction (Fig. S19). When the inducer was added at the beginning of cultivation (T=0), the growth of strain 1A751-pVio was markedly inhibited, and the cell concentration was almost halved compared with that of the negative control. In contrast, when the inducer was added after 6 h of cultivation (T=6), the violacein-producing 1A751-pVio strain initially grew at a rate similar to that of the control, which was followed by retardation of cell growth after induction due to the activation of violacein biosynthesis. These results demonstrate that the violacein pathway could be stringently regulated by the MATE-ON system.

Second, as the key substrate of violacein biosynthesis, the L-tryptophan pool in the host cell needs to be increased together with the *vioABCDE* artificial operon. Thus, the expression of the genes responsible for L-tryptophan synthesis was inducibly upregulated by the MATE-ON system, while the *aroH* gene responsible for the conversion of chorismate to prephenate was inducibly downregulated by the MATE-OFF system (Fig. 7E). To achieve this, the native promoter of the *trpEDFCAB* operon in the genome was replaced with the P_{malA}-Δcre3 promoter. Additionally, the region of transcription attenuation that binds to a regulatory protein, the *trp* RNA-binding attenuation protein (TRAP), was removed to prevent the inhibitory effects of excess L-tryptophan on transcription and translation(27), generating the TrpOp-pVio strain. After that, the expression of *aroH* encoding a key enzyme in the L-phenylalanine and L-tyrosine shunt pathways, chorismate mutase AroH, which competes for chorismate in the L-tryptophan pathway, was inducibly repressed by the MATE-OFF system (Fig. 7E). The native promoter of the *aroH* gene was replaced by the maltose-repressible promoter from pMATE-g1M2. To avoid the influence of the cryptic promoter in the *aroB* gene, insulator elements (double fd terminators from the bacteriophage fd) were inserted upstream and downstream of the *aroH* gene (Fig. 7F), generating the Chor-pVio strain.

Next, to demonstrate whether the MATE system can be used to fine-tune the tryptophan pathway for violacein production, elements from the MATE system with different activation and repression strengths were employed to control the metabolic flux of the tryptophan biosynthetic pathway. Five maltose-inducible activation promoters with low to high levels of activation strength (P_{malA}-M5, P_{malA}-M6, P_{malA}-M9, P_{malA}-M12, and P_{malA}-M15) were each used to regulate the *trp* operon, and five maltose-inducible repression promoters with low to high levels of repression strength (P_{malA}-q12, P_{malA}-b6, P_{malA}-g1, P_{malA}-g1-06-1, and P_{malA}-g1M2) were selected to regulate the *aroH* gene.

Fig. S1.

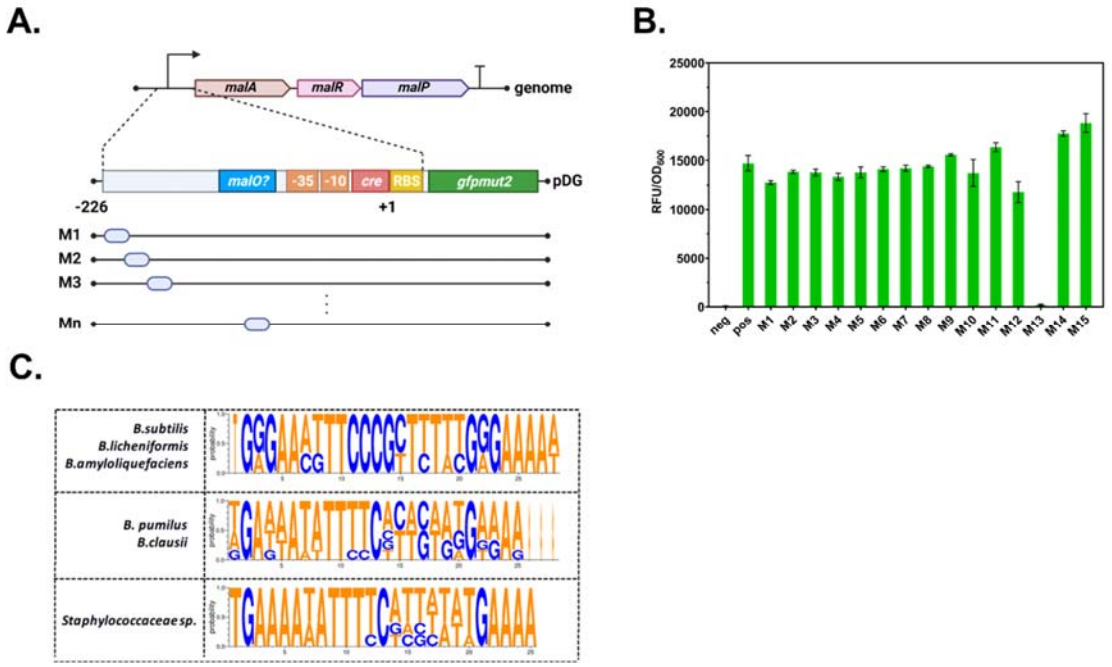

Figure S1. Determination of the *malO* operator that binds with the transcription activator MalR in the P_{malA} promoter of *B. subtilis* by truncated analysis and homologous sequence alignment. (A) Illustration of the construction of different truncated mutants of P_{malA} to locate the position of *malO*. Fifteen promoter truncated mutants (M1–M15) were constructed and their promoter activities were determined by GFP fluorescence in the presence of maltose. (B) Fluorescence detection results from a reporter gene *gfpmut2* driven by different promoter mutants in *B. subtilis*. (C) Alignment results of the predicted *malO* operator sequence in *B. subtilis, B. licheniformis, B. amyloliquefaciens, B. pumilus, B. clausii* and a *Staphylococcaceae sp.* by MEGA 5 software. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S2.

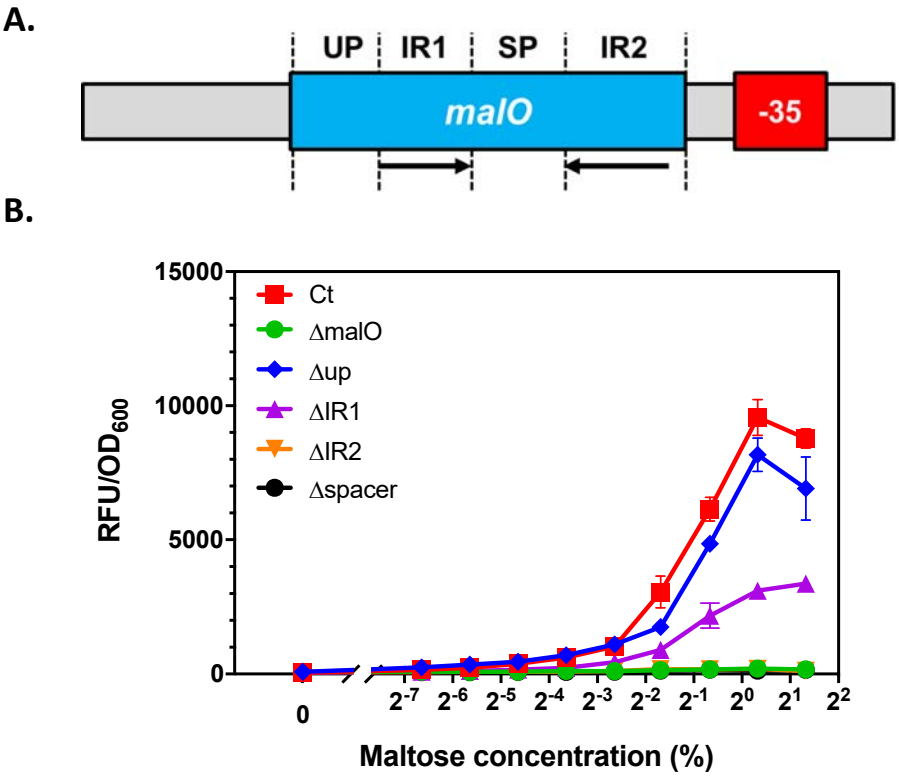

Figure S2. Mutagenesis and analysis of the predicted *malO* operator in *B. subtilis* using a GFP reporter protein. (A) The conserved sequence of the predicted operator was divided into four regions (UP, IR1, Spacer and IR2) to determine the importance of different regions to the activity of the promoter P_{malA}. (B) Fluorescence detection results of promoter mutants that are deficient in different *malO* regions. To avoid potential interference from flanking regions, the original sequences of each region were mutated to their complementary nucleotides. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S3.

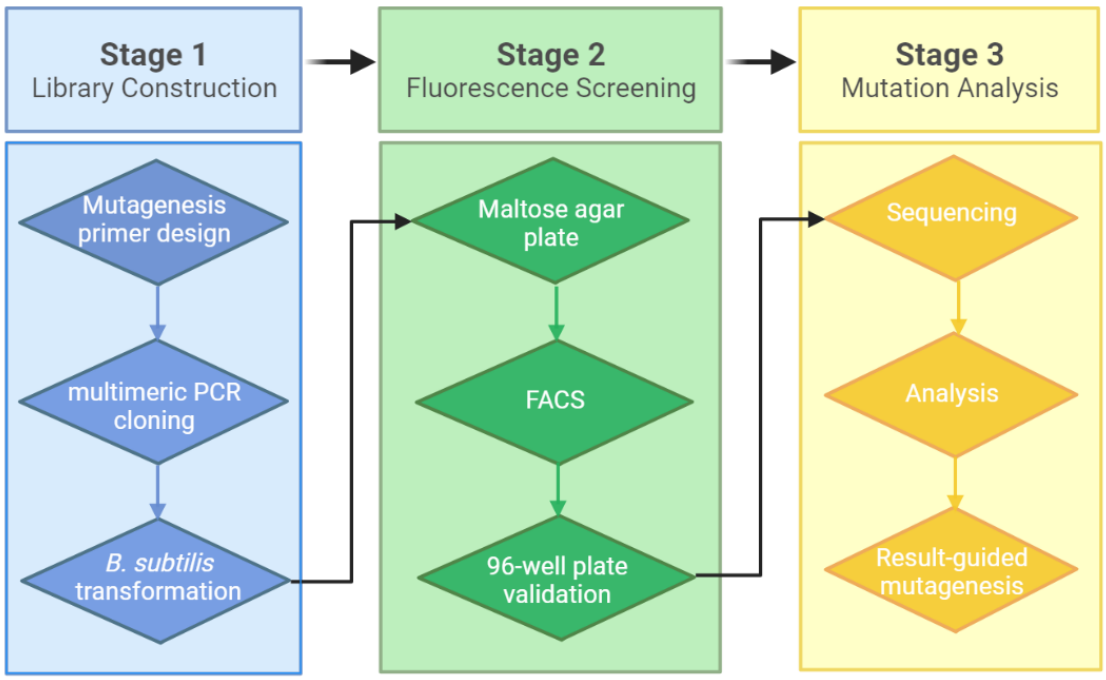

Figure S3. Schematic of the workflow for *malO* operator mutagenesis and a high-throughput screening procedure. We depict the general scheme for designing, constructing and selecting promoter mutants with superior expression characteristics. To construct the promoter library of *malO* operator mutations, degenerated primer-based mutagenesis was employed using the plasmid pDG-P_{malA} as a template. To eliminate the influence of copy number on fluorescence signal strength, library was established based on pDG-P_{malA} integrative vector, and then transformed and integrated into *amyE* locus of genome. According to the consensus of *malO* operator, we chose the UP and Spacer region as focal sites for random mutagenesis, while the rest of the operator sequence were designed of degenerated nucleotides based on the alignment results from the six different gram-positive bacteria (*B. subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus clausii* and *Staphylococcaceae* sp.). The screening method employed a combination of agar plate-based fast screening and FACS-based high-throughput screening. The resulting colonies were used to inoculate media in 96-well plates for validation, and the integrated promoter mutant was amplified and sequenced for further analysis.

Fig. S4.

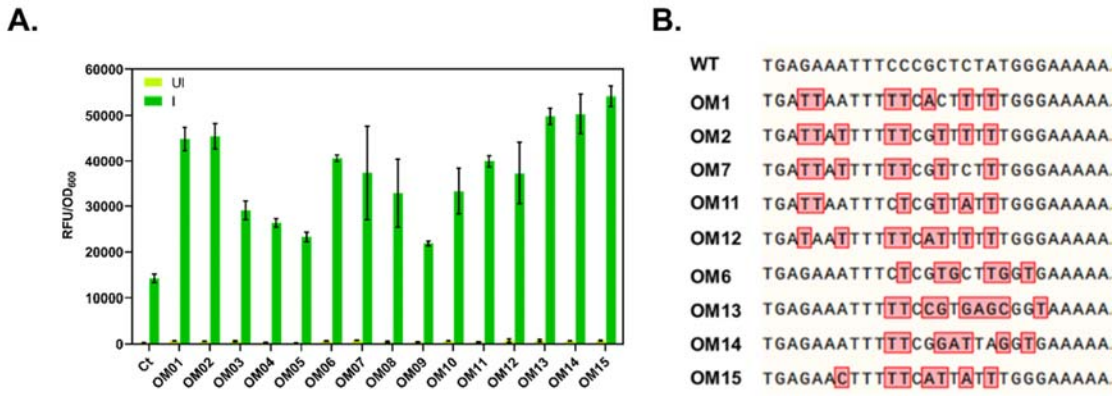

Figure S4. High-throughput screening results of the *malO* operator mutants that exhibited improved promoter activity in the presence of maltose. (A) Fluorescence assay of 15 selected strains harboring different promoter mutants with (I) or without (UI) the induction of maltose. (B) Sequence alignment of the *malO* operator region in different promoter mutants. The red box indicates the mutation site of the operator in comparison to the corresponding site in the wild-type promoter. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S5.

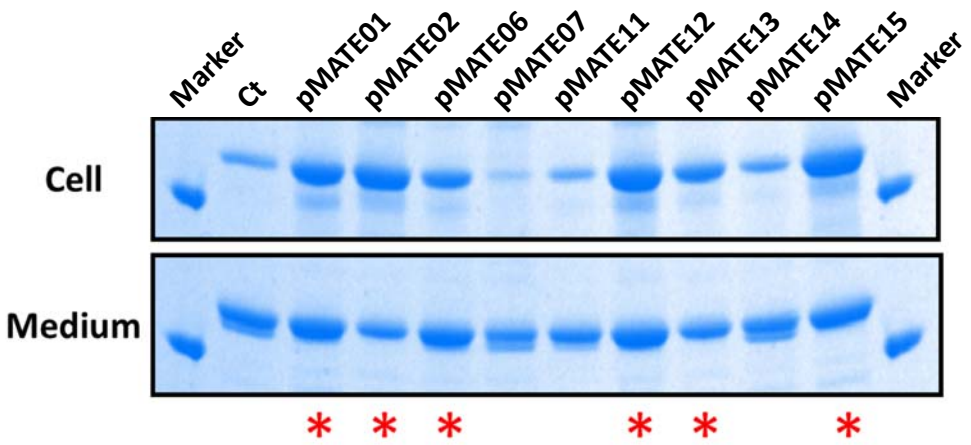

Figure S5. Evaluation of the strength of the promoter mutants based on the expression of GFP reporter protein. The promoter mutants were individually cloned into an episomal plasmid pMA5 to analyze their capacity for expressing the GFP in *B. subtilis.* The red star indicates the six promoter mutants that achieved highest expression levels of GFP than the control group.

Fig. S6.

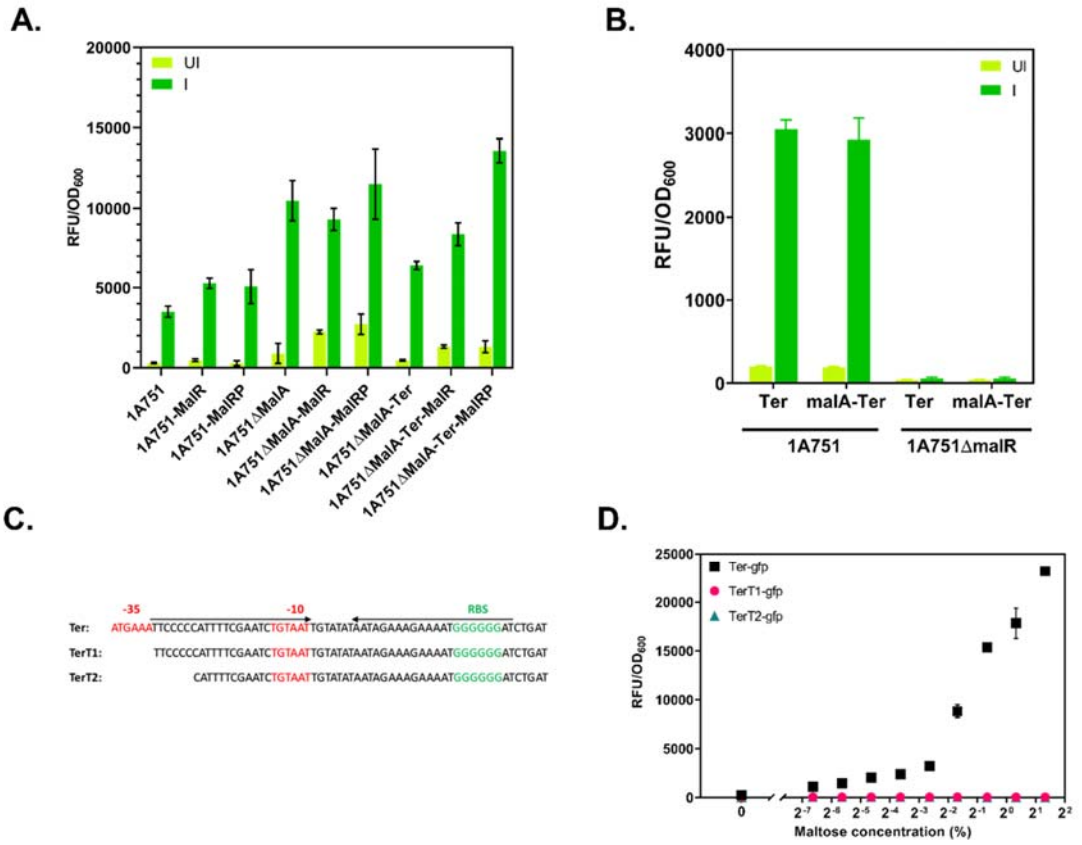

Figure S6. Fluorescence evaluation of different chassis cells and the potential terminator-like promoter between genes *malA* and *malR* in *B. subtilis*. (A) Fluorescence assay of different engineered host strains with or without the inducer using *B. subtilis* 1A751 harboring pMATE15 as a control. (B) Evaluation of the activity of the potential Ter promoter between gene *malA* and *malR*. (C) Construction of the truncated mutants of the potential Ter promoter. The core regions of the Ter promoter were predicted by BPROM and highlighted in red and green colors, and the arrows indicates the stem region of the transcript secondary structure. (D) Validation of the promoter core region of the potential Ter promoter. The Ter promoter and two truncated mutants, TerT1 and TerT2, were inserted into the promoter-probe plasmid pDG to test promoter activity.

Fig. S7.

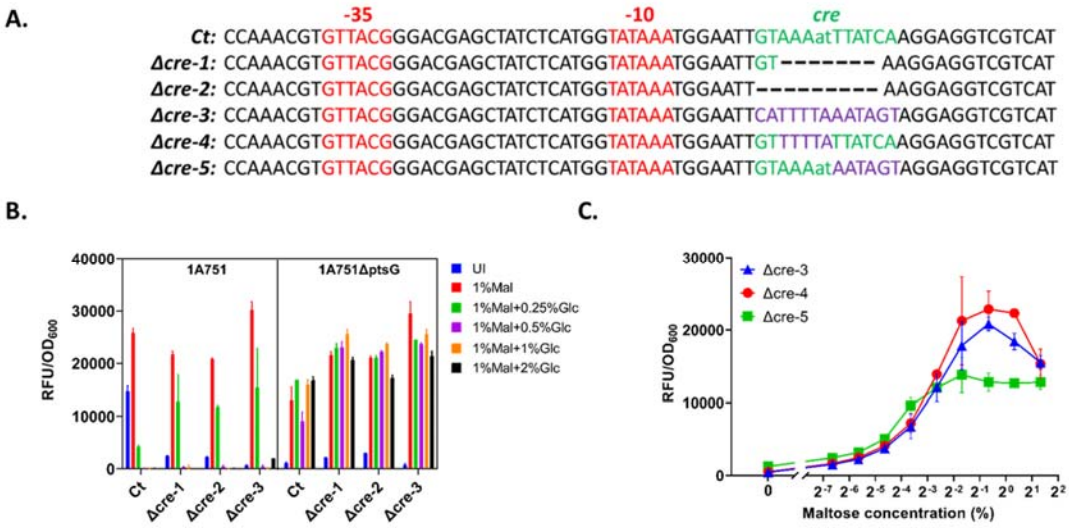

Figure S7. Mutagenesis of the catabolite response element (cre) in the promoter P_{malA} to alleviate the carbon catabolite repression (CCR) effect in *B. subtilis*. (A) Design of the mutation site in *cre* of P_{malA} that interrupts CcpA-binding in the presence of glucose. (B) Determination of induction activity of promoter mutants in the presence of different concentrations of glucose within the host cells 1A751 and 1A751ΔptsG. (C) Analysis of promoter activity of the mutants Δcre-3, Δcre-4 and Δcre-5 that with different mutation in *cre* box in response to different concentrations of a maltose inducer. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S8.

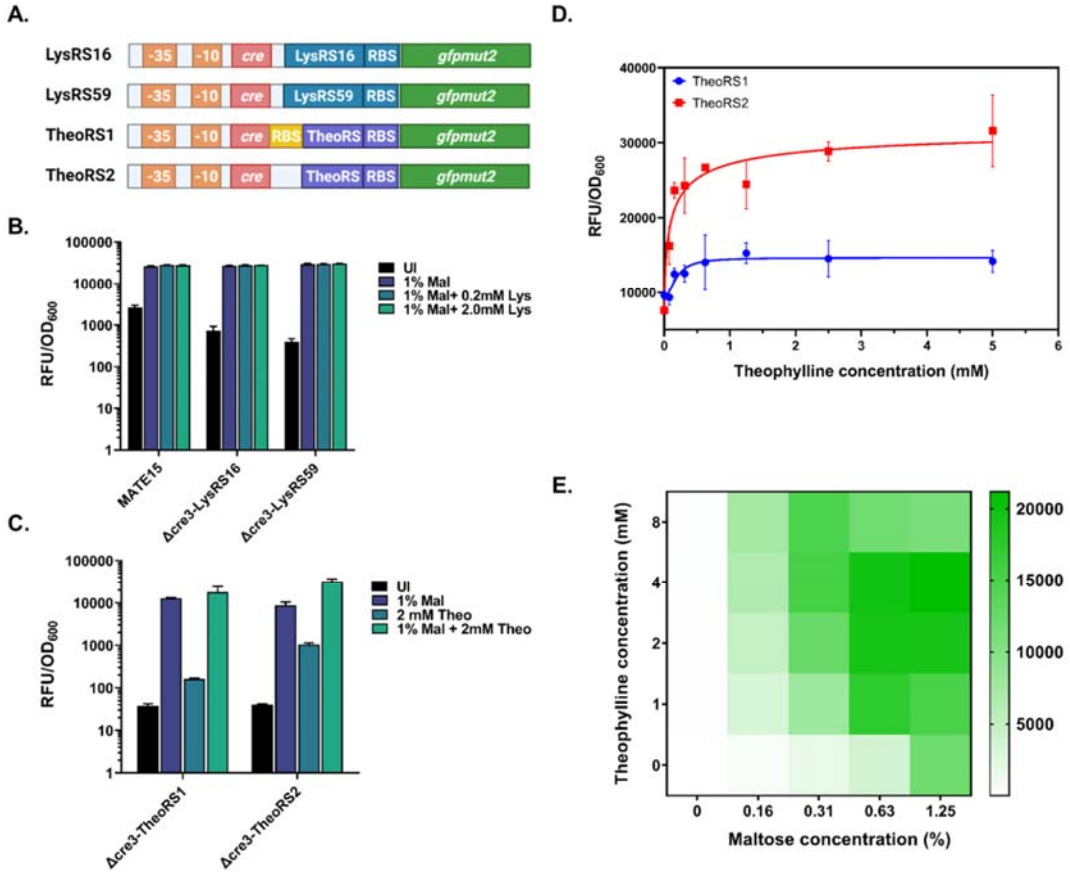

Figure S8. Transcriptional and translational dual controlling of the stringency of promoter P_{malA} by intercalating the lysine and theophylline inducible riboswitch. (A) Construction of promoter mutants that intercalated the lysine-activated riboswitch or theophylline-activated riboswitch. Two lysine-activated riboswitches LysRS16 and LysRS59 was placed between the P_{malA} promoter and *gfpmut2* gene. The theophylline-activated riboswitch was placed between the P_{malA} promoter and *gfpmut2* gene with or without the native RBS of P_{malA}. (B) Fluorescence assay of promoter mutants Δcre3-LysRS16 and Δcre3-LysRS59 upon the induction of maltose and theophylline. (C) Fluorescence detection of promoter mutants Δcre3-TheoRS1 and Δcre3-TheoRS2 upon induction with maltose and theophylline. (D) Dose-dependent activity of promoter mutants TheoRS1 and TheoRS2 induced by theophylline. (E) Determination of optimal concentrations of inducers maltose and theophylline for the second-generation of MATE-ON system.

Fig. S9.

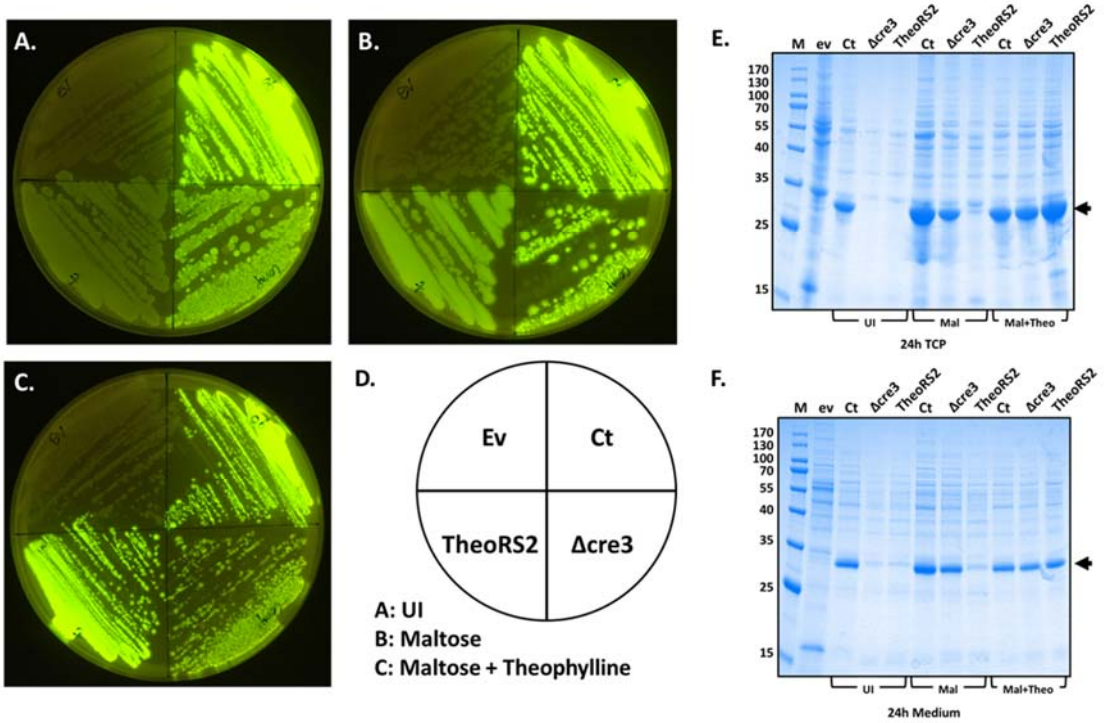

Figure S9. Stringency analysis of different promoter mutants Δcre3 and Δcre3-TheoRS2. (A-D) The leaky expression of the reporter gene *gfpmut2* in different strains were analysis by agar-plate supplemented with or without inducers. Ev, empty vector of pMATE15; Ct, pMATE15 plasmid harboring reporter gene *gfpmut2*. (E-F) the expression level of reporter gene *gfpmut2* in different strains were analysis by SDS-PAGE with different conditions of cultivation. The arrow indicates the target protein of GFPmut2.

Fig. S10.

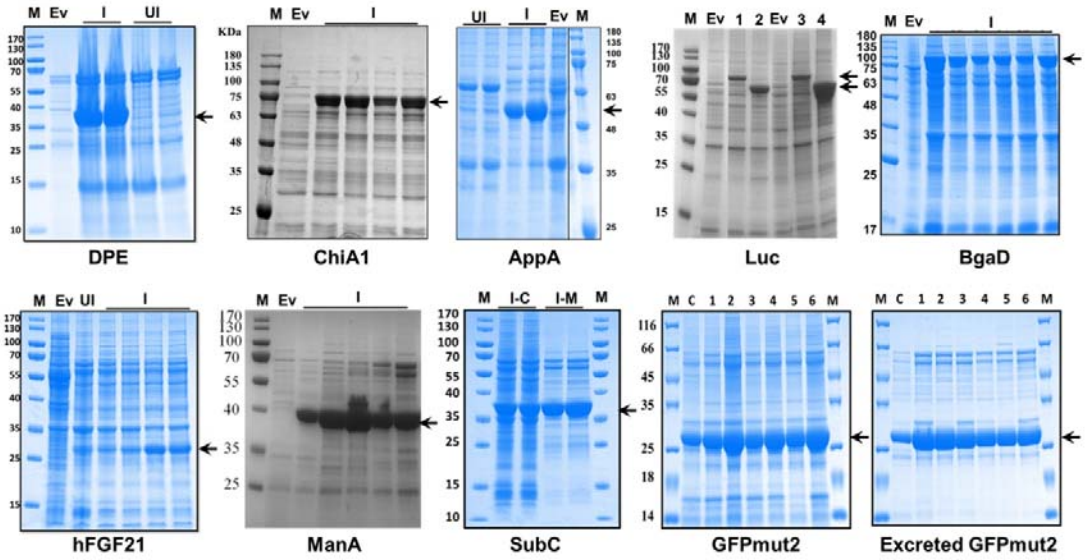

Figure S10. Robustness analysis of MATE system. Expression tests of the MATE-ON system using nine heterologous proteins (D-psicose 3-epimerase from *Ruminococcus* sp., DPE; chitinase from *Bacillus circulans*, ChiA1; phytase from *E. coli*, AppA; luciferase from firefly, Luc; β-galactosidase from *Bacillus circulans*, BgaD; fibroblast growth factor 21 from *homo sapiens*, hFGF21; β-mannanase from *B. licheniformis*, ManA; alkaline protease from *B. licheniformis*, SubC and green fluorescence protein from *Aequorea victoria*, GFPmut2) as reporter proteins. For PAGE analysis of Luc, lane 1 and 3 indicates the BgaB (from *B. licheniformis*) expression with pMA5 or pMATE15 plasmid respectively, lane 2 and 4 indicates the Luc expression with pMA5 or pMATE15 plasmid respectively. For PAGE analysis of GFPmut2 and excreted GFPmut2, lane C indicates the GFPmut2 expression with pMA5 plasmid, lane 1 and 2 indicates the expression of GFPmut2 with pMATE15 in 1A751ΔmalA-Ter-MalRP, lane 3 and 4 indicates the expression of GFPmut2 with pMATE15 in 1A751ΔmalA-Ter-MalR, lane 5 and 6 indicates the expression of GFPmut2 with pMATE15 in 1A751ΔmalA-Ter. For all PAGE analysis, lane M indicates protein marker, lane Ev indicates empty vector, lane UI indicates un-induced expression condition, lane I indicates induced expression condition, lane I-C indicates cell lysate fraction under the induced expression condition, lane I-M indicates medium fraction under the induced expression condition. The arrow indicates the target protein in PAGE pictures.

Fig. S11.

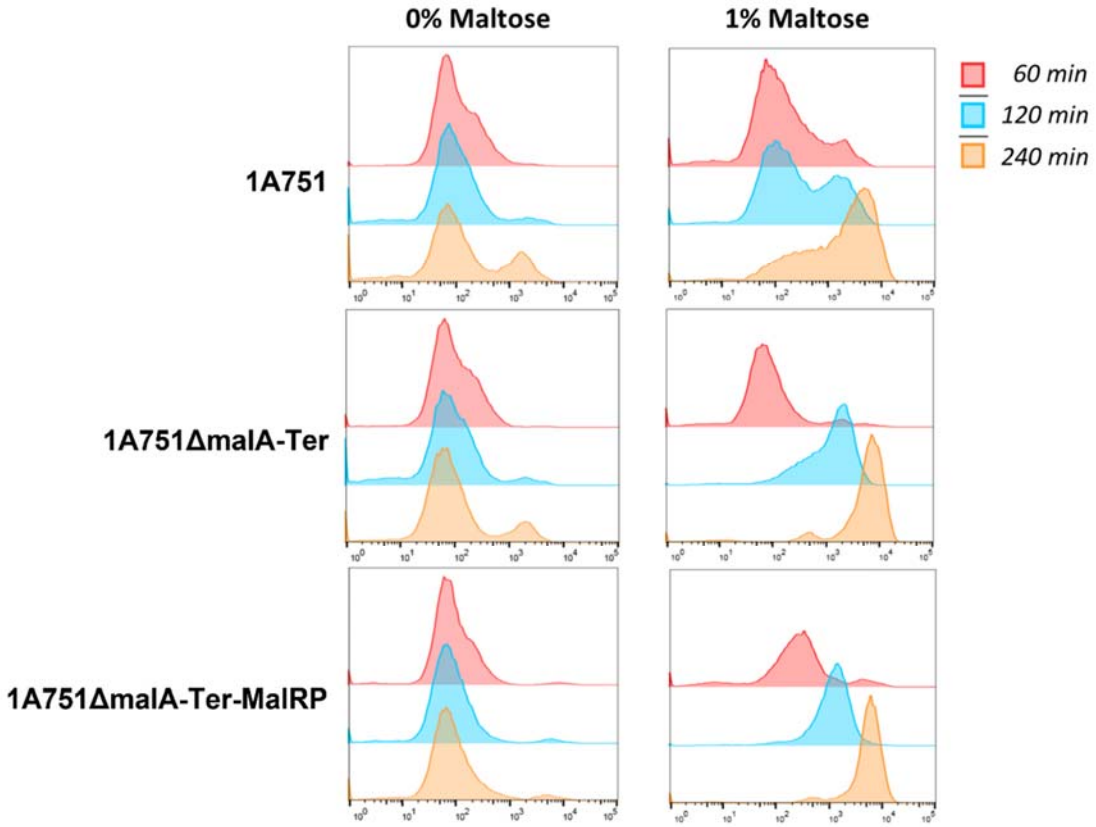

Figure S11. Homogeneity analysis of host cells of the MATE system. Homogeneity analysis of different host cells (1A751, 1A751ΔmalA-Ter and 1A751ΔmalA-Ter-MalRP) harboring the plasmid pMATE15-Δcre3. Fluorescence was assayed from cultures grown at different durations of incubation time, and fluorescence results obtained from three strains were plotted to compare the uniformity of GFP expression.

Fig. S12.

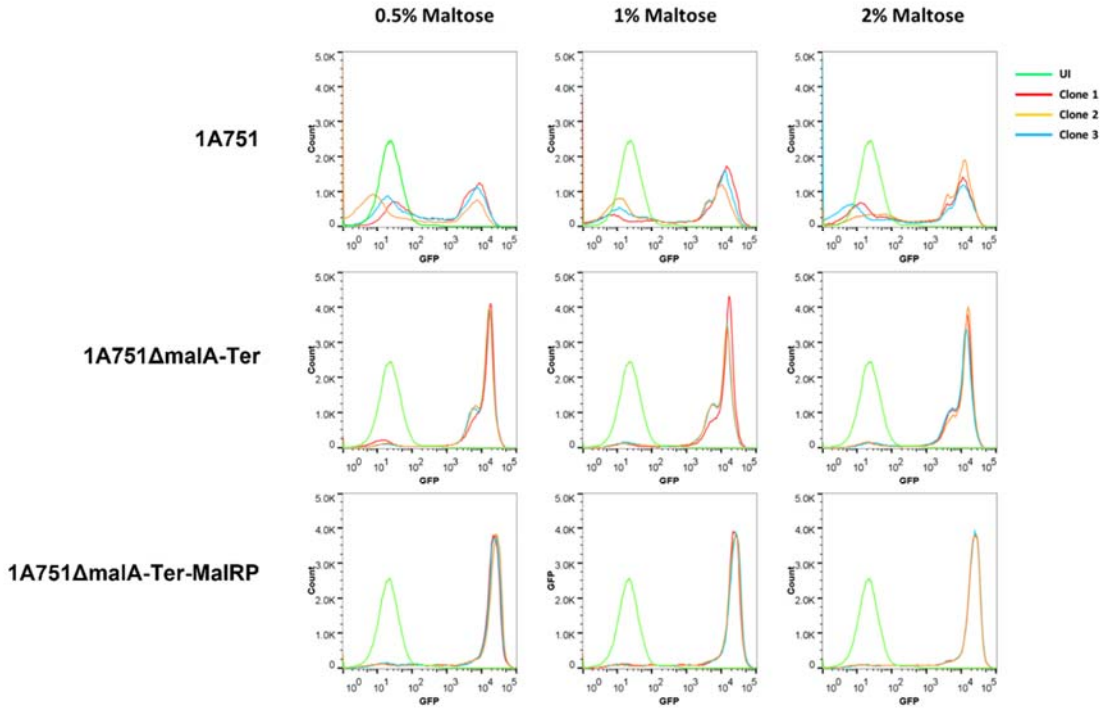

Figure S12. Reproducibility analysis of MATE system. Reproducibility analysis of different chassis cells with three different colonies. The overlapping red, yellow, or blue histograms of fluorescence data in each panel represent one of three replicates. UI indicates the control cells in the absence of inducer maltose.

Fig. S13.

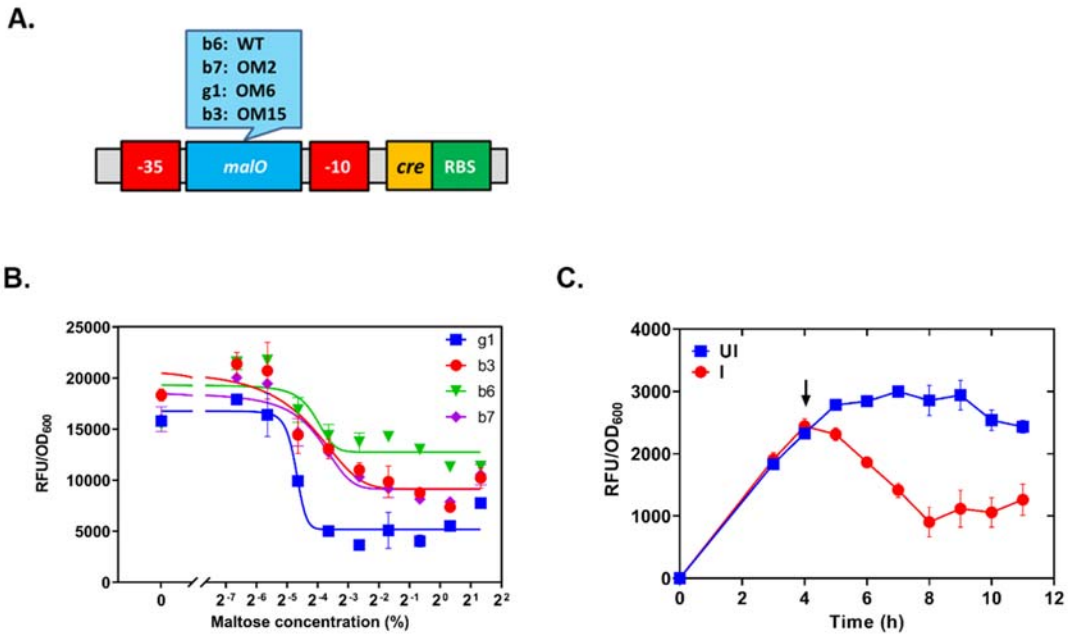

Figure S13. Design and characterization of the maltose-repressible gene regulatory system in *B. subtilis*. (A) Construction of the maltose-repressible promoter element by repositioning of the *malO* operator between consensus regions -35 and -10 of the promoter P_{malA}. This promoter mutant is constitutively expressed in the absence of the maltose inducer and upon exposure to the inducer, the transcription factor MalR binds to the operator region presumably causing steric hindrance in the binding of RNAP with the promoter, thereby repressing the transcription of the downstream gene. (B) Characterization of four different repressible promoter mutants in *B. subtilis* that were cloned into the pDG probe-vector. (C) Temporal characterization of the repressible-promoter mutants in pMATE-g1. To identify the repressible effect of the fluorescence signal, the *gfpmut2* reporter gene was fused to a ssrA-tag to prevent the accumulation of the GFP protein in host cells. All strains were cultivated for four hours in the absence of 1 % (w/v) maltose. Thereafter, one group was induced with maltose (I) and the other was not (UI), and fluorescence was monitored in strains with and without the addition of maltose. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S14.

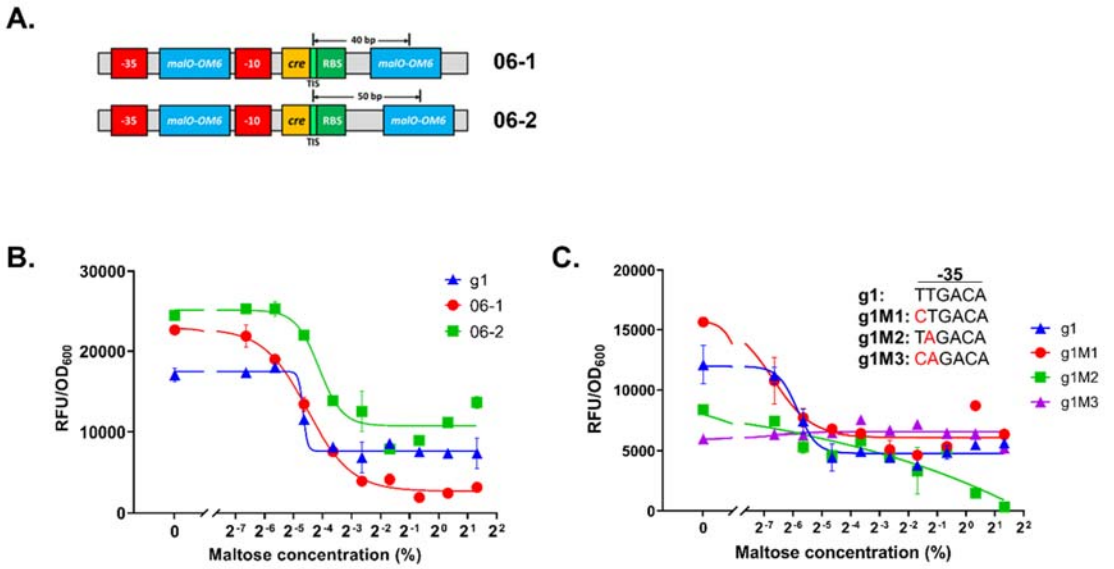

Figure S14. Stringency optimization of the maltose-repressible system in *B. subtilis*. (A) Illustration of the design of maltose-repressible mutant with higher stringency. (B) GFP fluorescence assay of two promoter mutants with an auxiliary *malO* operator. (C) GFP fluorescence assay of the maltose-repressible promoters with the -35 region mutation. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S15.

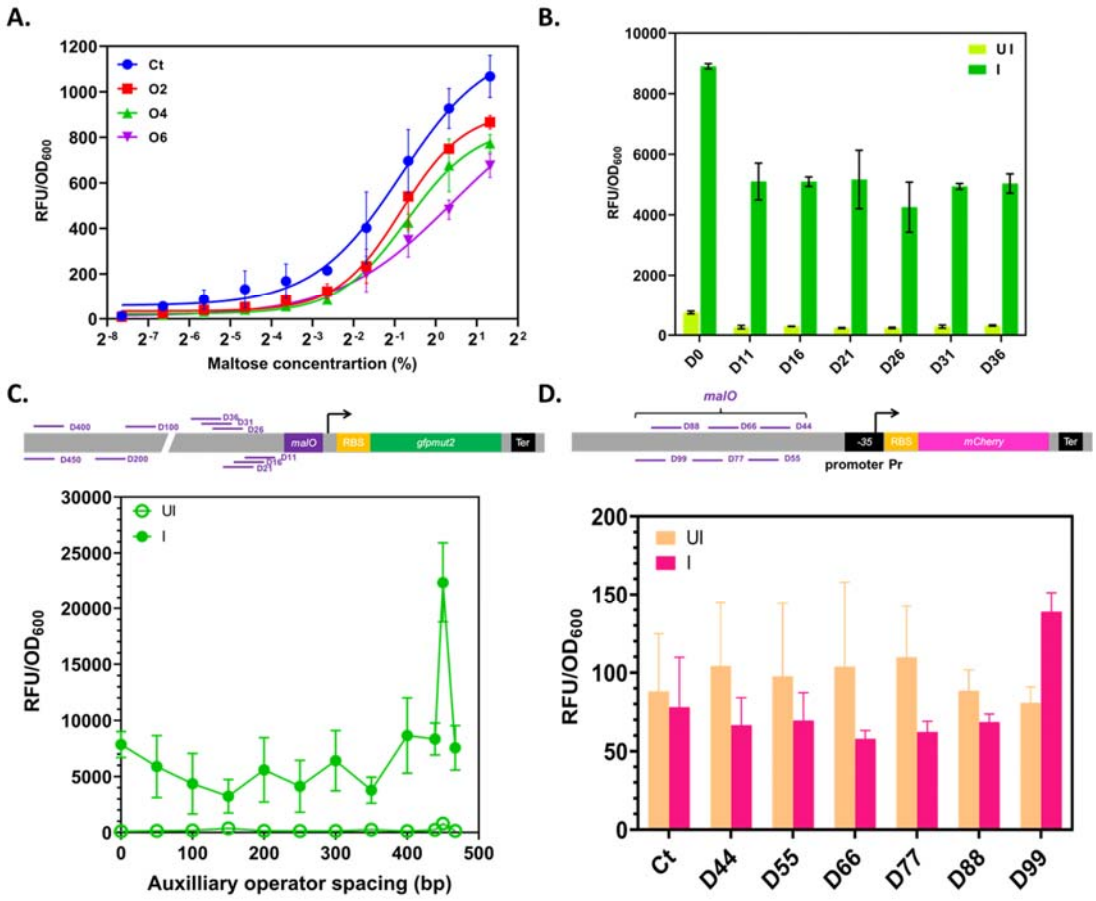

Figure S15. Characterization of the influence of different numbers of auxiliary *malO* operators and various distances between operators on the activity of the P_{malA} promoter. (A) Effect of different numbers of auxiliary *malO* on the activity of P_{malA}. The wild-type promoter (O1) was used as a control. (B) Effect of tandem *malO* operators (O2) of various short nucleotide distances between operators on the activity of PmalA. The wild-type promoter (D0) with one operator was used as a control. (C) Effect of an auxiliary *malO* operator at various long nucleotide distances on the activity of PmalA. (D) The activation effect of *malO* on the Pr promoter in *B. subtilis*. The wild-type promoter (Ct) without *malO* operator was used as a control. All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S16.

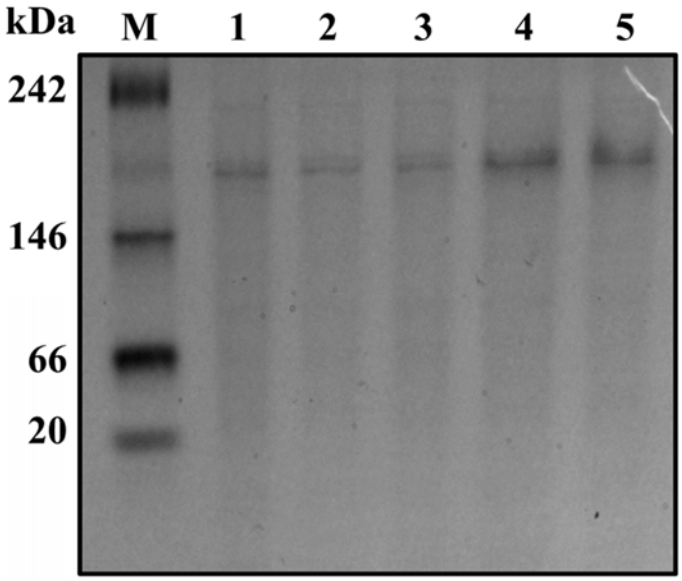

Figure S16. BN-PAGE analysis of the purified MalR protein from *B. subtilis*. To determine oligomeric states of the protein, BsMalR was expressed and purified from a 5-liter culture of *E. coli* BL21 (DE3) followed by His-tag affinity purification. The predicted molecular mass of BsMalR is about 29 kDa, while the molecular mass of the native MalR in native PAGE is between 146 kDa and 242 kDa, suggesting that MalR is functionally as an oligomer. Lane M shows the molecular weight marker; lane 1-5 shows the samples of purified MalR.

Fig. S17.

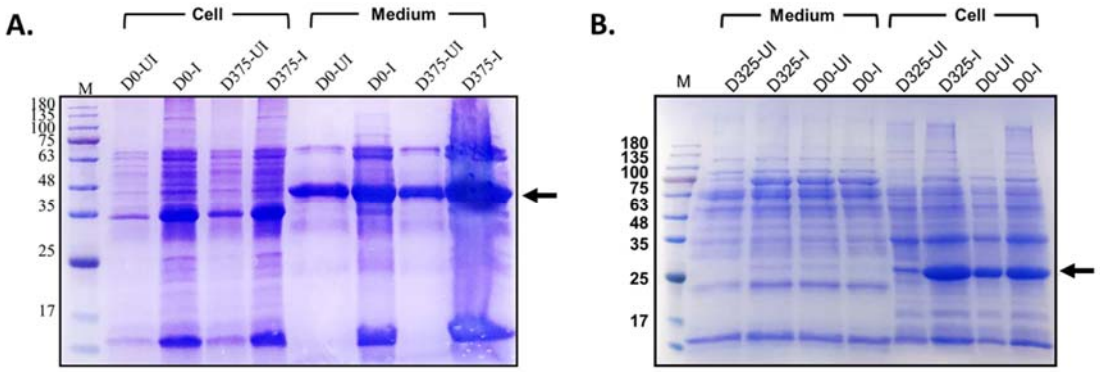

Figure S17. Examples of utilizing *malO* operator as promoter enhancer in optimization of protein production. PAGE analysis of the expression level of (A) α-amylase or (B) FGF21 in their producing strains with or without inserting *malO* operator upstream of chaperone gene *prsA* or *dnaK*. D0 indicates the control group without the *malO* operator upstreaming of chaperone gene, D375 or D325 indicates the optimized position of the inserting *malO* operator upstreaming of chaperone gene *prsA* or *dnaK*, respectively. UI and I indicates in the absence or in the presence of inducer maltose during cultivation. The arrow indicates two target proteins, AmyL and FGF21, respectively.

Fig. S18

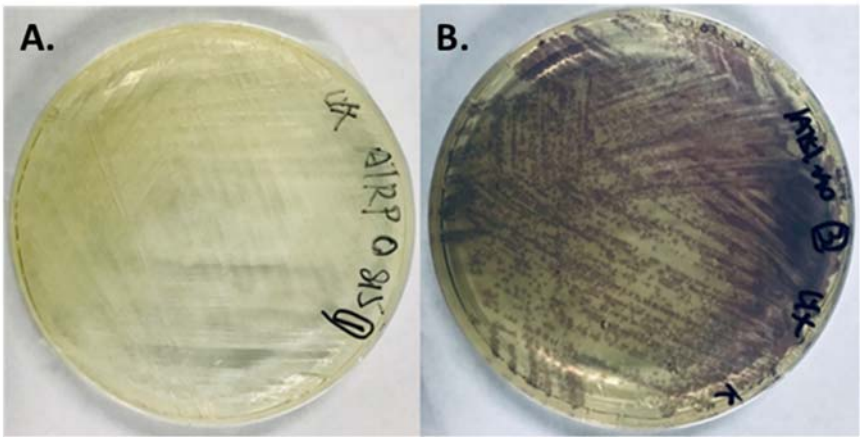

Figure S18. Agar plate of the violacein-producing strain with or without the induction of maltose. Violacein producing strain 1A751-pVio were cultivated in LB agar plate without (A) or with (B) the inducer maltose.

Fig. S19.

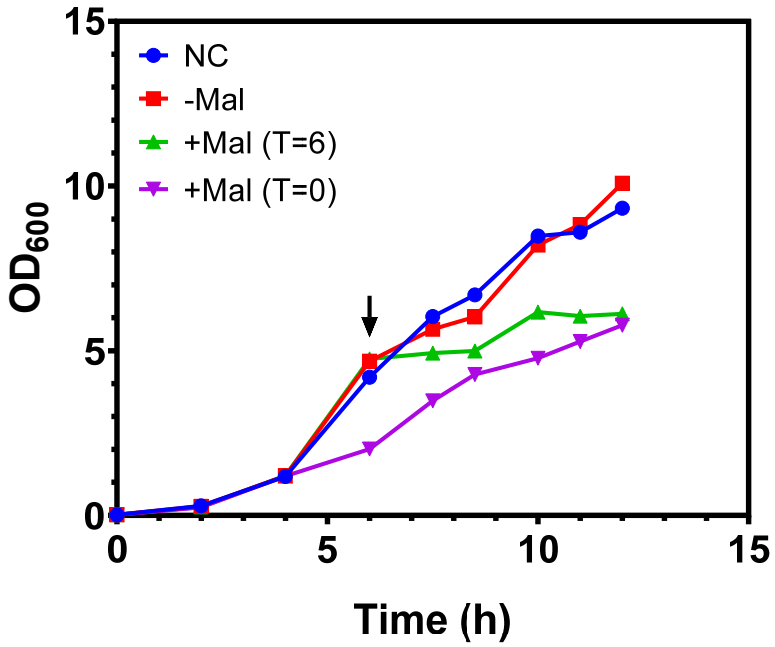

Figure S19. Growth curve of the violacein-producing strain with or without the induction of maltose. The inducer was supplemented at the beginning of cell cultivation (T=0) or after 6 hours of cultivation (T=6). The strain harboring an empty pMATE15 plasmid served as a negative control.

Fig. S20.

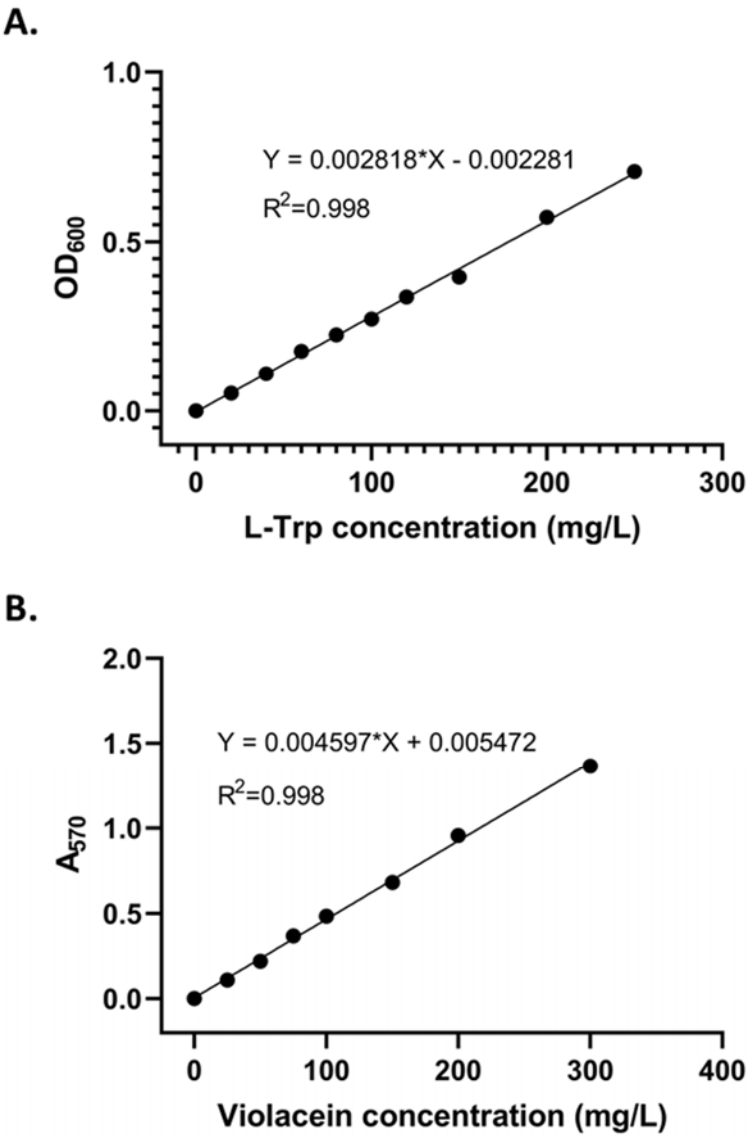

Figure S20. The correlation of absorbance and concentration of metabolite. (A) The correlation of absorbance at 600nm and concentration of L-tryptophan. (B) The correlation of absorbance at 570nm and concentration of crude violacein.

Fig. S21.

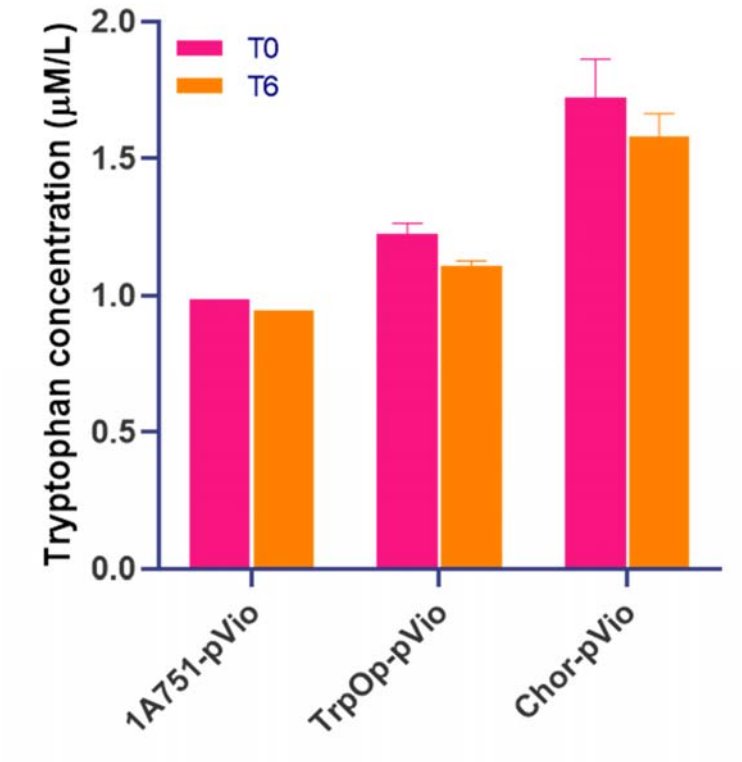

Figure S21. Evaluation of tryptophan titers of three violacein biosynthesis pathway-engineered strains. The maltose inducer was supplemented at the beginning of cell cultivation (T0) or after 6 hours of cultivation (T6). All the experiments were repeated at least three times, and the error bars represent SD.

Fig. S22.

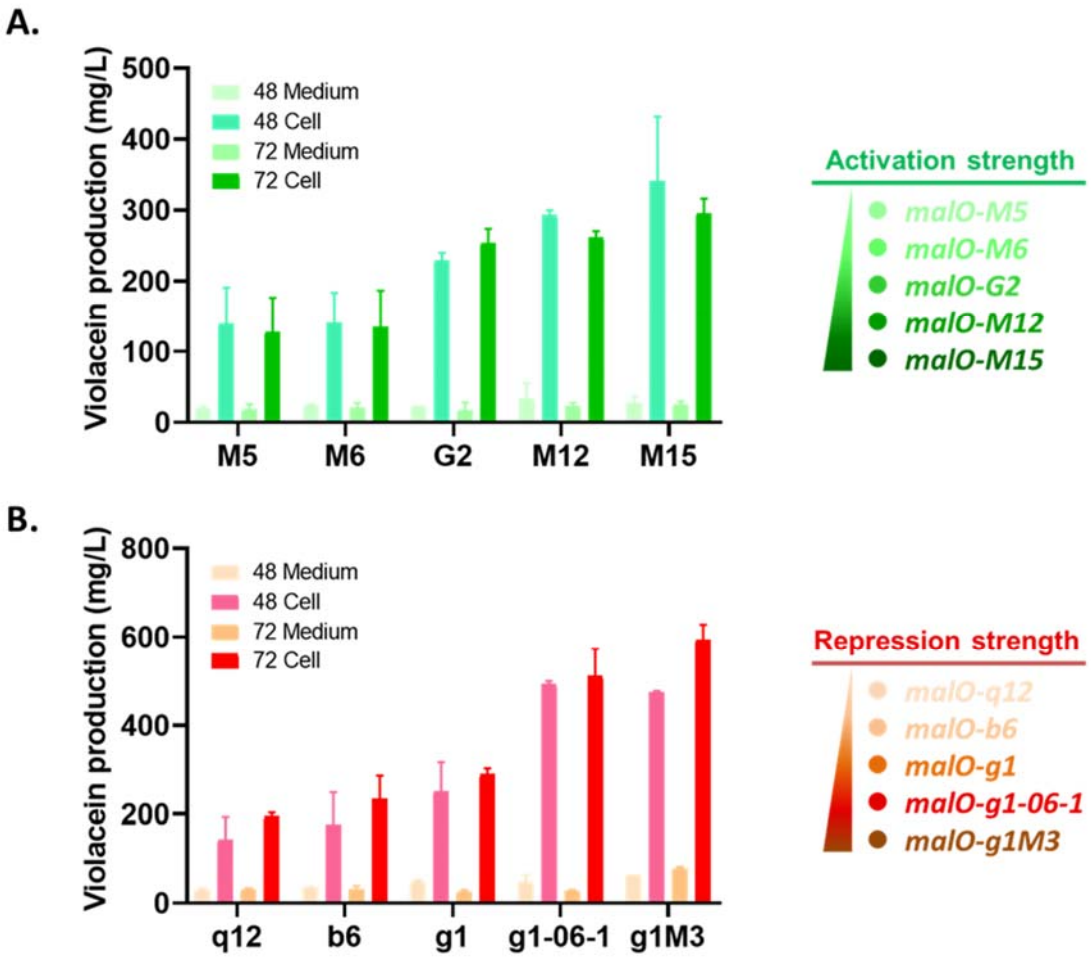

Figure S22. Fine-tuning the tryptophan biosynthesis pathway of violacein production by applying MATE-ON and MATE-OFF elements of varying strengths to affect the *trpEDCFBA* and *aroH* genes using *B. subtilis.* (A) *trpEDCFBA* operon: the native promoter was replaced by each of five activated P_{malA} promoters of increasing strengths determined by their respective *malO* operator. (B) *aroH* gene: each of five promoters of different repression strengths was inserted upstream of *aroH*, and the entire cassette was isolated by two insulators to avoid the influence of promoters from neighbor genes. All the experiments were repeated at least three times, and the error bars represent SD.

Table S1. *E. coli* and *B. subtilis* strains used in this study.

Strain name	Relevant features	Source
DH5*α*	Δ(argF-lac)169, φ80d lacZ58 (M15), ΔphoA8, glnX44 (AS), deoR481, rfbC1, gyrA96 (NalR), recA1, endA1, thiE1 and hsdR17.	Invitrogen Life Technologies
1A751	*his apr npr eglS(DELTA)102 bglT/bglS(DELTA)EV*	BGSC
1A751-MalR	1A751 *amyE*::P_{malA}-*malR*	This study
1A751-MalRP	1A751 *amyE*::P_{malA}-*malR-malP*	This study
1A751ΔmalA	1A751 Δ*malA*	This study
1A751ΔmalA-Ter	1A751 Δ*malA* ΔTer	This study
1A751ΔmalA-MalR	1A751 Δ*malA amyE*::P_{malA}-*malR*	This study
1A751ΔmalA-MalRP	1A751 Δ*malA amyE*::P_{malA}-*malR-malP*	This study
1A751ΔmalA-Ter-MalR	1A751 Δ*malA* ΔTer *amyE*::P_{malA}-*malR*	This study
1A751ΔmalA-Ter-MalRP	1A751 Δ*malA* ΔTer *amyE*::P_{malA}-*malR-malP*	This study
1A751Δ*malR*	1A751 *malR*:: *Kan*^{R}	This study
1A751Δ*ptsG*	1A751 Δ*ptsG*	This study
ΔATRPΔ*ptsG*	1A751ΔmalA-Ter-MalRP Δ*ptsG*	This study
1A751Δ*divIVA*	1A751 *divIVA*::*Kan*^{R}	This study
Δ*divIVA*-divOn	1A751Δ*divIVA*/ pDivOn	This study
Δ*divIVA*-divOff	1A751Δ*divIVA*/ pDivOff	This study
RibOn	ΔATRPΔ*ptsG* / pMATE-RibOn	This study
RibOn-RibCoff	ΔATRPΔ*ptsG* / fd Terminator-P_{malA}-g1M3-*ribC*- fd Terminator, pMATE-RibOn	This study
1A751-pVio	1A751ΔmalA-Ter-MalRP/ pVio	This study
TrpOp-pVio	1A751ΔmalA-Ter-MalRP P_{malA}-*trpEDCFBA*/ pVio	This study
Chor-pVio	1A751ΔmalA-Ter-MalRP P_{malA}-*trpEDCFBA* fd Terminator-P_{malA}-g1M3-*aroH*-fd Terminator/ pVio	This study

Table S2. Plasmids used in this study.

Plasmid name	Relevant features	Source
pDG	Integration promoter-probe vector containing *gfpmut2* reporter gene	(2)
pDG-P_{malA}	pDG vector containing promoter P_{malA} from *B. subtilis*	This study
pDG-P_{malA}-Δ*malO*	pDG-P_{malA} vector without operator *malO*	This study
pDG-P_{malA}-ΔUP	pDG-P_{malA} vector without the UP region of operator *malO*	This study
pDG-P_{malA}-ΔIR1	pDG-P_{malA} vector without the IR1 region of operator *malO*	This study
pDG-P_{malA}-ΔIR2	pDG-P_{malA} vector without the IR2 region of operator *malO*	This study
pDG-P_{malA}-ΔSpacer	pDG-P_{malA} vector without the Spacer region of operator *malO*	This study
pDG-Ter	pDG vector containing a potential promoter sequence that between gene *malA* and *malR*	This study
pDG-malA-Ter	pDG vector containing a potential promoter sequence that between gene *malA* and *malR* and 300 bp 5' truncated *malA* gene	This study
pDR	pDL vector containing gene *malR* controlled by promoter P_{malA}	This study
pDRP	pDL vector containing gene *malR* and *malP* controlled by promoter P_{malA}	This study
pMA5	Expression vector for *B. subtilis*	Lab stock
pMATE01	pMA5 vector containing P_{malA} promoter mutant OM01 and *gfpmut2* reporter gene	This study
pMATE02	pMA5 vector containing P_{malA} promoter mutant OM02 and *gfpmut2* reporter gene	This study
pMATE06	pMA5 vector containing P_{malA} promoter mutant OM06 and *gfpmut2* reporter gene	This study
pMATE12	pMA5 vector containing P_{malA} promoter mutant OM12 and *gfpmut2* reporter gene	This study
pMATE13	pMA5 vector containing P_{malA} promoter mutant OM13 and *gfpmut2* reporter gene	This study
pMATE15	pMA5 vector containing P_{malA} promoter mutant OM15 and *gfpmut2* reporter gene	This study
pMATE15-Δcre-1	pMATE15 vector that the catabolite response element (cre) of P_{malA} was deleted from 3rd nucleotide	This study
pMATE15-Δcre-2	pMATE15 vector that the catabolite response element (cre) of P_{malA} was deleted from 1st nucleotide	This study
pMATE15-Δcre-3	pMATE15 vector that the catabolite response element (cre) of P_{malA} was replaced by its complementary sequence	This study
pMATE15-Δcre-4	pMATE15 vector that the catabolite response element (cre) of P_{malA} was replaced by its complementary sequence from 3rd nucleotide to 7th nucleotide	This study
pMATE15-Δcre-5	pMATE15 vector that the catabolite response element (cre) of P_{malA} was replaced by its complementary sequence from 8th nucleotide to 13th nucleotide	This study
pMATE15-Δcre3-LysRS16	pMATE15-Δcre-3 vector containing a lysine activated riboswitch mutant LysRS16 downstream of promoter P_{malA}	This study
pMATE15-Δcre3-LysRS59	pMATE15-Δcre-3 vector containing a lysine activated riboswitch mutant LysRS59 downstream of promoter P_{malA}	This study
pMATE15-Δcre3-TheoRS1	pMATE15-Δcre-3 vector containing a theophylline activated riboswitch mutant TheoRS1 downstream of promoter P_{malA}	This study
pMATE15-Δcre3-TheoRS2	pMATE15-Δcre-3 vector containing a theophylline activated riboswitch mutant TheoRS2 downstream of promoter P_{malA}	This study

pMATE-c	pMA5 vector containing *gfpmut2* reporter gene controlled by P_{malA} promoter mutant that the -35 and -10 regions were mutated as “TTGACA” and “TATAAT”	This study
pMATE-b6	pMATE-c vector that the wild-type *malO* operator was repositioned between the -35 and -10 region	This study
pMATE-b7	pMATE-c vector that the *malO* operator mutant OM2 was repositioned between the -35 and -10 region	This study
pMATE-g1	pMATE-c vector that the *malO* operator mutant OM6 was repositioned between the -35 and -10 region	This study
pMATE-b3	pMATE-c vector that the *malO* operator mutant OM15 was repositioned between the -35 and -10 region	This study
pMATE-g1-06-1	pMATE-g1 vector containing an auxiliary OM6 *malO* operator downstream of -10 region at 40 bp	This study
pMATE-g1-06-2	pMATE-g1 vector containing an auxiliary OM6 *malO* operator downstream of -10 region at 50 bp	This study
pMATE-g1M1	pMATE-g1-06-1 vector that the -35 region was mutated to “CTGACA”	This study
pMATE-g1M2	pMATE-g1-06-1 vector that the -35 region was mutated to “TAGACA”	This study
pMATE-g1M3	pMATE-g1-06-1 vector that the -35 region was mutated to “CAGACA”	This study
pMATE-Ac	pMA5 vector containing *E2-crimson* red fluorescence protein reporter gene controlled by maltose activated promoter	This study
pMATE-Rg	pMA5 vector containing *gfpmut2* reporter gene controlled by maltose repressible promoter	This study
pMATE-AcRg	pMA5 vector containing *E2-crimson* red fluorescence protein reporter gene controlled by maltose activated promoter and *gfpmut2* reporter gene controlled by maltose repressible promoter	This study
pDG-O1	pDG-P_{malA} vector containing one native *malO* operator in P_{malA} promoter	This study
pDG-O2	pDG-P_{malA} vector containing one auxiliary *malO* upstreaming of native *malO* operator in P_{malA} promoter	This study
pDG-O4	pDG-P_{malA} vector containing three auxiliary *malO* upstreaming of native *malO* operator in P_{malA} promoter	This study
pDG-O6	pDG-P_{malA} vector containing five auxiliary *malO* upstreaming of native *malO* operator in P_{malA} promoter	This study
pDG-Dx	pDG-P_{malA} vector containing an auxiliary *malO* upstreaming of native *malO* operator with different spacing (11-467 bp) in P_{malA} promoter	This study
pDG-P_{prsA}-Dx	pDG vector containing an auxiliary *malO* upstreaming of promoters P_{prsA} with different spacing (50-375 bp)	This study
pDG-P_{hrcA}-Dx	pDG vector containing an auxiliary *malO* upstreaming of promoters P_{hrcA} with different spacing (-40-325 bp)	This study
pDivOn	pDL vector containing *divIVA* gene controlled by maltose activated promoter from pMATE15-Δcre-3	This study
pDivOff	pDL vector containing *divIVA* gene controlled by maltose activated promoter from pMATE-g1M2	This study
pMATE-RibOn	pMATE15-Δcre-3 vector containing five rib operon genes (*ribD, ribE, ribA, ribH* and *ribT*)	This study
pVio	pMATE15-Δcre-3 vector containing violacein biosynthesis genes *vioABCDE* from *C. violaceum*	This study

Table S3. DNA sequences used in this study.

Name	Sequence	Notes/Ref.
gfpmut2 gene	atgagtaaaggagaagaacttttcactggagttgtcccaattcttgttgaattaga tggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggtgatgc aacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttc catggccaacacttgtcactactttctcttatggtgttcaatgcttttcaagataccc agatcatatgaaacagcatgactttttcaagagtgccatgcccgaaggttatgt acaggaaagaactatatttttcaaagatgacgggaactacaagacacgtgct gaagtcaagtttgaaggtgatacccttgttaatagaatcgagttaaaaggtattg attttaaagaagatggaaacattcttggacacaaattggaatacaactataact cacacaatgtatacatcatggcagacaaacaaaagaatggaatcaaagtta acttcaaaattagacacaacattgaagatggaagcgttcaactagcagaccat tatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccatt acctgtccacacaatctgccctttcgaaagatcccaacgaaaagagagacca catggtccttcttgagtttgtaacagctgctgggattacacatggcatggatgaac tatacaaatag	(2)
E2-crimson gene	atggatagcactgagaacgtcatcaagcccttcatgcgcttcaaggtgcacat ggagggctccgtgaacggccacgagttcgagatcgagggcgtgggcgagg gcaagccctacgagggcacccagaccgccaagctgcaagtgaccaaggg cggccccctgcccttcgcctgggacatcctgtccccccagttcttctacggctcc aaggcgtacatcaagcaccccgccgacatccccgactacctcaagcagtcct tccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgt ggtgaccgtgacccaggactcctccctgcaggacggcaccctcatctaccac gtgaagttcatcggcgtgaacttcccctccgacggccccgtaatgcagaagaa gactctgggctgggagccctccactgagcgcaactacccccgcgacggcgtg ctgaagggcgagaaccacatggcgctgaagctgaagggcggcggccacta cctgtgtgagttcaagtccatctacatggccaagaagcccgtgaagctgcccg gctaccactacgtggactacaagctcgacatcacctcccacaacgaggacta caccgtggtggagcagtacgagcgcgccgaggcccgccaccacctgttcca gtag	(28)
AmyS gene	atgaaacaacaaaaacggctttacgcccgattgctgacgctgttatttgcgctc atcttcttgctgcctcattctgcagcagcggcggccgcacccttaacggcacca tgatgcagtactttgaatggtatttgccagatgatggcacattatggaccaaagt agccaatgaagccaataacttatcctctcttggcattaccgctctttggctgccgc ccgcttacaaaggaacatcacgcagcgacgtagggtacggagtatacgactt gtatgacctcggcgaattcaatcaaaaagggaccgtccgcacaaaatatgga acaaaagctcaatatcttcaagccattcaagccgcccatgccgctggaatgca agtctacgccgatgtcgtgtttgaccataaaggcggcgctgacggcacggaat gggtggacgccgtcgaagtcaatccgtccgaccgcaatcaagaaatttcggg cacctatcaaatccaagcatggacgaaatttgattttccggggcggggcaaca cctactccagctttaagtggcgctggtatcactttgacggcgttgattgggacga aagcaggaaattaagccgcatctacaaattcagaggcatcggcaaagcgtg ggattgggaagtagacacagaaaacggaaactatgactacttaatgtatgca gaccttgatatggatcatcccgaagtcgtgaccgagctgaaaaactgggggga aatggtatgtcaacacaacgaacattgatgggtttcggcttgatgcagtcaaac atattaagtttcaatttttccctgattggttgtcgtatgtgcgttctcagactggcaaa ccgctctttaccgtcggggaatattggagctatgacatcaacaagttgcacaatt acattacgaaaacaaacggaacgatgtctttgtttgatgcaccgttacacaaca agttctacacggcttccaaatcaggggcgcatttgatatgagaacgctgatga caaatactctcatgaaagatcagccgacattggcggtcacattcgttgataatc atgacacagaacctggccaggcgctgcagagctgggtggacccgtggttca aaccgttggcttacgcgttcatcctgactcggcaggaaggatacccgtgcgtctt ttatggtgactattatggcattccacagtataatattccttcgctgaaaagcaaaa tcgatccgctcctcatcgcgagaagggattatgcgtacggaacgcagcatgat tatcttgatcactccgacatcatcgggtggacaagggagggtgttactgagaa	(29)

	accaggatcaggtctggcggcactgatcacagatggtccgggaggaagcaa gtggatgtatgttggcaagcagcatgcgggaaaggtgttctatgatcttacagg aaatcggtcagatacggtaacgatcaattcagatggatggggtgagttcaagg tgaacggaggttcggtttcagtttgggttcctagaaagacgacggtttctacgat agcgagaccgataacaacgcgtccgtggactggtgagtttgtacgttggacgg agcctagactggtggcatggccttaa	
hFGF21 gene	atgaaatacctattgcctacggcagccgctggattgctattactcgcggctcaac ccgcaatggcccatccgattccggatagcagcccgctgctgcagtttggtggtc aggtgcgtcagcgttatctgtataccgatgatgcgcagcagaccgaagcaca cctggaaattcgtgaagatggtaccgtgggcggtgcggcggatcagagcccg gaaagcctgctgcagctgaaagcgctgaaaccgggtgtgattcagattctggg tgttaaaaccagccgttttctgtgccagcgtccggatggtgcgctgtatggcagc ctgcattttgatccggaagcgtgcagctttcgtgaactgctgctggaagatggtta taacgtgtatcagagcgaagcgcatggtctgccgctgcatctgccgggtaaca aaagcccgcatcgtgatccggcgccgcgtggtccggcgcgttttctgccgctgc cgggtctgccgccggctctgccggaaccgccgggtattctggctccgcagccg ccggatgttggtagctctgatccgctgtctatggtgggcccgagccagggtcgt agcccgagctatgcgagccatcatcatcatcattaa	(30)
DPE gene	atgaaatatggtatttattacgcttattgggaaaaggaatggaatggagattaca aatattatatagataaaatttcaaaattaggttttgatattctggaaatttcttgcgg cgctttttctgactattacacgaaagatcaggagttaattgatattggaaaatatg cgaaagaaaaaggcgtaacattgacagcagggtatggacctcattttaatga aagcctgtcatcttcagaacccaatacgcagaaacaagcaatcagtttttgga aagagacgctccggaaattgaagttaatggatattcatattgttggaggcgcac tctatggttattggcctgtagattattccaaacctttgataagaaaagggatttag agaattccattaaaaacatgaaaattattagtcagtatgctgaagaatatgacat aatgatggggatggaagttcttaaccgttttgaaggctatatgttgaatacatgcg atgaagcgttggcatacgttgaagaggttggctcttctaatgttggtgttatgttag atacttttcacatgaatatagaggaagataatatagcagcagccattcgtaaag caggagataggctttatcacttccatataggagaaggaaatcgtaaagtacca ggaaaaggtatgcttccttggaatgagataggacaggcattgcgagatataaa ctaccaacatgcagcagttatggagccatttgtaatgcagggaggaacagta gggcatgacattaaaatatggagagatatcattggaaactgttctgaagttacat tagatatggacgctcaaagtgcgttgcactttgtaaaacatgtatttgaagtctaa	(31)
subC gene	atgatgcgcaaaaaaagcttctggctgggcatgctgacggccttcatgctggtc ttcacgatggccttctccgatagcgctagcgccgcccaaccagccaagaacgt cgagaaggactacatcgtcggctttaagagcggcgtcaagacagccagcgt caagaaggacatcatcaaggagtccggcggaaaggtcgacaagcagttcc gcatcatcaacgccgccaaagccaaactggacaaggaggctcttaaggaa gtcaaaaacgatccggacgtcgcttacgtcgaagaggatcacgttgcccatg cccttgcccagacggttccgtacggcatcccgctgattaaagccgacaaagtc caagcccaaggcttcaaaggcgctaacgtcaaggtcgccgttcttgatacgg gcatccaagccagccatccggatctgaatgtcgtcggcggagctagcttcgttg ccggcgaagcctataacacggatggcaatggccatggcacacatgtcgctg gcacagttgctgcccttgacaatacaacgggcgttcttggcgttgccccaagcg tcagcctttatgccgtcaaggttcttaatagcagcggcagcggcacatactccg gcattgttagcggcattgagtgggctacgacgaatggcatggacgtcatcaac atgagccttggaggcccaagcggatccacggctatgaagcaagctgtcgac aacgcctatgccagaggagttgttgttgtcgccgctgctggcaatagcggctcc agcggcaatacgaacacgatcggctacccggccaaatacgatagcgttattg ccgtcggcgctgtcgacagcaacagcaatagagccagcttcagctccgttgg cgctgagcttgaagttatggctccgggcgccggcgtctacagcacatacccga cgagcacgtatgccacacttaatggcacgtccatggcctccccgcatgttgctg gagctgctgctcttattcttagcaaacacccgaatctgtccgccagccaagttcg caatcgccttagctccacagccacgtatctgggctccagcttctactacggcaa gggacttatcaacgttgaagctgccgcccag	(32)

ChiA1 gene	atggcatacgacagtcgttttgatgaatgggtacagaaactgaaagaggaaa gctttcaaaacaatacgtttgaccgccgcaaatttattcaaggagcggggaag attgcaggactttctcttggattaacgattgcccagtcggttggggcctttgaagta aatgctgctccaaccgcaccaagtatcgatatgtacggttccaataaccttcaat tttctaaaattgaattggcaatggaaaccacatctggctacaacgacatggtca aataccatgaactggctaagatcaaagtgaaatttaaccagtggagtggcac atctggcgacacttacaacgtctactttgacggtgtaaaagtggcaacaggcg ctatcactggcagtcaaaccacagcttcgtttgaatatggtcaaggcggcttgta ccaaatggaaatcgaagcgtgtgacgcaacaggttgttctaagagcgctccg gtagaaattaccattgcagatacagacggctcacacttgaagcctctgacgat gaatgttgatccgaacaacaagagctacaacaccgatccaagtatcgtgatg ggtacttattttgttgaatggggcatctacggtcgtgattacactgtcgacaacat gccagttgataacctaactcacatcctttacggctttatcccaatttgtggtccaaa cgaatcagtaaaatcagttggtggtaacagctttaatgcactgcaaacggcat gtcgtggtgtgaatgattacgaagtggttatccatgacccgtgggcagcttatca gaagagcttccctcaagcaggtcatgaatacagcacgccaatcaagggtaa ctacgcaatgctaatggcgttgaaacaacgtaacccggatctaaaaattatcc catctatcggtggttggacactttctgacccattctacgacttcgttgataagaag aatcgtgacacgtttgtcgcgtcagttaagaaattcctgaaaacttggaaatttta cgacggcgtagatattgactgggaattccctggtggcggcggcgctgcagca gataagggtgaccctgtaaacgatggtcctgcatacattgcattgatgcgtgaa ctacgtgtaatgctagatgaacttgaagcagaaacaggtcgtacttacgagct aacttcagcaatcggtgttggttacgacaagattgaagacgtagattacgcag acgcggttcagtacatggactacatctttgcgatgacttacgacttctacggcgg ctggaacaacgttcctggtcaccaaactgctctttactgtggctcattcatgcgtc ctggtcagtgtgatggcggcggcgtggatgaaaacggcgaaccgtacaaag gtccagcatacactgcagataacggtatccagcttcttctagcgcaaggtgttcc tgcaaataaactggttcttggtacagcgatgtatggtcgtggttgggaaggtgta acacctgatacgctaacagatccaaatgacccaatgaccggtactgcaacag gcaaactgaaaggcagcacagctcaaggtgtttgggaagatggcgtaatcg actacaaaggtattaagtcatttatgctaggtgcgaacaacactggcatcaacg gctttgaatacggctatgatgcgcaagcagaagcaccttgggtgtggaaccgtt cgactggtgagctaatcacatttgacgatcatcgttctgtgttagcgaaaggcaa ctacgcaaaatctctaggtctagcaggtctattctcttgggagattgatgcagata acggcgacatccttaatgcaatgcacgaaggtatggctggcggcgtcgttacc ccaccaaaccgtaaaccaacggcagcagcaggcgcagaccaagcagtaa caggtcctgcaagcgttgtgcttgatggcagcaactcaactgactctgacggta caatcgcaagttacgcttgggagcaagtatcaggtactgcagtcgttctatctgg cgcgaacacagcaacagcaagctttgatgcggtagaagtcgcagctgaaga gcaactaacgtttaagctgaccgtaacagataacgaaggcgcaacggcttct gatcttgttgtagtcaccgttaagccagcaggcgtgattgatccaccaaacaca gcgccagttgcacaggtttctgcaccagcaacggcaagcgctggtgacgtgg ttgtgattgatgcttcagcttctagcgatgctgacaacgacacactaacgttcgat tggacgctaccacaaggtctaaatgctacggtcaacggtgcgaaagtaacgtt cacagcagcagaatacccacaagacacaagcctaagcttcacggtaagcgt aagtgatggtcaagcagtatcaactgcaagtgcaaccgttgttgtagcgaagc acagcacaggcggtggtacttgtactaacgcatgggatgcaacggcagtttac actggtggtgatcaagtgacttacgcaggtaaaacttgggaagcgaaatggtg ggactcaagggtcaagatcctagcaagtctggcgaatggggtgtttggaaag aagttggtccatctaactgtaactaaacactgccatcatcatcatcatcattaa	(33)
appA gene	atgcaaagcgaaccggagctgaaactggagaacgtggttatcgtgagccgc cacggagtgagagcgccgacgaaatttacgcagctgatgcagtgcgttacgc cggatgcgtggcctacgtggccggttaaactgggcgaacttacaccgagagg ctgcgaactgattgcgtatctgggccactattggcgccaacgtttagtggcagat gagctgctgccgaaatgcggatgtccgcaaagcggacaagttgcgattattgc agatgtggatgaacgcacgcgcaaaactggtgaatgctttgcggctggtctgg	(34)

	cgccggattgcgcgattacagtgcatcatcaagctgatacgagcagccccgat ccgctgtttaacccgctgaaaacgggcgtgtgccagctggatgtggcaaacgt gacacgcgcaattctggaaagagcgggaggcagcatcgcggatttcactggt cattaccagacggcgtttcgcgaactggagcgcgttctgaatttcccgcagagc aattgctgtttaaagcgcgaaaaacaagatgaaagctgctctttaacacaagc tctgccttcagagcttaaggtgagcgcggacaatgtgagccttacgggcgcag tgtctttagcatcaatgctgacagagatctttctgctgcaacaagctcaaggcat gccggaaccgggctggggacgcattacagatagccaccagtggaacacgc tgctttctttacacaatgcgcagtttgaccttctgcagagaacaccggaagtggc aagatcaagagcgacgccgctgctggatctgattaaaacggcactgacgcc gcatcctccgcagaaacaagcttatggagtgacgctgccgacaagcgttctgt tcattgcgggccatgacacgaatctggcgaaccttggaggagcgcttgaactg aactggacactgccgggccaaccggataatacaccgcccggtggagaactt gtttttgagcgctggagacgtttaagcgataacagccagtggattcaagtgtctttt agtgttccagacacttcagcagatgcgcgacaaaacaccgctgtctttaaaca caccgccgggagaagtgaaacttacgctggctggttgcgaagaaagaaacg cgcaaggtatgtgtagccttgctggttttacgcagatcgtgaacgaggcacgca ttccggcgtgcagcctttaataa	
luc gene	atggaagacgccaaaaacataaagaaaggcccggcgccattctatccgctg gaagatggaaccgctggagagcaactgcataaggctatgaagagatacgc cctggttcctggaacaattgcttttacagatgcacatatcgaggtggacatcactt acgctgagtacttcgaaatgtccgttcggttggcagaagctatgaaacgatatg ggctgaatacaaatcacagaatcgtcgtatgcagtgaaaactctcttcaattctttt atgccggtgttgggcgcgttatttatcggagttgcagttgcgcccgcgaacgac atttataatgaacgtgaattgctcaacagtatgggcatttcgcagcctaccgtggt gttcgtttccaaaaaggggttgcaaaaaattttgaacgtgcaaaaaaagctcc caatcatccaaaaaattattatcatggattctaaaacggattaccagggatttca gtcgatgtacacgttcgtcacatctcatctacctcccggttttaatgaatacgatttt gtgccagagtccttcgatagggacaagacaattgcactgatcatgaactcctct ggatctactggtctgcctaaaggtgtcgctctgcctcatagaactgcctgcgtga gattctcgcatgccagagatcctatttttggcaatcaaatcattccggatactgcg attttaagtgttgttccattccatcacggttttggaatgtttactacactcggatatttg atatgtggatttcgagtcgtcttaatgtatagatttgaagaagagctgtttctgagg agccttcaggattacaagattcaaagtgcgctgctggtgccaaccctattctcctt cttcgccaaaagcactctgattgacaaatacgatttatctaatttacacgaaattg cttctggtggcgctcccctctctaaggaagtcggggaagcggttgccaagagg ttccatctgccaggtatcaggcaaggatatgggctcactgagactacatcagct attctgattacacccgagggggatgataaaccgggcgcggtcggtaaagttgt tccatttttgaagcgaaggttgtggatctggataccgggaaaacgctgggcgtt aatcaaagaggcgaactgtgtgtgagaggtcctatgattatgtccggttatgtaa acaatccggaagcgaccaacgccttgattgacaaggatggatggctacattct ggagacatagcttactgggacgaagacgaacacttcttcatcgttgaccgcct gaagtctctgattaagtacaaaggctatcaggtggctcccgctgaattggaatc catcttgctccaacaccccaacatcttcgacgcaggtgtcgcaggtcttcccga cgatgacgccggtgaacttcccgccgccgttgttgttttggagcacggaaaga cgatgacggaaaaagagatcgtggattacgtcgccagtcaagtaacaaccg cgaaaaagttgcgcggaggagttgtgtttgtggacgaagtaccgaaaggtctt accggaaaactcgacgcaagaaaaatcagagagatcctcataaaggccaa gaagggcggaaagatcgccgtgtaa	(35)
bgaB gene	atgggcaattctgttagttacgacggcgaacgtcgcgttaacttcaacgaaaac tggcgcttccaacgcgaaaccaacggttctattgcaggtgcacagaatccgg gttttgacgatagcagttggcgtaaactgaatctgccgcacgattggagcatcg aactggacttcaacaaaaacagcctggcaacccacgaaggcggttatctgg acggcggcattggttggtatcgtaaaaccttcaccatcccggaaagcatgaaa ggcaaacgcatcagcctggattttgacggcgtctacatgaacagcaccaccta tctgaacggcgaagttctgggtacctatccgtttggctataacgcgttcagctac	(36)

	gacatcagcgacaaactgtacaaagatggtcgcgcaaacgttctggtcgttaa agttaacaatacccagccgagcagccgttggtattctggcagcggcatttatcg caacgtctatctgaccgttaccgatccgattcacgttgcacgttacggtacctttgt taccaccccgaacctggagaaaagcatcaaagaggatcgcgcggacgtca acatcaaaaccaaaatcagtaatgacgcagcggaagcgaaacaggttaaa atcaaaagcaccatctacgacggcgcaggtaataccgttcagaccgtcgaa accgaagaaaaaaccgcagcagcaggtaccgttaccccgtttgaacagaa caccgtcatcaaacagccgaaactgtggagcatcgacaaaccgtaccgctat aacctggtcaccgaagttatcgtcggcggtcaaaccgttgatacctacgaaac caaattcggcgtccgctacttcaaattcgacgaaaacgaaggcttcagtctga acggcgaatacatgaaactgcacggcgtttccatgcatcatgatctgggcgca ctgggcgcagcaaccaacgcacgcggcgttgaacgtcaaatgcaaatcatg aaagacatgggcgtcaacgcgattcgcgttacccataatccggcaagtccgg aactgctggaagcagcaaacaaactgggtctgttcatcatcgaagaagcgttc gatagctgggcacagagcaaaaaaccgtacgactacggtcgctttttcaacg cctgggcggaacacgatatcaaagagatggtggatcgcggcaaaaacgaa ccggccattattatgtggagcatcggtaacgaaatctacgataccaccaacgc ggcaggcgttgaaaccgcacgtaatctggttggttgggtcaaagaaattgata ccacccgtccgaccaccattggcgaagataaaacccgcggcgataaagtca acgtcaccccgatcaacagctacatcaaagaaatttttaacatcgtggatgtgg ttggcctgaactacagcgaaaacaactacgacggctaccataaacagaatc cgagctggaaactgtacggcagcgaaaccagttctgcaacccgtagtcgcg gcgtttatacccatccgtaccagtacaaccagagcaccaaatacgcggattta cagcagagcagctacgataacgattacgttggttggggtcgtaccgcagaag acgcttggaaatacgatcgcgacctgaaacatattgcgggtcagtttatctgga ccggtttcgattacattggcgaaccgaccccgtattacaacagctatccggcga aaagcagctactttggcgcagttgataccgcaggttttccgaaagacatcttcta ctactaccagagccagtggaaaaaagagccgatggtccatctgctgccgcat tggaattggaaagagggcgagaaagttcgtgttctggcatataccaacgcaa gcaaagtcgaactggttctgaacggcgaaagcctgggcgagaaaaactacg acaacaaacagaccagttggggcgcaccgtataaagaaaccaaagacgg caaaacctacctggagtgggcagtaccgtttaaaccgggtaaactggaagcg gttgcgaaagacgagaacggcaaagtcatcgcacgtgatcaagttgttaccg caggcgaaccggcatctgtacgtctgaccgcagatcgtaaagtcgttaaagc ggacggtaccgatctgagctttattaccgcggacatcgtcgatagcaaaggtat cgttgttccggacgcggatcatctgattacctttaacgtcaccggtcaaggcgaa ctggcaggcgttgataacggtaacgcaagcagcgttgagcgctacaaagac aacaaacgtaaagcgtttagcggtaaagcactggcaattgtccagagcagca aactgagcggcaaaattaccgtacacgcgagcgtggcaggtctgagcagcg atagcaccagcgtttttaccgttaccccgtaa	
manA gene	atgaaatttgtaaaaagaaggatcattgcacttgtaacaattttgatgctgtctgtt acatcgctgtttgcgttgcagccgtcagcaaaagccgctcacactgtcagccct gtaaaccctaacgcacaaccgactactaaggcagtgatgaactggctggca catctgccaaaccgtactgagagccgtgtaatgtccggtgcattcggtggttac agcctggatactttcagcactgctgaggcagaccgtatcaagcaggcaactg gtcagctgccggctatctacggttgtgattacgctcgtggttggctggaaccaga gaaaatcgctgataccatcgattactcctgcaaccgtgacctgattgcttactgg aaaagcggtggtatcccgcagattagcatgcacctggctaacccagcgttcac ctctggtcattacaaaacgcagatttccaactcccagtacgaacgtatcctgga ctcctccaccccggagggcaaacgtctggaagcgatgctgtctaaaatcgcg gatggtctgcaggaactggagaacgaaggtgttccggttctgttccgcccgctg cacgaaatgaatggcgaatggttctggtggggtctgacccaatataatcagaa agactctgaacgcatctctctgtacaaacagctgtacgtgaaaatctacgacta catgaccaaaacccgtggcctggaccacctgctgtgggtgtacgcgccggat gccaaccgcgactttaaaacggacttctatccgggcgcgtcttacgtggatatc gtaggcctggatgcttacttcgacgacccgtatgccattgatggctatgaagaa	(37)

	ctgacctctctgaacaagccgttcgcgtttaccgaagtcggcccgcagaccac caatggcggtctggattatgcgcgcttcattcacgccatcaaagaaaaatatcc gaaaacgacctattttctggcgtggaacgacgaatggtctccggccgttaaca agggcgcggataccctgtatctgcacccgtggacgctgaacaaaggcgaaa tttgggacggcgactctctgaccccggttgttgaataa	
vioABCDE expression cassette	*taagattacaaaaaaggtaaaaaaaccaaatctctcagacataaggcaaat gagaaatttctcgtgcttggtgaaaaaacactaaagttgatcaaatgacctaag tgcgccaaacgtgttacgggacgagctatctcatggtataaatggaattgttttta aatagaaggaggtcgtcat***atgaagcactcttctgacatctgtatcgttggt gctggtatctctggtttgacctgtgcttctcacttgttggactctccagcttgt agaggtttgtctttgagaatcttcgacatgcaacaagaagctggtggtag aatccgatctaagatgttggacggtaaggcttctatcgaattgggtgctg gtagatactctccacaattgcacccacacttccaatctgctatgcaacact actctcaaaagtctgaagtttacccattcacccaattgaagttcaagtctc acgttcaacaaaagttgaagagagctatgaacgaattgtctccaagattg aaggaacacggtaaggaatctttcttgcaattcgttagtagataccaagg tcacgactctgctgttggtatgatccgatctatgggttacgacgctttgttct tgccagacatctctgctgaaatggcttacgacatcgttggtaagcaccca gaaatccaatctgttaccgacaacgacgctaaccaatggttcgctgctg aaacaggtttcgctggtttgatccaaggtatcaaggctaaggttaaggct gctggtgctagattctctttgggttacagattgttgtctgttagaaccgacg gtgacggttacttgttgcaattggctggtgacgacggttggaagttggaa cacagaaccagacacttgatcttggctatcccaccatctgctatggctgg tttgaacgttgacttcccagaggcttggtctggtgctagatacggttctttg ccattgttcaagggtttcttgacctacggtgaaccatggtggttggactac aagttggacgaccaagttttgatcgttgacaacccattgagaaaaatcta cttcaagggtgacaagtacttgttcttctacaccgactctgaaatggctaa ctactggagaggttgtgttgctgaaggtgaagacggttacttggaacaaa tcagaacccacttggcttctgctttgggtatcgttagagaaagaatcccac aaccattggctcacgttcacaagtactgggctcacggtgttgagttctgta gagactctgacatcgaccacccatctgctttgtctcacagagactctggt atcatcgcttgttctgacgcttacaccgaacactgtggttggatggaaggt ggtttgttgtctgctagagaggctagtagattgttgttgcaaagaatcgctg ctttgagaagatagcggtgaaaggaggaataattatgagcattctggattt cccgcgtatccacttccgtggctgggcccgtgtcaatgcgccgaccgc gaaccgcgatccgcacggccacatcgatatggccagcaataccgtgg cgatggcgggtgagccgttcgacctggcacgccatcctacggagttcc accgtcacctgcgctccctgggtccgcgcttcggcttggatggtcgtgct gacccggaaggcccgttcagcctggccgagggctacaacgctgccg gtaacaaccacttttcgtgggagagcgcaaccgttagccacgtgcaatg ggatggcggtgaggcggatcgtggtgacggtctggtcggtgctcgtttg gcactgtggggtcactacaatgattatctgcgtaccaccttcaatcgtgct cgttgggtcgacagcgacccgacgcgccgtgacgctgcacaaatctat gcgggccaattcaccattagcccggctggtgccggtccgggtacgccg tggctgtttacggcagacattgatgatagccatggtgcacgttggacgcg tggcggccacattgcagagcgtggcggccacttcttggatgaagagttt ggtctggcacgcctgtttcagttctctgtgccgaaagatcacccacatttt ctgtttcacccgggtccgtttgattccgaggcctggcgtcgtctgcaattg gctctggaggatgacgacgttctgggtctgaccgtgcaatatgcgttgtt caatatgagcaccccgcctcagccgaacagcccggtttttcacgatatg gtcggtgttgtcggtctgtggcgtcgtggtgaactggcgagctacccgg ctggtcgtctgctgcgtccgcgtcaaccgggtctgggtgacctgaccct gcgcgtcaacggtggtcgcgttgcgctgaatttggcgtgtgccattccgt tcagcactcgtgccgcgcagccaagcgcaccggaccgcctgacccc ggacctgggtgccaaactgccgctgggcgatctgctgctgcgtgatga ggacggcgcactgttggcacgtgtgccgcaggctctgtaccaagacta*	*P_{malA}-OM15-Δcre-3,* ***vioABCDE***, fd Terminator

	ttggacgaatcacggtattgtggacctgccgctgctgcgcgaaccgcgt **ggtagcttgaccctgagcagcgaactggcggagtggcgtgagcaaga** **ctgggtcacccaaagcgacgcgtctaacctgtacctggaggcaccgg** **atcgccgtcacggtcgctttttccctgagagcatcgcgctgcgcagctac** **tttcgcggtgaagcgcgtgcgcgtccggatatcccgcatcgtatcgagg** **gcatgggcctggtcggcgtcgaatctcgtcaggatggcgacgctgcgg** **aatggcgtctgacgggtctgcgtccgggtccggcacgcattgttctgga** **cgatggtgccgaggcgatccctctgcgtgttctgcctgacgattgggcg** **ctggatgacgcgaccgtcgaagaagtggattacgccttttgtaccgcc** **acgttatggcgtattacgagctggtgtatccattcatgagcgacaaggtgt** **tttccctggctgatcgttgcaaatgtgaaacgtacgcacgtctgatgtggc** **agatgtgtgatccgcagaaccgcaacaagtcctattacatgccgagcac** **ccgcgaactgtcggcaccgaaagctcgtttgttcttgaagtatctggccc** **acgtggaaggccaggcacgcctgcaagcacctccgccagcgggtcc** **ggcacgcattgaatctaaagcccagttggcggcagagctgcgtaaagc** **cgtcgacctggagctgtctgtgatgctgcaatacctgtacgcggcgtata** **gcattccgaactatgcacagggccaacaacgtgttcgtgacggtgcgtg** **gaccgccgagcagctgcaactggcgtgcggtagcggtgaccgtcgcc** **gtgatggcggtattcgtgcagcactgctggaaattgctcatgaagaaatg** **attcattacctggtcgttaacaacctgctgatggccctgggcgagccgtt** **ctacgcgggtgtcccgctgatgggcgaagcggcacgtcaggcgtttgg** **cctggacaccgagttcgctctggaaccgtttagcgaaagcacgctggc** **acgttttgttcgtctggaatggccgcactttatcccagcaccgggcaaat** **ccatcgcggactgctatgccgccattcgtcaggcgtttttggatctgccg** **gacttgtttggtggcgaggcaggtaagcgtggcggtgaacaccacctgt** **tcctgaatgagctgaccaaccgtgcgcatccgggttatcaactggaagtt** **ttcgatcgcgactcggcgctgtttggtattgcatttgtgaccgatcagggc** **gaaggtggcgctctggacagcccgcactacgaacatagccattttcaa** **cgtctgcgtgaaatgagcgcgcgtatcatggctcaaagcgcaccgttcg** **aaccggcgctgccggcgttgcgtaatccggttctggatgagagcccgg** **gttgccaacgtgtcgcagacggtcgtgcgcgtgcgctgatggcattgta** **ccaaggcgtttatgagctgatgtttgcgatgatggcgcagcacttcgccg** **tgaaaccgctgggtagcttgcgtcgcagccgcctgatgaacgcagcaa** **tcgatctgatgaccggtctgttgcgtccgctgagctgcgcgctgatgaac** **ctgccaagcggcatcgccggtcgcacggccggtccgccgctgccgg** **gtccggttgacacccgtagctatgacgactacgcgctgggctgtcgcat** **gctggcacgccgttgcgagcgtctgctggagcaggcgagcatgctgg** **aaccgggttggctgccggatgcgcagatggagctgctggatttctatcg** **tcgccaaatgctggacttggcgtgcggcaaactgagccgcgaggccta** **agtgcgaaaggaggtttgacaatgaaaagagctatcatcgttggtggtg** **gtttggctggtggtttgaccgctatctacttggctaagagaggttacgaag** **ttcacgttgttgaaaagagaggtgacccattgagagacttgtcttcttacgt** **tgacgttgtttctagtagagctatcggtgtttctatgaccgttagaggtatca** **agtctgttttggctgctggtatcccaagagctgaattggacgcttgtggtg** **aaccaatcgttgctatggctttctctgttggtggtcaatacagaatgagag** **aattgaagccattggaagacttcagaccattgtctttgaacagagctgctt** **tccaaaagttgttgaacaagtacgctaacttggctggtgttagatactactt** **cgaacacaagtgtttggacgttgacttggacggtaagtctgttttgatcca** **aggtaaggacggtcaaccacaaagattgcaaggtgacatgatcatcgg** **tgctgacggtgctcactctgctgttagacaagctatgcaatctggtttgag** **aagattcgagttccaacaaaccttcttcagacacggttacaagaccttgg** **ttttgccagacgctcaggctttgggttacagaaaggacaccttgtacttctt** **cggtatggactctggtggtttgttcgctggtagagctgctaccatcccag** **acggttctgtttctatcgctgtttgtttgccatactctggttctccatctttgac** **caccaccgacgaaccaaccatgagagctttcttcgacagatacttcggt** **ggtttgccacgcgacgctcgcgacgaaatgttgagacaattcttggcta**	

	agccatctaacgacttgatcaacgttcgatcttctaccttccactacaagg gtaacgttttgttgttgggtgacgctgctcacgctaccgctccattcttggg tcaaggtatgaacatggctttggaagacgctagaaccttcgttgaattgtt ggacagacaccaaggtgaccaagacaaggctttcccagagttcaccg aattgagaaaggttcaagctgacgctatgcaagacatggctagagctaa ctacgacgttttgtcttgttctaacccaatcttcttcatgagagctagataca ccagatacatgcactctaagttcccaggtttgtacccaccagacatggct gaaaagttgtacttcacctctgaaccatacgacagattgcaacaaatcca aagaaagcaaaacgtttggtacaagatcggtagagttaactagagcgg aaaggaggaataattatgaaaatcttggttatcggtgctggtccagctggt ttggttttcgcttctcaattgaagcaagctagaccattgtgggctatcgaca tcgttgaaaagaacgacgaacaagaagttttgggttggggtgttgttttgc caggtagaccaggtcaacacccagctaacccattgtcttacttggacgc tccagaaagattgaacccacaattcttggaagacttcaagttggttcacc acaacgaaccatctttgatgtctaccggtgttttgttgtgtggtgttgaaag aagaggtttggttcacgctttgagagacaagtgtcgatctcaaggtatcg ctatcagattcgaatctccattgttggaacacggtgaattgccattggctg actacgacttggttgttttggctaacggtgttaaccacaagaccgctcact tcaccgaggctttggttccacaagttgactacggtagaaacaagtacatc tggtacggaacctctcaattgttcgaccaaatgaacttggttttcagaacc cacggtaaggacatcttcatcgctcacgcttacaagtactctgacaccat gtctaccttcatcgttgaatgttctgaagaaacctacgctagagctagatt gggtgaaatgtctgaagaggcttctgctgaatacgttgctaaggttttcca agctgaattgggtggtcacggtttggtttctcaaccaggtttgggttggag aaacttcatgaccttgtctcacgacagatgtcacgacggtaagttggtttt gttgggtgacgctttgcaatctggtcacttctctatcggtcacggaaccac aatggctgttgttgttgctcaattgttggttaaggctttgtgtaccgaagac ggtgttccagctgctttgaagagattcgaagaaagagctttgccattggtt caattgttcagaggtcacgctgacaacagtagagtttggttcgaaaccgt tgaagaaagaatgcacttgtcttctgctgagttcgttcaatctttcgacgct agaagaaagtctttgccaccaatgccagaggctttggctcaaaacttga gatacgctttgcaaagatagatgggaaaggaggaataattatggaaaac agagaaccaccattgttgccagctagatggtcttctgcttacgtttcttact ggagtccaatgttgccagacgaccaattgacctctggttactgttggttcg actacgaaagagacatctgtagaatcgacggtttgttcaacccgtggtct gaaagagacaccggttacagattgtggatgtctgaagttggtaacgctg cttctggtagaacctggaagcaaaaggttgcttacggtagagaaagaac cgctttgggtgaacaattgtgtgaaagaccattggacgacgaaaccggt ccattcgctgaattgttcttgccacgcgacgttttgagaagattgggtgct agacacatcggtagaagagttgttttgggtagagaagctgacggttgga gataccaaagaccaggtaagggtccatctaccttgtacttggacgctgct tctggtaccccattgagaatggttaccggtgacgaggctagtagagcttc tttgagagacttcccaaacgtttctgaagctgaaatcccagacgctgtttt cgctgctaagagatagcatatgagttatgcagtttgtagaatgcaaaaagtg aaatcaggggatcctctagagtcgagctcaagctagcttggtacgtaccaga tctgagatcacgcgttctagaggtcgaaattcacctcgaaagcaagctgataa accgatacaattaaaggctccttttggagccttttttttggagattttcaacgtgaa aaaattattattcgcaattccaagctaattcacctcgaaagcaagctgataaac cgatacaattaaaggctccttttggagccttttttttggagattttcaacgtgaaaa aattattatt**	
Lysine activated riboswitch LysRS16	gtactacctgcgctagcgcaggccagaagaggcgcgttgcccaagtaacgg tgttggaggagccagtcctgtgataacacctgaggggtgcatcgccgaggtg attgaacggctggccacgttcatcatcggctacaggggctgaatcccctgggtt gtcaccagaagcgttcgcagtcgggcgtttcgcaagtggtggagcacttctgttt atcgaggagcatcgcgacac	(38)

Lysine activated riboswitch LysRS59	gtactacctgcgctagcgcaggccagaagaggcgcgttgcccaagtaacgg tgttggaggagccagtcctgtgataacacctgaggggtgcatcgccgaggtg attgaacggctggccacgttcatcatcggctacaggggctgaatcccctgggtt gtcaccagaagcgttcgcagtcgggcgtttcgcaagtggtggagcacttcttgtt tacgaacgtgactacttcgatttggcgacac	(38)
Theophylline activated riboswitch TheoRS1	aaggaggtcgtcatggtgataccagcatcgtcttgatgcccttggcagcaccct gctaaggaggaataatt	(39)
Theophylline activated riboswitch TheoRS2	ggtgataccagcatcgtcttgatgcccttggcagcaccctgctaaggaggaat aatt	(39)

All functional elements are indicated by lowercase letters. The italic and bold sequences denote the promoter and protein coding sequence, respectively.

Table S4. Primers used in this study.

Name	Sequence	Notes
P_{malA}-F	CGCGGATCCCTTTTGTCCCCTGCCTTTTCT	Construction of pDG-P_{malA}
P_{malA}-R	CGGGGTACCATGACGACCTCCTTGATAAATTTTACAATTCCATTTA	
pDG2_linear-1.for	TCTAAATTCACGCACAATTGGATGT	Construction of the 15 truncated mutants of the P_{malA} promoter
pDG2_linear-1.rev	GGATCCCCGGGAATTCTCATG	
pDG2-linear-2.for	TGGATGTTTTATATAAATGATTATAAATAATTCGG	
pDG2_linear-2.rev	AAAGGCAGGGGACAAAAGG	
pDG2-linear-3.for	GATTATAAATAATTCGGCATGTATCCG	
pDG2_linear-3.rev	ATTGTGCGTGAATTTAGAAAAGGC	
pDG2-linear-4.for	ATGTATCCGAATCGTACAAAAGAACC	
pDG2_linear-4.rev	ATTTATATAAAACATCCAATTGTGCGTG	
pDG2-linear-5.for	AAAGAACCTTTTCATAAGAATTGGAAG	
pDG2_linear-5.rev	GCCGAATTATTTATAATCATTTATATAAAACATCC	
pDG2-linear-6.for	AATTGGAAGGGCGTATATTCACT	
pDG2_linear-6.rev	TGTACGATTCGGATACATGCC	
pDG2-linear-7.for	TCACTTAAAATTCACAGTTGGTGAG	
pDG2_linear-7.rev	CTTATGAAAAGGTTCTTTTGTACGATTCG	
pDG2-linear-8.for	TGGTGAGACTTTAAGATTACAAAAAAGGT	
pDG2_linear-8.rev	ATATACGCCCTTCCAATTCTTATGA	
pDG2-linear-9.for	ACAAAAAAGGTAAAAAAACCAAATCTCTC	
pDG2_linear-9.rev	ACTGTGAATTTTAAGTGAATATACGCC	
pDG2-linear-10.for	CCAAATCTCTCAGACATAAGGCAAATG	
pDG2_linear-10.rev	AATCTTAAAGTCTCACCAACTGTGAA	
pDG2-linear-11.for	AGGCAAATGAGAAATTTCCCG	
pDG2_linear-11.rev	TTTTTTTACCTTTTTTGTAATCTTAAAGTCTC	
pDG2-linear-12.for	CCGCTCTATGGGAAAAAACACT	
pDG2_linear-12.rev	TATGTCTGAGAGATTTGGTTTTTTTACC	
pDG2-linear-13.for	CACTAAAGTTGATCAAATGACCTAAGT	
pDG2_linear-13.rev	GAAATTTCTCATTTGCCTTATGTCTG	
pDG2-linear-14.for	GACCTAAGTGCGCCAAACG	
pDG2_linear-14.rev	TTTTTTCCCATAGAGCGGGAAA	
pDG2_linear-15.for	GTGTTACGGGACGAGCTATC	
pDG2_linear-15.rev	ATTTGATCAACTTTAGTGTTTTTTCCCA	
malO-up-F	TTTCTCATTTCCCGCTCTATGGGAAAAAAC	Construction of the *malO* truncated mutants in pDG-P_{malA}
malO-up-R	TTTGCCTTATGTCTGAGAGATTTG	
malO-IR1-F	GGGAAAGCTCTATGGGAAAAAACACTAAAGT	
malO-IR1-R	TTTCTCATTTGCCTTATGTCTGAG	
malO-IR2-F	TTTCCCAAACACTAAAGTTGATCAAATGACCT	
malO-IR2-R	ATAGAGCGGGAAATTTCTCATTTG	

malO-spacer-F	CGAGATAGGGAAAAAACACTAAAGTTGATC	
malO-spacer-R	GGGAAATTTCTCATTTGCCTTATG	
malO-F	ACACTAAAGTTGATCAAATGACCT	
malO-R	TTTGCCTTATGTCTGAGAGATTTG	
*malO*2-F	TGAGAAATTTCTCGTGCTTGGTGAAAAACAGACA TAAGGCAAATGAGAAA	Construction of the mutants of P_{malA} promoter with different numbers of tandem *malO* operators
*malO*2-R	AGAGATTTGGTTTTTTTACCTTTTTTGTAATCTTAA A	
*malO*4-F	AAAAGGTAATGAGAAATTTCTCGTGCTTGGTGAAA AAAAAAACCAAATCTCTTGAGAAAT	
*malO*4-R	TTGTAATTTTTCACCAAGCACGAGAAATTTCTCAT CTTAAAGTCTCACCAACTGTGAATT	
*malO*6-F	CACAGTTTGAGAAATTTCTCGTGCTTGGTGAAAA AGGTGAGACTTTAAGA	
*malO*6-R	AATTTTAATTTTTCACCAAGCACGAGAAATTTCTCA GTGAATATACGCCCTTCCAATTC	
*malO*2d11-F	GAGAAATTTCCCGCTCTATGGGAAAAAATAAGGC AAATGAGAAATTTCCCGCT	Construction of the duplicated *malO* mutants of P_{malA} promoter with different distance of operators
*malO*2d11-R	GTCTGAGAGATTTGGTTTTTTTACCTTTTTTGTAAT C	
*malO*2d16-F	GAGAAATTTCCCGCTCTATGGGAAAAACAGACAT AAGGCAAATGAGAAATTTCC	
*malO*2d16-R	AGAGATTTGGTTTTTTTACCTTTTTTGTAATCTT	
*malO*2d21-F	GAGAAATTTCCCGCTCTATGGGAAAAATCTCTCA GACATAAGGCAAATGAGAAATT	
*malO*2d21-R	TTTGGTTTTTTTACCTTTTTTGTAATCTTAAAGTCT C	
*malO*2d26-F	GAGAAATTTCCCGCTCTATGGGAAAAACCAAATCT CTCAGACATAAGGCAAATG	
*malO*2d26-R	TTTTTTTACCTTTTTTGTAATCTTAAAGTCTCACCA ACTG	
*malO*2d31-F	GAGAAATTTCCCGCTCTATGGGAAAAAAAAAACC AAATCTCTCAGACATAAGGC	
*malO*2d31-R	TTACCTTTTTTGTAATCTTAAAGTCTCACCAACTG	
*malO*2d36-F	GAGAAATTTCCCGCTCTATGGGAAAAAGGTAAAA AAACCAAATCTCTCAGACATAAGG	
*malO*2d36-R	TTTTTTGTAATCTTAAAGTCTCACCAACTGTGAATT TTAAG	
malO-mut-F1	CAGACATAAGGCAAATGADWAHTTTYYCRYTHTW TGGGAAAAAACACTAAAGTTGATC	Construction of the *malO* operator mutant library
malO-mut-F2	CAGACATAAGGCAAATGAGAAATTTYYCNNNNNN NGDDAAAAAACACTAAAGTTGATC	
malO-mut-R	TTTGCCTTATGTCTGAGAGATTTG	
Ter-F	TATAATAGAAAGAAAATGGGGGGATCTGATATGAG TAAAGGAGAAGAACTTTTC	Construction of plasmid pDG-Ter and its variants
Ter-R	ACAGATTCGAAAATGGGGGAATTTCATAATTATTC CCCCTAGCTAATTTTCGT	
TerT1-R	ACAGATTCGAAAATGGGGGAAAATTATTCCCCCTA GCTAATTTTCGT	
TerT2-R	ACAGATTCGAAAATGAATTATTCCCCCTAGCTAATT TTCGT	
MalA-Ter-F	TTCGCGGATCCCCGATTACCGTTGGCACCATTC	

MalA-Ter-R	TTCGGGGTACCATCAGATCCCCCCATTTTCTTTCTATTATATACAATTACA	
pDR-IF	AAGGAGGTCGTCATGGTACCATGCAGCTCGAAGAACTGATCAAT	Construction of plasmid pDR
pDR-IR	AGGAGCTCGGAGGGCAGTCTCTACTCATTGTCATTTTTTTCTGCCTCTTTGT	
pDR-VF	AAAAAAATGACAATGAGTAGAGACTGCCCTCCGAGCTC	
pDR-VR	ATCAGTTCTTCGAGCTGCATGGTACCATGACGACCTCCTTGA	
pDRP-IF	ATTAGCTAGGGGGAATAATTATGCAGCTCGAAGAACTGATCAAT	Construction of plasmid pDRP
pDRP-IR	ACTGTCGGAACGAGACTTCTATGGCGGTCTTTTTGACGGC	
pDRP-VF	AGAAGTCTCGTTCCGACAGTTGG	
pDRP-VR	AATTATTCCCCCTAGCTAATTTTCGTT	
malA_UP-F	GGATTCAAAAGCACTCTTGCCGC	Construction of the fragment for malA gene knockout
malA_UP-R	GGGGGAATTTCATATGACGACCTCCTTGATAACGTTTAC	
malA_DN-F	GGAGGTCGTCATATGAAATTCCCCCATTTTCGAATCTGTAATTG	
malA_DN-R	GGTGCTTTAGTTGAAGAAAATAAAGATGGCTCCGATGATGTTGG	
malA_CR-F	GGAGCCATCTTTATTTTCTTCAACTAAAGCACCCATTAGTTCAAC	
malA_CR-R	TGAGAATGATTTTTTCTTCATTTATTCATTCAGTTTTCGTGCGGACT	
malA_G-F	CACGAAAACTGAATGAATAAATGAAGAAAAAATCATTCTCAATCGTAATAGC	
malA_G-R	GATGCATGGCACCTCAACCATC	
malA-Ter_UP-F	GGATTCAAAAGCACTCTTGCCGC	Construction of the fragment for malA-Ter gene knockout
malA-Ter_UP-R	TTCGAGCTGCATATGACGACCTCCTTGATAACGTTTAC	
malA-Ter_DN-F	GAGGTCGTCATATGCAGCTCGAAGAACTGATCAAT	
malA-Ter_DN-R	GGTGCTTTAGTTGAAGAAAATAAAGATGGCTCCGATGATGTTGG	
malA-Ter_CR-F	GGAGCCATCTTTATTTTCTTCAACTAAAGCACCCATTAGTTCAAC	
malA-Ter_CR-R	TGAGAATGATTTTTTCTTCATTTATTCATTCAGTTTTCGTGCGGACT	
malA-Ter_G-F	CACGAAAACTGAATGAATAAATGAAGAAAAAATCATTCTCAATCGTAATAGC	
malA-Ter_G-R	TAATTGTATATAATAGAAAGAAAATGGGGGGATCTGT	
ptsG_UP-F	ATGACAAAGGAGCTGAGGATCGTG	Construction of the fragment for ptsG gene knockout
ptsG_UP-R	GCGTACTAACACCCAAGAATTGACCTCCTCTTTTTACTAGTCTGAC	
ptsG_DN-F	GAGGTCAATTCTTGGGTGTTAGTACGCCGTGC	
ptsG_DN-R	GGTGCTTTAGTTGAAGAATGGGTCAATAATGACATCGCCAT	

ptsG_CR-F	GTCATTATTGACCCATTCTTCAACTAAAGCACCCATTAGTTCAACAA	
ptsG_CR-R	CCGAATAATGCTTTAAACATTTATTCATTCAGTTTTCGTGCGGACT	
ptsG_G-F	CACGAAAACTGAATGAATAAATGTTTAAAGCATTATTCGGCGTTCTTCA	
ptsG_G-R	TTATTTTTCAATCTTCACAATATCTTCTTGTTCTCTGTTGAC	
Cre1-F	AGGAGGTCGTCATGGTACCTAAATT	Construction of plasmid pDG-P_{malA} variants with different cre-box truncation
Cre1-R	ACAATTCCATTTATACCATGAGA	
Cre2-F	AGGAGGTCGTCATGGTACCT	
Cre2-R	AATTCCATTTATACCATGAGATAGC	
Cre3-F	TGATAAATTTTACAGGAGGTCGTCATGGTACCT	
Cre3-R	AATTCCATTTATACCATGAGATAGC	
Cre4-F	ATTTTACTTATCAAGGAGGTCGTCATGGT	
Cre4-R	AATTCCATTTATACCATGAGATAGCTCGT	
Cre5-F	GTAAAATTGATAAAGGAGGTCGTCATGGTACC	
Cre5-R	AATTCCATTTATACCATGAGATAGCT	
LysRS16-F	AGGTCGTCATGGTACCTAAAGTACTACCTGCGCTAGCGC	Construction of plasmid pMATE15-Δcre3-LysRS16 and pMATE15-Δcre3-LysRS59
LysRS16-R	ACTCATAATTATTCCCCCTAGTGTCGCGATGCTCCTCG	
LysRS59-F	AGGTCGTCATGGTACCTAAAGTACTACCTGCGCTAGCGC	
LysRS59-R	ACTCATAATTATTCCCCCTAGTGTCGCCAAATCGAAGT	
LysRS-VF	TAGGGGGAATAATTATGAGTAAAGGAGAAGAACT	
LysRS-VR	TTTAGGTACCATGACGACCTCCTTGA	
TheoRS1-F	CCTTGGCAGCACCCTGCTAAGGAGGAATAATTATGAGTAAAGGAGAAGAACTTTTC	Construction of plasmid pMATE15-Δcre3-TheoRS1 and pMATE15-Δcre3-TheoRS2
TheoRS1-R	GCATCAAGACGATGCTGGTATCACCATGACGACCTCCTTGATAAATTTTACA	
TheoRS2-F	CCTTGGCAGCACCCTGCTAAGGAGGAATAATTATGAGTAAAGGAGAAGAACTTTTC	
TheoRS2-R	GCATCAAGACGATGCTGGTATCACCATGACGAGATAAATTTTACA	
divIVA-F	ATTAGCTAGGGGGAATAATTATGCCATTAACGCCAAATGAT	Construction of the plasmids pDivON and pDivOff
divIVA-R	ACTGTCGGAACGAGACTTCTTTATTCCTTTTCCTCAAATACAGCG	
pDiv-VF	AGAAGTCTCGTTCCGACAGTTGG	
pDiv-VR	AATTATTCCCCCTAGCTAATTTTCGTT	
Crimson-IF1	ATTAGCTAGGGGGAATAATTATGGATAGCACTGAGAACGTCATCAAG	Construction of the plasmid pMATE-Ac
Crimson-IR1	ACTGTCGGAACGAGACTTCTCTACTGGAACAGGTGGTGGCG	
Crimson-VF	AGAAGTCTCGTTCCGACAGT	
Crimson-VR	AATTATTCCCCCTAGCTAATTTTCGTTTAATTAT	

GFP-IF	TGAGCGCAACGCAATTAATGCTTTTGTCCCCTGC CTTTTCTAAAT	Construction of the plasmid pMATE-Rg
GFP-IR	TCTGTAAGCGGATGCCGGGACACCGAAACGCGC GAGG	
GFP-VF	TCCCGGCATCCGCTTACAGAC	
GFP-VR	CATTAATTGCGTTGCGCTCACTG	
Crimson-IF2	CGGTCTTTAAAAAGGCCGTCTCTATTTGTATAGTT CATCCATGCC	Construction of the plasmid pMATE-AcRg
Crimson-IR2	TTAGCTGGATATTCTTTTGTCCCCTGCCTTTTCTA	
AcRg-VF	AATATCCAGCTAAACGGTCTGGT	
AcRg-VR	ACGGCCTTTTTAAAGACCGTAAAGAAA	
Rib-F	ATTAGCTAGGGGGAATAATTATGGAAGAGTATTATA TGAAGCTGG	Construction of the plasmid pMATE-RibOn
Rib-R	ACTGTCGGAACGAGACTTCTTTATTCAAATGAGC GGTTTAAATTTG	
pRib-VF	AGAAGTCTCGTTCCGACAGTTG	
pRib-VR	AATTATTCCCCCTAGCTAATTTTCGTTTAATTAT	
Vio-F	ATGAAGCACTCTTCTGACATCTGTATCG	Construction of the plasmid pVio
Vio-R	CTATCTCTTAGCAGCGAAAACAGCG	
pVio-VF	CGCTGTTTTCGCTGCTAAGAGATAGAGAAGTCTC GTTCCGACAGTTG	
pVio-VR	GATGTCAGAAGAGTGCTTCATAATTATTCCCCCTA GCTAATTTTCGTTTAATTAT	

The underlined sequences denote the restricted enzyme site in the primer sequence.

SI References

1. J. Spizizen, Transformation of biochemically deficient strains of *Bacillus Subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U. S. A.* **44**, 1072-1078 (1958).
2. Y. Song *et al.*, Promoter screening from *Bacillus subtilis* in various conditions hunting for synthetic biology and industrial applications. *PLoS One* **11**, e0158447 (2016).
3. T. Morimoto, K. Ara, K. Ozaki, N. Ogasawara, A new simple method to introduce marker-free deletions in the *Bacillus subtilis* genome. *Genes Genet. Syst.* **84**, 315-318 (2009).
4. C. You, X. Z. Zhang, Y. H. P. Zhang, Simple cloning via direct transformation of PCR product (DNA Multimer) to *Escherichia coli* and *Bacillus subtilis*. *Appl. Environ. Microbiol.* **78**, 1593-1595 (2012).
5. R. Higuchi, B. Krummel, R. K. Saiki, A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351-7367 (1988).
6. I. Wittig, H.-P. Braun, H. Schägger, Blue native PAGE. *Nature Protocols* **1**, 418-428 (2006).
7. M. Y. Fang *et al.*, High crude violacein production from glucose by Escherichia coli engineered with interactive control of tryptophan pathway and violacein biosynthetic pathway. *Microbial cell factories* **14**, 8 (2015).
8. H. Wang *et al.*, Optimization of culture conditions for violacein production by a new strain of Duganella sp. B2. *Biochemical Engineering Journal* **44**, 119-124 (2009).
9. G. Wang *et al.*, Integrated whole-genome and transcriptome sequence analysis reveals the genetic characteristics of a riboflavin-overproducing Bacillus subtilis. *Metabolic Engineering* **48**, 138-149 (2018).
10. T. Shi *et al.*, Deregulation of purine pathway in Bacillus subtilis and its use in riboflavin biosynthesis. *Microbial cell factories* **13**, 101 (2014).
11. H. Yamamoto, M. Serizawa, J. Thompson, J. Sekiguchi, Regulation of the glv operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is repressed through CcpA and cre. *J. Bacteriol.* **183**, 5110-5121 (2001).
12. K. A. Egland, E. P. Greenberg, Conversion of the *Vibrio fischeri* transcriptional activator, LuxR, to a repressor. *J. Bacteriol.* **182**, 805-811 (2000).
13. H. Ludwig, C. Meinken, A. Matin, J. Stulke, Insufficient expression of the ilv-leu operon encoding enzymes of branched-chain amino acid biosynthesis limits growth of a *Bacillus subtilis* ccpA mutant. *J. Bacteriol.* **184**, 5174-5178 (2002).
14. B. R. Belitsky, H. J. Kim, A. L. Sonenshein, CcpA-dependent regulation of *Bacillus subtilis* glutamate dehydrogenase gene expression. *J. Bacteriol.* **186**, 3392-3398 (2004).
15. I. T. Paulsen, S. Chauvaux, P. Choi, M. H. Saier, Characterization of glucose-specific catabolite repression-resistant mutants of *Bacillus subtilis*: identification of a novel hexose:H+ symporter. *J. Bacteriol.* **180**, 498-504 (1998).
16. K. Jahreis, E. F. Pimentel-Schmitt, R. Bruckner, F. Titgemeyer, Ins and outs of glucose transport systems in eubacteria. *FEMS Microbiol. Rev.* **32**, 891-907 (2008).
17. H. Fiegler, J. Bassias, I. Jankovic, R. Bruckner, Identification of a gene in *Staphylococcus xylosus* encoding a novel glucose uptake protein. *J. Bacteriol.* **181**, 4929-4936 (1999).
18. S. Schonert *et al.*, Maltose and maltodextrin utilization by *Bacillus subtilis*. *J. Bacteriol.* **188**, 3911-3922 (2006).
19. L. B. Zhou, A. P. Zeng, Engineering a lysine-ON Riboswitch for metabolic control of lysine production in *Corynebacterium glutamicum*. *ACS Synth. Biol.* **4**, 1335-1340 (2015).
20. S. Topp *et al.*, Synthetic riboswitches that induce gene expression in diverse bacterial species. *Appl. Environ. Microbiol.* **76**, 7881-7884 (2010).
21. A. Khlebnikov, K. A. Datsenko, T. Skaug, B. L. Wanner, J. D. Keasling, Homogeneous expression of the P(BAD) promoter in Escherichia coli by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology (Reading)* **147**, 3241-3247 (2001).
22. R. M. Morgan-Kiss, C. Wadler, J. E. Cronan, Long-term and homogeneous regulation of the *Escherichia coli* araBAD promoter by use of a lactose transporter of relaxed specificity. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7373-7377 (2002).
23. P. J. Choi, L. Cai, K. Frieda, X. S. Xie, A stochastic single-molecule event triggers phenotype switching of a bacterial cell. *Science* **322**, 442-446 (2008).
24. A. Khlebnikov, J. D. Keasling, Effect of lacY expression on homogeneity of induction from

the P(tac) and P(trc) promoters by natural and synthetic inducers. *Biotechnol. Prog.* **18**, 672-674 (2002).
25. A. M. Albiniak *et al.*, High-level secretion of a recombinant protein to the culture medium with a *Bacillus subtilis* twin-arginine translocation system in *Escherichia coli*. *FEBS J.* **280**, 3810-3821 (2013).
26. N. A. Becker, J. P. Peters, T. A. Lionberger, I. I. I. L. J. Maher, Mechanism of promoter repression by Lac repressor–DNA loops. *Nucleic Acids Res.* **41**, 156-166 (2013).
27. P. Gollnick, P. Babitzke, A. Antson, C. Yanofsky, Complexity in regulation of tryptophan biosynthesis in *Bacillus subtilis*. *Annu. Rev. Genet.* **39**, 47-68 (2005).
28. E. Balleza, J. M. Kim, P. Cluzel, Systematic characterization of maturation time of fluorescent proteins in living cells. *Nat Methods* **15**, 47-51 (2018).
29. G. Fu, J. Liu, J. Li, B. W. Zhu, D. Zhang, Systematic screening of optimal signal peptides for secretory production of heterologous proteins in Bacillus subtilis. *Journal of agricultural and food chemistry* **66**, 13141-13151 (2018).
30. D. Li, G. Fu, R. Tu, Z. Jin, D. Zhang, High-efficiency expression and secretion of human FGF21 in Bacillus subtilis by intercalation of a mini-cistron cassette and combinatorial optimization of cell regulatory components. *Microbial cell factories* **18**, 17 (2019).
31. J. Chen *et al.*, High-level intra- and extra-cellular production of D-psicose 3-epimerase via a modified xylose-inducible expression system in Bacillus subtilis. *J Ind Microbiol Biotechnol* **43**, 1577-1591 (2016).
32. A. M. Mirończuk, A. Krasowska, A. Murzyn, M. Płachetka, M. Łukaszewicz, Production of the Bacillus licheniformis SubC protease using Lactococcus lactis NICE expression system. *SpringerPlus* **1**, 54 (2012).
33. S. Wang *et al.*, High-Efficiency Secretion and Directed Evolution of Chitinase BcChiA1 in Bacillus subtilis for the Conversion of Chitinaceous Wastes Into Chitooligosaccharides. *Front Bioeng Biotechnol* **8**, 432 (2020).
34. J. Li, X. Li, Y. Gai, Y. Sun, D. Zhang, Evolution of E. coli Phytase for Increased Thermostability Guided by Rational Parameters. *Journal of microbiology and biotechnology* **29**, 419-428 (2019).
35. J. Hanna *et al.*, Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc Natl Acad Sci U S A* **107**, 9222-9227 (2010).
36. X. Duan, S. Hu, X. Qi, Z. Gu, J. Wu, Optimal extracellular production of recombinant Bacillus circulans β-galactosidase in Escherichia coli BL21(DE3). *Process Biochemistry* **53**, 17-24 (2017).
37. Y. Song *et al.*, High-Efficiency Secretion of beta-Mannanase in Bacillus subtilis through Protein Synthesis and Secretion Optimization. *J Agric Food Chem* **65**, 2540-2548 (2017).
38. L. B. Zhou, A. P. Zeng, Engineering a Lysine-ON Riboswitch for Metabolic Control of Lysine Production in Corynebacterium glutamicum. *ACS Synth Biol* **4**, 1335-1340 (2015).
39. S. Topp *et al.*, Synthetic riboswitches that induce gene expression in diverse bacterial species. *Appl Environ Microbiol* **76**, 7881-7884 (2010).