SUPPLEMENTARY DATA

MATERIAL AND METHODS

Media and Bacterial strains

Complex culture medium (853) consisted of 10 g bacto-tryptone, 5 g yeast extract, 0.1% glucose, 5 g NaCl, 0.7 g K_2HPO_4 and 0.3 g KH_2PO_4 in 1 liter water. Antibiotics were added to the media when needed as indicated (unless otherwise mentioned), kanamycin (60 μg/mL), chloramphenicol (25 μg/mL) and ampicillin (100 μg/mL).

B. subtilis subsp. subtilis wild type (LMG 7135) was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG, Gent, Belgium). *E. coli* MG1655 [λ^- F^- *rph*-1 *rfb*-50 *ilvG*$^-$] was obtained from the Netherlands Culture Collection of Bacteria (NCCB, Utrecht, The Netherlands). *E. coli* MG1655 knock-in derivatives bearing a heterologous sigma factor in the genome were made through homologous recombination according to the method of Datsenko and Wanner (1). However, instead of using pKD3 or pKD4 (that are dependent on expression of the *pir* gene) to join the antibiotic resistance cassette to the sequence to be inserted, a pUC18 derivative bearing the chloramphenicol resistance cassette flanked by FRT sites was used for this purpose (VUB – MICR lab). Homology regions used for insertion in the genome are: 5'-GCGAAATCCTGCAAACGCAGGGGCTGAATATCGAAGCGCTGTTCCGCGAGTA-3' and 5'-TTACCGGATTCTTAATTACCTGGTGCGTATGGGCGGTAATTTGACCTTAATAAAA AGGTC-3'. Overview of used primers and resulting strains are listed in Table S2 and S3.

Cloning, growth and fluorescence reporters

Plasmid pTrc99a was obtained from Pharmacia Biotech (Uppsala, Sweden) and sigma factors from *B. subtilis* and *E. coli* were placed under control of the IPTG-inducible promoter. Plasmid pSC101-mKate2 (UGent – Memo Group) was used as fluorescent reporter construct to measure the activity of the *B. subtilis* and *E. coli* promoters. All derivatives of these plasmids were constructed in a seamless ligation reaction, protocol adapted from Zhang *et al.* (2). One raction consisted of 1 μL 10X buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT), 1 μL cell extract prepared from *E. coli* DH10B transformed with plasmid pKD46 expressing the lambda red recombination system (1), 100 ng of linearized vector, PCR fragments or synthetic oligonucleotide duplexes to be inserted (with overlapping sequences of 40-50 nucleotides) at a molar ratio of 1:5 (vector:inserts) and ddH_2O up to a total volume of 10 μL in PCR tubes. Reaction mixes were incubated for 1 h at 37 °C in a water bath and 1-2 μL reaction mix was subsequently transformed into 100 μL *E. coli* MG1655 competent cells. A list of all constructs with primers and enzymes (for linearisation of plasmid) used is given in Table S2 and S3. Composition of the *B. subtilis* and *E. coli* promoters with their sequences are given in Table S1. All constructs were verified by DNA sequencing. Competent cells for DNA transformation were prepared by the standard $CaCl_2$ treatment (3).

Growth analysis

All strains (*E. coli* MG1655 knock-in derivatives and MG1655 transformants bearing a pTrc99a derivative) were tested in triplicate (different colonies = biological triplicates) on microtiter plates and experiments were repeated at least twice (independent plates). Pre-cultures (150 μL of 853 medium) were grown for 24 h with appropriate antibiotics in 96-well plates at 37 °C while shaking (700 rpm in an Eppendorf Thermomixer). Cultures were diluted 150-fold (by serial dilution) in fresh medium with appropriate antibiotics (none for knock-in mutants and ampicillin for transformants bearing pTrc99a or derivatives thereof) and grown in a Biotek Synergy Mx Multi-Mode reader at 30 °C while shaking (fast mode). OD at 600 nm was measured every 10 min for 18 h. Growth curve data were analyzed with the Grofit package in R (4) after subtracting the background signal (OD of wells with only medium). Growth parameters given were derived from fitting the Gompertz growth model:

$$\log\left(\frac{\mathrm{OD}_t}{\mathrm{OD}_0}\right) = \mathrm{A} * \mathrm{e}^{-\mathrm{e}^{\left(\frac{(\mu * e)}{\mathrm{A} * (\lambda - \mathrm{t})} + 1\right)}}$$

with OD_0 the OD at time 0, *e* is Euler's number, λ representing the length of the lag phase, growth rate is given by the maximum slope μ, and the maximum cell growth is A.

Fluorescence analysis

Each 96-well plate contained the same controls; a media blank (853 medium + kanamycin and/or ampicillin), *E. coli* MG1655 cells that do not produce mKate2 (transformed with control plasmid pSC101-P_{no}) and *E. coli* MG1655 cells that constitutively produce mKate2 (transformed with plasmid pSC101-P_{high}). All strains/constructs were tested in triplicate (different transformed colonies = biological triplicates) on a microtiter plate and experiments were repeated at least two times (independent plates). Pre-cultures (150 μL 853 medium with kanamycin and/or ampicllin) were grown for 24 h in 96-well plates at 37 °C while shaking (700 rpm in an Eppendorf Thermomixer). Cultures were diluted 150-fold (by serial dilution) in fresh medium with kanamycin (and/or ampicillin) in black 96-well plates (150 μL) and grown in a Biotek Synergy Mx Multi-Mode reader at 30 °C while shaking (fast mode). OD at 600 nm and fluorescence (FL) (excitation: 588 nm and emission: 633 nm) were measured every 10 min for 24 h. OD and FL values at each time point were first corrected by subtracting the corresponding values of the media blank at that same time point. Further the ratio of FL to OD (FL/OD) was calculated for each well and the mean FL/OD of *E. coli* MG1655 cells with pSC101-P_{no} in the same growth phase was subtracted to correct for autofluorescence of the cells. To be able to combine all data measured from independent plates all data is expressed relative to the FL/OD of wild type MG1655 carrying a pSC101 plasmid with the strong constitutive σ^{70} promoter (P_{high}) directing transcription of the fluorescent reporter gene.

Promoter library construction

A detailed overview of our general pLibrary vector and DNA sequence is depicted in SI Figure S2. We constructed all libraries using a 2-piece CPEC assembly with sequence overlaps situated adjacent to the promoter library site and in the SC101 *ori* region. Randomized library sequences are included in PCR primers and the pLibrary vectors containing the respective original promoter were used as PCR templates. PCR primers (purchased from IDT, Leuven, Belgium) are shown in Table S2. Assembled libraries were cloned into Top10 electrocompetent cells (Invitrogen) and after overnight growth in 10 mL LB supplied with kanamycin (37 °C), plasmid DNA was isolated with a Qiagen Plasmid Mini Kit to be stored at -20 °C for later use.

Flow cytometry preparation and analysis

E. coli MG1655 wild type and derivatives containing either the gene for heterologous sigma factor B, W or F, were freshly made electrocompetent and subdivided in aliquots of 50 μL for transformation with 1.8 μL of the previously stored libraries. Transformants were grown at 30 °C with shaking in 1 mL prewarmed LB for 1.5 h and subsequently transferred to 50 mL tubes, containing 9 mL 853 medium supplied with kanamycin. After 3 h of incubation, 5 mL was removed and stored as a 35% glycerol stock at -80 °C for future work. The remaining 5 mL was further incubated overnight at 30 °C for a total of 12 h. Subsequently, cultures were chilled on ice, centrifuged and resuspended in 5 mL ice-cold and filtered PBS. OD was measured and the final flow cytometry samples were prepared by dilution in PBS to contain approximately 10^7 CFU/mL. At least 100,000 events were recorded for each sample on a BD Influx Cell Sorter, after calibration with Rainbow Calibration Particles (8 peaks, 3.0 – 3.4 μm). The diluted samples of the wild type strains containing the different libraries were also used as a template for high fidelity PCR and subsequent Sanger sequencing (Macrogen Inc., Amsterdam, The Netherlands) to verify the randomization of the sequences (Figure S3).

Flow cytometry data processing

Flow cytometry data was processed with a custom written R script. Data files were imported with the Bioconductor flowCore package (5) and subsequently the raw fluorescence was transformed with an inverse hyperbolic sine function as follows:

$$\operatorname{asinh}\left(\frac{fluorescence}{cofactor}\right)$$

This transformation is a basic form of the more general logicle transformations, which is preferred over the classically used logarithmic transformation for flow cytometry fluorescence data (6-8). The transformed fluorescence data from the constitutively expressed control gene, *sfgfp*, and the forward scatter were used for automatic gating with the Bioconductor flowPeaks package (9) to differentiate relevant cells from cells not harboring a pLibrary plasmid, cell debris and other artifacts. Gated data with the cluster boundaries are presented in Figure S4 A. The same package is used to remove outliers from the data (Figure S4 B). A minimum of 94% of all measured events in each sample is situated in the retained cluster and between 0.01% and 2.81% of those events are identified as outliers. Subsequently, data

presented in this study were obtained by calculating the ratio of red fluorescence over green fluorescence, applying the asinh transformation and adjusting these values by subtraction with the mean value of the blank control vector. This results in corrected and transformed fluorescence ($Fluorescence_{CT}$) values:

$$\operatorname{asinh}\left(\frac{red\ fluorescence}{green\ fluorescence} * \frac{1}{cofactor}\right) - mean\left(asinh\left(\frac{red\ fluorescence}{green\ fluorescence} * \frac{1}{cofactor}\right)_{sfGFP-only}\right)$$

Library promoter selection and characterization

Libraries were assembled proportional to the maximum amount of different sequences present in a single library, limited by the amount of obtained transformants displayed in Table 1. Preparation of cells for transformation, electroporation and FACS samples was performed as described above in flow cytometry analysis. A BD Influx Cell Sorter, calibrated with Rainbow Calibration Particles (8 peaks, 3.0 – 3.4 μm), was used for cell sorting. sfGFP expression was taken into account for bin selection to exclude artifacts, especially in the lower mKate2 expression regions. Sorted cells were collected in collection tubes with 1 mL 853 medium supplemented with kanamycin. In the subsequent screening, strains with sfGFP expression outside the median±10% interval (separately for each library) were excluded prior to selection. Plasmid DNA extraction of selected library strains was performed on 200 μL cultures on microtiter plate. A Qiagen Plasmid Mini Kit was used for lysis and neutralization with 1/6 buffer volumes. Next DNA was purified by addition of 75 μL isopropanol to the supernatant from previous lysis, centrifugation and a wash step with 100 μL cold 70% EtOH. After resuspension in 20 μL elution buffer, the DNA was transformed on microtiter plate scale by adding 2 μL of DNA to 10 μL chemically competent cells prepared in TSS buffer (5g PEG 8000, 1.5 mL 1M $MgCl_2$ and 2.5 mL DMSO supplemented with LB to 50 mL total) and a 45s heat shock. Pre-cultures for analysis (150 μL 853 medium with kanamycin) were grown for 24 h in 96-well plates at 37 °C while shaking (850 rpm in a Compact Digital Microplate Shaker, ThermoFisher Scientific). Cultures were diluted 300-fold (by serial dilution) in fresh medium with kanamycin in black 96-well plates (150 μL) and incubated similarly to pre-cultures. OD at 600 nm, mKate2 fluorescence (excitation: 588 nm and emission: 633 nm) and sfGFP fluorescence (excitation: 480 nm and emission: 520 nm) were measured after reaching stationary phase in a Tecan Infinite m200 Pro plate reader. mKate2 and sfGFP signals from media (blank) were substracted from the signals measured for the strains, and subsequently mKate2 fluorescence was corrected through division by sfGFP fluorescence ($Fluorescence_C$).

References

1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.

2. Zhang Y, Werling U, Edelmann W (2012) SLiCE: a novel bacterial cell extract-based DNA cloning method. *Nucleic Acids Res* 40(8). doi:10.1093/nar/gkr1288.

3. Dagert M, Ehrlich SD (1979) Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* 6(1):23–28.

4. Kahm M, Hasenbrink G, Lichtenberg-Fraté H, Ludwig J, kschischo M (2010) Grofit: fitting biological growth curves with R. *Journal of Statistical Software* 33(7).

5. Hahne F, et al. (2009) flowCore: a bioconductor package for high throughput flow cytometry. *BMC Bioinformatics* 10:106.

6. O'Neill K, Aghaeepour N, Spidlen J, Brinkman R (2013) Flow cytometry bioinformatics. *PLoS Comput Biol* 9(12):e1003365.

7. Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* 7(7):681–685.

8. Parks DR, Roederer M, Moore WA (2006) A new “Logicle” display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. *Cytometry A* 69(6):541–551.

9. Ge Y, Sealfon SC (2012) flowPeaks: a fast unsupervised clustering for flow cytometry data via K-means and density peak finding. *Bioinformatics* 28(15):2052–2058.

10. Davis JH, Rubin AJ, Sauer RT (2011) Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Res* 39(3):1131–1141.

11. Mutalik VK, et al. (2013) Precise and reliable gene expression via standard transcription and translation initiation elements. *Nature Methods* 10(4):354–360.

12. Cambray G, et al. (2013) Measurement and modeling of intrinsic transcription terminators. *Nucleic Acids Res* 41(9):5139–5148.

13. Pédelacq J-D, Cabantous S, Tran T, Terwilliger TC, Waldo GS (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol* 24(1):79–88.

14. Salis HM, Mirsky EA, Voigt CA (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat Biotechnol* 27(10):946–950.

15. Borujeni AE, Mishler DM, Wang J, Huso W, Salis HM (2016) Automated physics-based design of synthetic riboswithces from diverse RNA aptamers. *Nucleic Acids Res* 44(1):1-13.

16. Shcherbo D, et al. (2009) Far-red fluorescent tags for protein imaging in living tissues. *Biochem J* 418(3):567.

SUPPLEMENTARY FIGURES AND TABLES

Table S1: Information on the promoter sequences originating from *B. subtilis* and the four control constructs for *E. coli*. Name of construct, original gene under control of the promoter and sequence of the promoter is given. For the sequence: preceding insulator region (10) is depicted in blue, original promoter is given in black with the consensus regions in red, and the translation initiation region (BCD19 - (11)) with its bicistronic design containing two Shine-Dalgarno motifs is given in green (start codon in bold). The four first constructs are controls for *E. coli* and based on synthetic promoters from Davis *et al.* (10).

Sigma factor	Promoter construct	Original gene	Sequence
	P_{no}	no promoter	-
70	P_{low}	proB (Davis *et al.*, 2011)	TTCTAGAGCACAGCTAACACCACGTCGTCC CTATCTGCTGCCCTAGGTCTATGAGTGGTT GCTGGATAACTTTACGGGCATGCATAAGGC TCGTAATATATTCAGGG
	P_{mid}	proC (Davis *et al.*, 2011)	TTCTAGAGCACAGCTAACACCACGTCGTCC CTATCTGCTGCCCTAGGTCTATGAGTGGTT GCTGGATAACTTTACGGGCATGCATAAGGC TCGTATGATATATTCAGGG
	P_{high}	proD (Davis *et al.*, 2011)	TTCTAGAGCACAGCTAACACCACGTCGTCC CTATCTGCTGCCCTAGGTCTATGAGTGGTT GCTGGATAACTTTACGGGCATGCATAAGGC TCGTATAATATATTCAGGG
B	P_{B1}	*ctc*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTACTGTTCAGCTAAAC CATTTTTCGAGGTTTAAATCCTTATCGTTAT GGGTATTGTTTGTAATGGGCCCAAGTTCAC TTAAAA**AGGAG**ATCAACAATGAAAGCAATT TTCGTACTGAAACATCTTAATCATGCTA**TG GAGG**TTTTCTA**ATG**
	P_{B2}	*gspA*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATGTTTAAAAAAATG TCGGAGAACGTGTTTATTTTTTTGAAAAAG GGTATGTAACTTGTAGGGCCCAAGTTCACT TAAAA**AGGAG**ATCAACAATGAAAGCAATTT TCGTACTGAAACATCTTAATCATGCTA**TGG AGG**TTTTCTA**ATG**
	P_{B3}	*trxA*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAGCTTCATGCCGGC GCTCTTTTTCAGGTTTTAAAACAGCTCCGG CAGGGCATGGTAAAGTACGGGCCCAAGTT CACTTAAAA**AGGAG**ATCAACAATGAAAGCA ATTTTCGTACTGAAACATCTTAATCATGCTA **TGGAGG**TTTTCTA**ATG**

Sigma factor	Promoter construct	Original gene	Sequence
F	P_{F1}	*spoIIR*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATTGCTAGATTTTTTT CACCCTGCACGTTTATCCCAGGCTCTCCTT GTCCATAATAGGGCTAGAAGGGCCCAAGT TCACTTAAAA**AGGAG**ATCAACAATGAAAGC AATTTTCGTACTGAAACATCTTAATCATGCT A**TGGAGG**TTTTCTA**ATG**
	P_{F2}	*spoIIQ*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTACTAAAAAAGTTTTTT TGGATAGGTTGTATATATTTTCAGAAAAGT GTTCAGAATGTTGCTGAGGGGGCCCAAGT TCACTTAAAA**AGGAG**ATCAACAATGAAAGC AATTTTCGTACTGAAACATCTTAATCATGCT A**TGGAGG**TTTTCTA**ATG**
	P_{F3}	*ywhE*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAGTAAAGATGCGTCC TGTTCTGCGATGTTTAAAAACGATCTTTTTT TCTCATAATAGTAGAAACAGGGCCCAAGTT CACTTAAAA**AGGAG**ATCAACAATGAAAGCA ATTTTCGTACTGAAACATCTTAATCATGCTA **TGGAGG**TTTTCTA**ATG**
G	P_{G1}	*yoaR*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAATTCAAACAAACGA TGGGAAGAAATACATCAAAGGATAAGCGG CTGTTCATACTAATGATTGGGAGGGCCCAA GTTCACTTAAAA**AGGAG**ATCAACAATGAAA GCAATTTTCGTACTGAAACATCTTAATCAT GCTA**TGGAGG**TTTTCTA**ATG**
	P_{G2}	*yozQ*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATGGCCAAAGCGCG AATGAAAAAAGTGCATGAATACCTGCCCAA CAGACAGAATAAGAAGAGTTGGGGCCCAA GTTCACTTAAAA**AGGAG**ATCAACAATGAAA GCAATTTTCGTACTGAAACATCTTAATCAT GCTA**TGGAGG**TTTTCTA**ATG**
	P_{G3}	*yvaB*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATCTATAATAAAGTC TAAGAGAGACAGAATAATCATTATGCATCT GTATGATAATAATTGATGTGTGGGCCCAAG TTCACTTAAAA**AGGAG**ATCAACAATGAAAG CAATTTTCGTACTGAAACATCTTAATCATG CTA**TGGAGG**TTTTCTA**ATG**
M	P_{M1}	*yfnI*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATTTTTTTATTTCTGA GAAAAAAATGTGAAACGAAATGAAGGTTTC TTTCGTCCAGTGATTGGGGGCCCAAGTTC ACTTAAAA**AGGAG**ATCAACAATGAAAGCAA TTTTCGTACTGAAACATCTTAATCATGCTA**T GGAGG**TTTTCTA**ATG**
	P_{M2}	*rodA*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATTCATTTGAAAAGT TTTGTGTCAATCGAAACATTTCGGTTTATG ATACGTCATATTTCGTGGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT

Sigma factor	Promoter construct	Original gene	Sequence
			TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{M3}	*divIC*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAATCCGTTTTATCGC GAAACAATGTTTGAAACTTCTTCCTGTGAA AATGCGTCTAACTTTTAGGGGCCCAAGTTC ACTTAAAA**AGGAG**ATCAACAATGAAAGCAA TTTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{M4}	*ywtF*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATTACTTGTATTTTTT TCAATGTCGCCGAAACATTTTACCTGCTGC GGCGTCCAATATAAGGGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
W	P_{W1}	*ybfO*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAAAGCTTTTTTTTGTT GGCAGGAAAAGGAAACTTTTTCTATATCTA TCTCGTAATGACTAGAGGGGCCCAAGTTC ACTTAAAA**AGGAG**ATCAACAATGAAAGCAA TTTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{W2}	*sigW*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTATGATAAACTTATTTT ATAAAAAAATTGAAACCTTTTGAAACGAAG CTCGTATACATACAGAGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{W3}	*ydjF*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAGAAATGTCATTTTT TATTAAAAAAGTGAAACTTTTAACGATAATA AATAGTATATGTAACAAGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{W4}	*yfhL*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTACGAGGCTTGTCTTT TTGCCTATGCATGAAACATTTCTTCTTTCTG CACGTAACAATGAGAAGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
X	P_{X1}	*lytR*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAATTTTAAAGAAAAA TTAAGAAACAATGAAACTTTTTTTTATAAAA AACGACTATTTTAGGAGGGCCCAAGTTCAC TTAAAA**AGGAG**ATCAACAATGAAAGCAATT TTCGTACTGAAACATCTTAATCATGCTA**TG** **GAGG**TTTTCTA**ATG**

Sigma factor	Promoter construct	Original gene	Sequence
	P_{X2}	*csbB*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAAAAATCATGAATGT CACCATAAAATTGTAACAAAAAACAGGTTT AAACGACTTTAAAAAAAGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**
	P_{X3}	*bcrC*	TTCTAGAGCACAGCTAACACCACGTCGTC CCTATCTGCTGCCCTAATTTCAGACAATCT CTATTTTTATTTGAAACTTTTCATGAGTAAG ATTAGTCTACTAAATATGGGCCCAAGTTCA CTTAAAA**AGGAG**ATCAACAATGAAAGCAAT TTTCGTACTGAAACATCTTAATCATGCTA**T** **GGAGG**TTTTCTA**ATG**

Table S2: List of primer sequences used for cloning purposes in this work.

Primer name	Sequence (5’ □ 3’)
IB0173	ATGGTTAGCGAGCTGATCAAAG
IB0174	CTTCGTAAATCTGGCGAGTG
IB0175	TGTGCATGTTTTCTTTGATCAGCTCGCTAACCATCATTAGAAAACCTCCATAGCATG
IB0176	GATGTCTGGCAGTTCCCCACTCGCCAGATTTACGAAGTTCTAGAGCACAGCTAACAC
IB0180	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGTGTAGGCTGGAGCTGCTTC
IB0181	AACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCTGGTCCATATGAATATCCTCCTTAG
IB0184	TTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAGGAGACCACAACGGTTTCCCTCTAC
IB0186	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTCATTCATCATTAACACCTCTATTATAAAGTGCTTTCAGCC
IB0198	TTACCGGATTCTTAATTACCTGGTGCGTATGGGCGGTAATTTGACCTTAATAAAAAGGTCTGGTCCATATGAATATCCTCCTTAG
IB0199	GCGAAATCCTGCAAACGCAGGGGCTGAATATCGAAGCGCTGTTCCGCGAGTAGGAGACCACAACGGTTTCCCTCTAC
IB0238	GAGTCACACAGGAAAGTACTAGATGACGATCGATGAAATTTACC
IB0245	TGAGCGGATAACAATTTCACACAGGAAACAGACCATGGAATTCGGAGACCACAACGGTTTCCCTCTAC
IB0249	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTCATTCATCATTAACACCTCTATTATAAAGTGCTTTCAGCCGCTGTC
IB0250	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGACACAACCATCAAAAACTACGAAACTAAC
IB0251	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTACATTAACTCCATCGAGGGATCTTC
IB0252	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGGATGTGGAGGTTAAGAAAAACGGCAAAAACG
IB0253	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGCTAGCCATCCGTATGATCCATTTGAACC
IB0254	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGGTGTCGAGAAATAAAGTCGAAATCTGCGGGGTGGATAC
IB0255	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTATTGATGAATATTTTTATTCATTTGTTTGATAGCCGCTTTTTCAAGTCTGGACACCTGCGCTTGAG
IB0256	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGGTGAATCTACAGAACAACAAGGGAAAATTCAAC
IB0257	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTACAAACTGATTTCGCGAATTTCCAAGTAC
IB0258	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGGAAATGATGATTAAAAAAAGAATTAAACAAGTCAAAAAAGGCGACCAG
IB0259	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTAAAGATCCCTTAATTGTTTTCTAAGAGCCTCTCTG
IB0260	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGGAAGAAACCTTTCAATTATTATATGATACATATCATCAAGATTTG
IB0261	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTAACTGCCGGAAGTTGACTTAACAACTC
IB0262	CTAGTACTTTCCTGTGTGACTC
IB0268	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTTACATTAACTCCATCGAGGG

IB0269	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTCTAGCCATC CGTATGATCC
IB0270	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTTATTGATGA ATATTTTTATTCATTTGTTTGATAGCC
IB0271	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTTACAAACTG ATTTCGCGAATTTCC
IB0272	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTTAAAGATCC CTTAATTGTTTTCTAAGAGC
IB0273	GTATAGGAACTTCGAAGCAGCTCCAGCCTACACGGGGATCTTTAACTGCC GGAAGTTGACTTAACAACTCCTTTATCTG
IB0476	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGAAGCAAGGTTTGC AACTCAGGCTTAG
IB0477	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTCAAACG AGTTGTTTACGCTGGTTTGAC
IB0478	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGGTGAATTCACTCTATAC CGCTGAAGGTG
IB0479	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTATAAC TTACCCAGTTTAGTGCGTAACC
IB0480	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGACTGACAAAATGCA AAGTTTAG
IB0481	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTTACGCT TCAATGGCAGCAC
IB0482	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGTCTGACCGCGCCA CTAC
IB0483	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTCATAAC CCATACTCCAGACGGAACAG
IB0484	TTAACTTTTACTAGAGTCACACAGGAAAGTACTAGATGAGCGAGCAGTTAA CGGAC
IB0485	CCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTCAACGC CTGATAAGCGGTTGAAC
Fw_BB	GGATCTCGTAACCGAACTTG
Fw_LibB_1	GCCCTATGTTTAAAAAAATGTCGGAGAACGTGTTTAT**NNNNNNNNNNNN**GG GTATGTAACTTGTAGGGCC
Fw_LibB_2	GCCCTATGTTTAAAAAAATGTCGGAGAACGTGTTTATTTTTTTT**NNNNNN**GGG TATGTAACTTGTAGGGCC
Fw_LibB_3	GCCCTATGTTTAAAAAAATGTCGGAGAACGTGTTTAT**NNNNNN**GAAAAAGG GTATGTAACTTGTAGGGCC
Fw_LibF_1	GATGCGTCCTGTTCTGCGATGTTTA**NNNNNNNNNNNNNNNK**CTCATAATAG TAGAAACAGGGCC
Fw_LibF_2	GATGCGTCCTGTTCTGCGATGTTTAAAAACGAT**NNNNNNNK**CTCATAATAGT AGAAACAGGGCC
Fw_LibF_3	GATGCGTCCTGTTCTGCGATGTTTA**NNNNNNNN**CTTTTTTTCTCATAATAGT AGAAACAGGGCC
Fw_LibW_1	CTATCTGCTGCCCTATGATAAACTTATTTTATAAAAAAATTGAAAC**NNNNNNN NNNNNNNNN**CGTATACATACAGAGGGCC
Fw_LibW_2	CTATCTGCTGCCCTATGATAAACTTATTTTATAAAAAAATTGAAACCTTTTGA A**NNNNNNNN**CGTATACATACAGAGGGCC
Fw_LibW_3	CTATCTGCTGCCCTATGATAAACTTATTTTATAAAAAAATTGAAAC**NNNNNNN N**ACGAAGCTCGTATACATACAGAGGGCC
Fw_LibproD_1	GGTTGCTGGATAACTTTACG**NNNNNNNNNNNNNN**TCGTATAATATATTCAG GGAGAGCACAAC
Fw_LibproD_2	GGTTGCTGGATAACTTTACG**NNNNNNNNNNNNNNNNN**TATAATATATTCAG

	GGAGAGCACAAC
Rv_BB	CTGGTTGTTCTCAAGTTCGG
Rv_LibB	CGACATTTTTTTAAACATAGGGCAG
Rv_LibF	CATCGCAGAACAGGACGCATC
Rv_LibW	GTTTATCATAGGGCAGCAGATAG
Rv_LibproD	CGTAAAGTTATCCAGCAACC

Table S3: Overview of all plasmid constructs and linear cassettes (for integration in the genome) made for this work, apart from the libraries construction.

Construct	Linearised vector - restriction enzymes	Template for insert	Primers for amplification of insert
pUC18-Cml	pUC18 - - - SmaI	pKD3	IB0180 & IB0181
pTrc99a-σ^{B} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0250 & IB0251
pTrc99a-σ^{F} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0252 & IB0253
pTrc99a-σ^{G} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0254 & IB0255
pTrc99a-σ^{H} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0256 & IB0257
pTrc99a-σ^{M} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0238 & IB0249
pTrc99a-σ^{W} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0258 & IB0259
pTrc99a-σ^{X} *B. subtilis*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *B. subtilis*	1) IB0245 & IB0262 2) IB0260 & IB0261
pTrc99a-σ^{E} *E. coli*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *E. coli* MG1655	1) IB0245 & IB0262 2) IB0484 & IB0485
pTrc99a-σ^{F} *E. coli*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *E. coli* MG1655	1) IB0245 & IB0262 2) IB0478 & IB0479
pTrc99a-σ^{H} *E. coli*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *E. coli* MG1655	1) IB0245 & IB0262 2) IB0480 & IB0481
pTrc99a-σ^{N} *E. coli*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *E. coli* MG1655	1) IB0245 & IB0262 2) IB0476 & IB0477
pTrc99a-σ^{FecI} *E. coli*	pTrc99a - - - SmaI and EcoRI	1) pSC101-mKate2 2) genomic DNA *E. coli* MG1655	1) IB0245 & IB0262 2) IB0482 & IB0483
pUC18-Cml-σ^{B}	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^{B}	IB0184 & IB0268
pUC18-Cml-σ^{F}	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^{F}	IB0184 & IB0269
pUC18-Cml-σ^{G}	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^{G}	IB0184 & IB0270
pUC18-Cml-σ^{H}	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^{H}	IB0184 & IB0271
pUC18-Cml-σ^{M}	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^{M}	IB0184 & IB0186

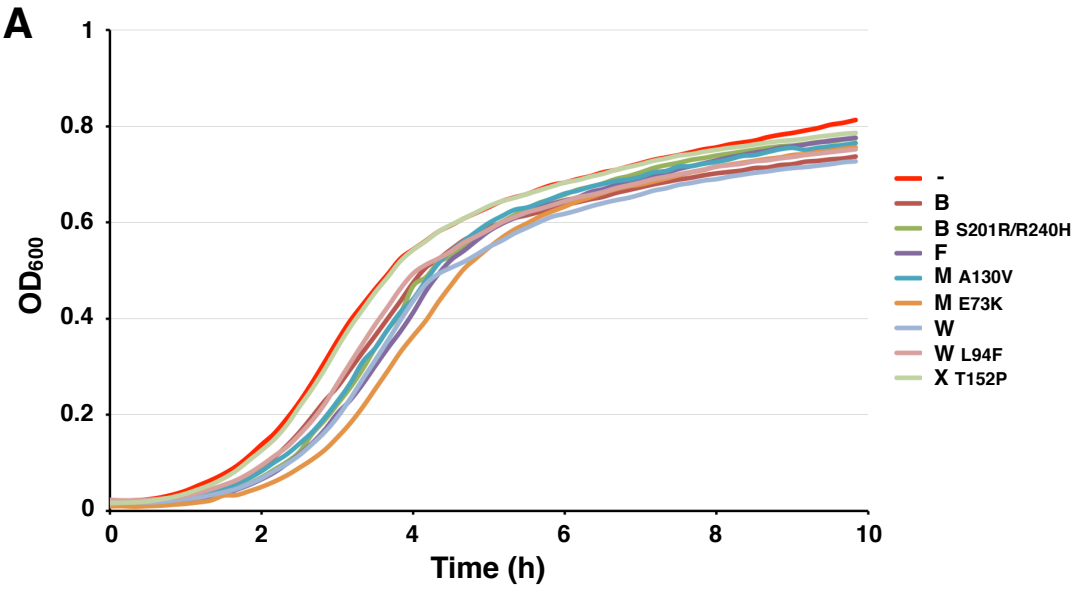
pUC18-Cml-σ^W	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^W	IB0184 & IB0272
pUC18-Cml-σ^X	pUC18-Cml - - - BamHI & HindIII	pTrc99a-σ^X	IB0184 & IB0273
Linear cassette in genome behind *rpoS* gene	/	linearized pUC18-Cml derivatives (XmnI or EcoO109I or EcoRI - single cutter outside area of interest)	IB0198 & IB0199
pSC101 *Bacillus* promoter constructs	pSC101-mKate2 (linearized by PCR: IB0173 & IB0174)	Synthetic oligos different promoters (see Table S3)	IB0175 & IB0176

Table S4: Overview of all promoter spacer DNA sequences of the representative set of promoters for each library. -35 and -10 conserved regions are marked in red.

Sigma factor	Promoter	Sequence
B	P_{B2}	GTTTATTTTTTGAAAAAGGGTAT
	$P_{B2.1}$	GTTTATCAAATGGTGCTGGGGTAT
	$P_{B2.2}$	GTTTATCGTTTAATCTGTGGGTAT
	$P_{B2.3}$	GTTTATAGGTCCTCAATTGGGTAT
	$P_{B2.4}$	GTTTATCAAAAGGCACATGGGTAT
	$P_{B2.5}$	GTTTATTCCCCAGTTTTGGGGTAT
	$P_{B2.6}$	GTTTATTTGTTCGAAAGGGGGTAT
	$P_{B2.7}$	GTTTATCATATGCAAAACGGGTAT
	$P_{B2.8}$	GTTTATTCTGGGAAAATCGGGTAT
	$P_{B2.9}$	GTTTATCTGTGGTAAAACGGGTAT
	$P_{B2.10}$	GTTTATGTTTTTTCTGTACAGGGTAT
F	P_{F3}	GTTTAAAAACGATCTTTTTTTCTCATAAT
	$P_{F3.1}$	GTTTAAGCTATTGAGGGTATTCTCATAAT
	$P_{F3.2}$	GTTTATGCCAAATGGCAGGTGCTCATAAT
	$P_{F3.3}$	GTTTATTGACGGATATCGCTGCTCATAAT
	$P_{F3.4}$	GTTTAGTGATGTGTCACGATGCTCATAAT
	$P_{F3.5}$	GTTTATTTGAAGGGATGAGTGCTCATAAT
	$P_{F3.6}$	GTTTAGTTTTAATTATAACTGCTCATAAT
	$P_{F3.7}$	GTTTAAAAACGATGCGTTGTGCTCATAAT
	$P_{F3.8}$	GTTTACATAATTTAATTTTGGCTCATAAT
	$P_{F3.9}$	GTTTACTTTTATGTGTTTATGCTCATAAT
W	P_{W2}	TGAAACCTTTTGAAACGAAGCTCGTA
	$P_{W2.1}$	TGAAACTTATTTACCCTCGTA
	$P_{W2.2}$	TGAAACCTTTTGAGCAGCTTTCGTA
	$P_{W2.3}$	TGAAACGAGCCCGGGATTTCGCGTA
	$P_{W2.4}$	TGAAACCTTTTGAAAGGATTTGCGTA
	$P_{W2.5}$	TGAAACCTTTTGAACGTTTGCACGTA
	$P_{W2.6}$	TGAAACGGAAAAATGGAGCGGGCGTA
	$P_{W2.7}$	TGAAACCGATCGTCTGCGGACGCGTA
	$P_{W2.8}$	TGAAACGCGGAAAAACGAAGCTCGTA
	$P_{W2.9}$	TGAAACGTCTCGGAGGGGTGTTCGTA

Figure S1: **A.** Representative growth curves of *E. coli* MG1655 and knock-in variants bearing a heterologous sigma factor in the genome. **B.** Overview of growth parameters. Data represent the mean of at least three replicates ± standard deviation.

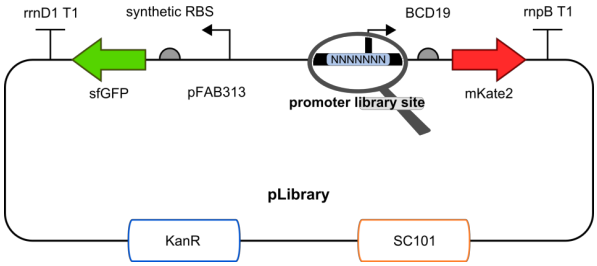
A



B

	Chromosomal insertion (single copy)		Plasmid-borne (pTrc99a; non-induced)	
Heterologous sigma factor	μ	A	μ	A
-	0.55 ± 0.02	1.48 ± 0.02	0.51 ± 0.02	1.58 ± 0.08
B	0.52 ± 0.04	1.58 ± 0.12	0.48 ± 0.03	1.67 ± 0.16
B S201R/R240H	0.57 ± 0.03	1.63 ± 0.17	0.55 ± 0.01	1.62 ± 0.14
F	0.53 ± 0.06	1.60 ± 0.19	0.48 ± 0.04	1.59 ± 0.07
G	0.53 ± 0.05	1.63 ± 0.14	0.46 ± 0.02	1.89 ± 0.31
M A130V	0.54 ± 0.07	1.70 ± 0.29	0.41 ± 0.12	1.65 ± 0.13
M E73K	0.53 ± 0.02	1.74 ± 0.11	0.55 ± 0.03	1.65 ± 0.12
W	0.56 ± 0.05	1.81 ± 0.18	0.49 ± 0.01	1.63 ± 0.02
W L94F	0.54 ± 0.06	1.60 ± 0.18	0.52 ± 0.03	1.77 ± 0.16
X T152P	0.58 ± 0.01	1.62 ± 0.14	0.51 ± 0.04	1.67 ± 0.30

Figure S2: Schematic overview and annotated DNA sequence (genbank format) of the general pLibrary in which the different engineered sigma factor-specific promoter libraries are inserted at the promoter library site. All library construct sequences are obtained by replacing 'NNNNNNN' with the sequences provided in Table 1. The relevant genetic parts of the construct are the *rrnD1* T1 (12) terminator, sfGFP (13) coding DNA sequence (CDS), a synthetic RBS (14, 15) with a translation initiation rate of 14921, pFAB313 (11) promoter from the biofab collection, promoter library site, an RBS with bicistronic design (BCD19) (11), *mKate2* (16) CDS and the *rnpB* T1 (12) terminator.



LOCUS pLibrary_construct 5882 bp ds-DNA circular
DEFINITION .
FEATURES Location/Qualifiers
CDS 1240..1941
/label="mKate2"
CDS complement(112..828)
/label="sfGFP"
promoter complement(864..899)
/label="pFAB313 "
terminator 1971..2052
/label="rnpB T1"
misc_feature 1145..1151
/label="promoter library site"
CDS 3062..4012
/label="repA"
RBS 1152..1239
/label="BCD19"
CDS complement(4662..5456)
/label="KanR"
RBS complement(829..863)
/label="synthetic RBS (TIR = 14921)"
terminator complement(1..91)
/label="rrnD1 T1"
ORIGIN
1 CGGGAGAGTG TTCACCGACA AACAACAGAT AAAACAAAAG GCCCAGTCTT CCGACTGAGC
61 CTTTTGTTTT ATTTGATGTC TGGCAGTTCC CCACTCGCCA GATTTACGAA GTTACTTATA
121 GAGTTCATCC ATGCCATGAG TAATCCCCGC TGCCGTGACG AATTCCAACA GGACCATATG
181 GTCACGCTTC TCGTTTGGGT CTTTACTAAG AACGCTTTGT GTAGACAGGT AATGATTATC
241 CGGGAGCAGA ACGGGGCCAT CGCCAATCGG AGTATTCTGC TGGTAATGAT CAGCCAGCTG
301 CACGGAACCG TCCTCTACGT TATGACGGAT TTTAAAGTTG GCTTTGATGC CATTTTTCTG
361 TTTATCCGCT GTAATGTATA CGTTGTGCGA ATTAAAGTTG TATTCCAATT TGTGCCCCAG
421 GATATTCCCA TCCTCTTTGA AATCGATACC TTTTAATTCA ATGCGGTTAA CTAAGGTATC
481 GCCTTCAAAT TTCACTTCCG CGCGGGTCTT ATACGTCCCA TCGTCTTTGA AGCTAATAGT
541 CCGTTCCTGC ACATAACCTT CAGGCATTGC GCTTTTAAAA AAGTCGTGGC GTTTCATGTG
601 ATCTGGATAG CGTGAAAAGC ATTGGACGCC ATACGTCAAC GTTGTGACCA GCGTCGGCCA

661 AGGCACGGGC AGCTTACCGG TGGTACAGAT GAACTTCAGG GTCAGCTTAC CATTGGTGGC
721 ATCTCCCTCT CCTTCGCCAC GAACAGAGAA CTTATGACCG TTCACGTCTC CGTCCAGTTC
781 TACTAAAATC GGCACAACAC CGGTAAAAAG CTCTTCGCCC TTGCCCATTG GTACCTCCTC
841 GATAATTCTC TTTGATTGGA TAATCCACAC ACCCTAAGAG CCGGATGATT AATTGTCAAT
901 CTTTGGGTTA ATGAGGCGCT TATCCATCAG CGCGCTTGAT GAAGTTTTGC TATATAAGAA
961 ACGAAAGTTT AGCGCCGCAT TTTCAGTTTC AACCAGGGAT TCGGCTCACA GAGTATAAGT
1021 GGCCGCTGCG AAGGCCGGAT TATCGCCTCT GTGTTTCATC AAAAGTAATC CTGCCGGCAT
1081 TAATTTCAGG GGGCTTGCCT TCTAGAGCGC AGCTAACACC ACGTCGTCCC TATCTGCTGC
1141 CCTANNNNNN NGGGCCCAAG TTCACTTAAA AAGGAGATCA ACAATGAAAG CAATTTTCGT
1201 ACTGAAACAT CTTAATCATG CTATGGAGGT TTTCTAATGA TGGTTAGCGA GCTGATCAAA
1261 GAAAACATGC ACATGAAACT GTATATGGAA GGCACCGTGA ATAACCACCA CTTTAAATGT
1321 ACCAGCGAAG GTGAAGGTAA ACCGTATGAA GGCACCCAGA CCATGCGTAT TAAAGCAGTT
1381 GAAGGTGGTC CGCTGCCGTT TGCATTTGAT ATTCTGGCAA CCAGCTTTAT GTATGGCAGC
1441 AAAACCTTTA TTAACCATAC CCAGGGTATC CCGGATTTTT TCAAACAGAG CTTTCCGGAA
1501 GGTTTTACCT GGGAACGTGT TACCACCTAT GAAGATGGTG GTGTTCTGAC CGCAACCCAG
1561 GATACCAGTC TGCAGGATGG TTGTCTGATT TATAATGTGA AAATTCGCGG TGTGAACTTT
1621 CCGAGCAATG GTCCGGTTAT GCAGAAAAAA ACCCTGGGTT GGGAAGCAAG CACCGAAACC
1681 CTGTATCCGG CAGATGGTGG TCTGGAAGGT CGTGCAGATA TGGCACTGAA ACTGGTTGGT
1741 GGTGGTCATC TGATTTGCAA TCTGAAAACC ACCTATCGTA GCAAAAAACC GGCAAAAAAT
1801 CTGAAAATGC CTGGCGTGTA TTATGTTGAT CGTCGTCTGG AACGTATTAA AGAGGCAGAT
1861 AAAGAAACCT ATGTGGAACA GCATGAAGTT GCAGTTGCAC GTTATTGTGA TCTGCCGAGC
1921 AAACTGGGTC ACCGCTGATA ACCATGGGCT AGCGGTTTGA AGGGTATTGG TCGGTCAGTT
1981 TCACCTGATT TACGTAAAAA CCCGCTTCGG CGGGTTTTTG CTTTTGGAGG GGCAGAAAGA
2041 TGAATGACTG TCCTTTTATT GGAGAGGTGG ACAAGTGGCA TCAGAGTTCA CTCTTAATTC
2101 TGAACATACC CGTCTTTTTC GCCTCTTTTA CGTGATTAAC TCCAGCGCTG GCGGCGGTTT
2161 TTAAAGGACA AAGACTCCGG TATTCAGACA TGACAACAAA TTACCAGGGT TTGGCTGCCG
2221 GACATAAAAT TTTGGTTTAC AGCAATTTAT ATATTCCAGT CGGGAAACCT GTCGTGCCAG
2281 CTGCATTAAT GAATCGGCCA ACGCGAATTC CCGACAGTAA GACGGGTAAG CCTGTTGATG
2341 ATACCGCTGC CTTACTGGGT GCATTAGCCA GTCTGAATGA CCTGTCACGG GATAATCCGA
2401 AGTGGTCAGA CTGGAAAATC AGAGGGCAGG AACTGCTGAA CAGCAAAAAG TCAGATAGCA
2461 CCACATAGCA GACCCGCCAT AAAACGCCCT GAGAAGCCCG TGACGGGCTT TTCTTGTATT
2521 ATGGGTAGTT TCCTTGCATG AATCCATAAA AGGCGCCTGT AGTGCCATTT ACCCCCATTC
2581 ACTGCCAGAG CCGTGAGCGC AGCGAACTGA ATGTCACGAA AAAGACAGCG ACTCAGGTGC
2641 CTGATGGTCG GAGACAAAAG GAATATTCAG CGATTTGCCC GAGCTTGCGA GGGTGCTACT
2701 TAAGCCTTTA GGGTTTTAAG GTCTGTTTTG TAGAGGAGCA AACAGCGTTT GCGACATCCT
2761 TTTGTAATAC TGCGGAACTG ACTAAAGTAG TGAGTTATAC ACAGGGCTGG GATCTATTCT
2821 TTTTATCTTT TTTTATTCTT TCTTTATTCT ATAAATTATA ACCACTTGAA TATAAACAAA
2881 AAAAACACAC AAAGGTCTAG CGGAATTTAC AGAGGGTCTA GCAGAATTTA CAAGTTTTCC
2941 AGCAAAGGTC TAGCAGAATT TACAGATACC CACAACTCAA AGGAAAAGGA CTAGTAATTA
3001 TCATTGACTA GCCCATCTCA ATTGGTATAG TGATTAAAAT CACCTAGACC AATTGAGATG
3061 TATGTCTGAA TTAGTTGTTT TCAAAGCAAA TGAACTAGCG ATTAGTCGCT ATGACTTAAC
3121 GGAGCATGAA ACCAAGCTAA TTTTATGCTG TGTGGCACTA CTCAACCCCA CGATTGAAAA
3181 CCCTACAAGG AAAGAACGGA CGGTATCGTT CACTTATAAC CAATACGCTC AGATGATGAA
3241 CATCAGTAGG GAAAATGCTT ATGGTGTATT AGCTAAAGCA ACCAGAGAGC TGATGACGAG
3301 AACTGTGGAA ATCAGGAATC CTTTGGTTAA AGGCTTTGAG ATTTTCCAGT GGACAAACTA

3361 TGCCAAGTTC TCAAGCGAAA AATTAGAATT AGTTTTTAGT GAAGAGATAT TGCCTTATCT
3421 TTTCCAGTTA AAAAAATTCA TAAAATATAA TCTGGAACAT GTTAAGTCTT TTGAAAACAA
3481 ATACTCTATG AGGATTTATG AGTGGTTATT AAAAGAACTA ACACAAAAGA AAACTCACAA
3541 GGCAAATATA GAGATTAGCC TTGATGAATT TAAGTTCATG TTAATGCTTG AAAATAACTA
3601 CCATGAGTTT AAAAGGCTTA ACCAATGGGT TTTGAAACCA ATAAGTAAAG ATTTAAACAC
3661 TTACAGCAAT ATGAAATTGG TGGTTGATAA GCGAGGCCGC CCGACTGATA CGTTGATTTT
3721 CCAAGTTGAA CTAGATAGAC AAATGGATCT CGTAACCGAA CTTGAGAACA ACCAGATAAA
3781 AATGAATGGT GACAAAATAC CAACAACCAT TACATCAGAT TCCTACCTAC ATAACGGACT
3841 AAGAAAAACA CTACACGATG CTTTAACTGC AAAAATTCAG CTCACCAGTT TTGAGGCAAA
3901 ATTTTTGAGT GACATGCAAA GTAAGTATGA TCTCAATGGT TCGTTCTCAT GGCTCACGCA
3961 AAAACAACGA ACCACACTAG AGAACATACT GGCTAAATAC GGAAGGATCT GAGGTTCTTA
4021 TGGCTCTTGT ATCTATCAGT GAAGCATCAA GACTAACAAA CAAAAGTAGA ACAACTGTTC
4081 ACCGTTACAT ATCAAAGGGA AAACTGTCCA TATGCACAGA TGAAAACGGT GTAAAAAAGA
4141 TAGATACATC AGAGCTTTTA CGAGTTTTTG GTGCATTCAA AGCTGTTCAC CATGAACAGA
4201 TCGACAATGT AACAGATGAA CAGCATGTAA CACCTAATAG AACAGGTGAA ACCAGTAAAA
4261 CAAAGCAACT AGAACATGAA ATTGAACACC TGAGACAACT TGTTACAGCT CAACAGTCAC
4321 ACATAGACAG CCTGAAACAG GCGATGCTGC TTATCGAAGT CTGACGCTCA GTGGAACGAA
4381 AACTCACGTT AAGGGATTTT GGTCATGCAT ATGAATATCC TCCTTAGTTC CTATTCCGAA
4441 GTTCCTATTC TCTAGAAAGT ATAGGAACTT CAGAGCGCTT TTGAAGCTGG GGTGGGCGAA
4501 GAACTCCAGC ATGAGATCCC CGCGCTGGAG GATCATCCAG CCGGCGTCCC GGAAAACGAT
4561 TCCGAAGCCC AACCTTTCAT AGAAGGCGGC GGTGGAATCG AAATCTCGTG ATGGCAGGTT
4621 GGGCGTCGCT TGGTCGGTCA TTTCGAACCC CAGAGTCCCG CTCAGAAGAA CTCGTCAAGA
4681 AGGCGATAGA AGGCGATGCG CTGCGAATCG GGAGCGGCGA TACCGTAAAG CACGAGGAAG
4741 CGGTCAGCCC ATTCGCCGCC AAGCTCTTCA GCAATATCAC GGGTAGCCAA CGCTATGTCC
4801 TGATAGCGGT CCGCCACACC CAGCCGGCCA CAGTCGATGA ATCCAGAAAA GCGGCCATTT
4861 TCCACCATGA TATTCGGCAA GCAGGCATCG CCATGGGTCA CGACGAGATC CTCGCCGTCG
4921 GGCATGCGCG CCTTGAGCCT GGCGAACAGT TCGGCTGGCG CGAGCCCCTG ATGCTCTTCG
4981 TCCAGATCAT CCTGATCGAC AAGACCGGCT TCCATCCGAG TACGTGCTCG CTCGATGCGA
5041 TGTTTCGCTT GGTGGTCGAA TGGGCAGGTA GCCGGATCAA GCGTATGCAG CCGCCGCATT
5101 GCATCAGCCA TGATGGATAC TTTCTCGGCA GGAGCAAGGT GAGATGACAG GAGATCCTGC
5161 CCCGGCACTT CGCCCAATAG CAGCCAGTCC CTTCCCGCTT CAGTGACAAC GTCGAGCACA
5221 GCTGCGCAAG GAACGCCCGT CGTGGCCAGC CACGATAGCC GCGCTGCCTC GTCCTGCAGT
5281 TCATTCAGGG CACCGGACAG GTCGGTCTTG ACAAAAAGAA CCGGGCGCCC CTGCGCTGAC
5341 AGCCGGAACA CGGCGGCATC AGAGCAGCCG ATTGTCTGTT GTGCCCAGTC ATAGCCGAAT
5401 AGCCTCTCCA CCCAAGCGGC CGGAGAACCT GCGTGCAATC CATCTTGTTC AATCATGCGA
5461 AACGATCCTC ATCCTGTCTC TTGATCAGAT CTTGATCCCC TGCGCCATCA GATCCTTGGC
5521 GGCAAGAAAG CCATCCAGTT TACTTTGCAG GGCTTCCCAA CCTTACCAGA GGGCGCCCCA
5581 GCTGGCAATT CCGGTTCGCT TGCTGTCCAT AAAACCGCCC AGTCTAGCTA TCGCCATGTA
5641 AGCCCACTGC AAGCTACCTG CTTTCTCTTT GCGCTTGCGT TTTCCCTTGT CCAGATAGCC
5701 CAGTAGCTGA CATTCATCCG GGGTCAGCAC CGTTTCTGCG GACTGGCTTT CTACGTGTTC
5761 CGCTTCCTTT AGCAGCCCTT GCGCCCTGAG TGCTTGCGGC AGCGTGGGGG ATCTTGAAGT
5821 TCCTATTCCG AAGTTCCTAT TCTCTAGAAA GTATAGGAAC TTCGAAGCAG CTCCAGCCTA
5881 CA
//

Figure S3: For each of the P_{B2}, P_{F3} and P_{W2} promoters and the associated libraries, a PBS diluted sample, prepared for flow cytometry analysis, was used as a template for high fidelity PCR and subsequent Sanger sequencing. The sequence traces are shown for the original and randomized *B. subtilis* relevant promoter regions.

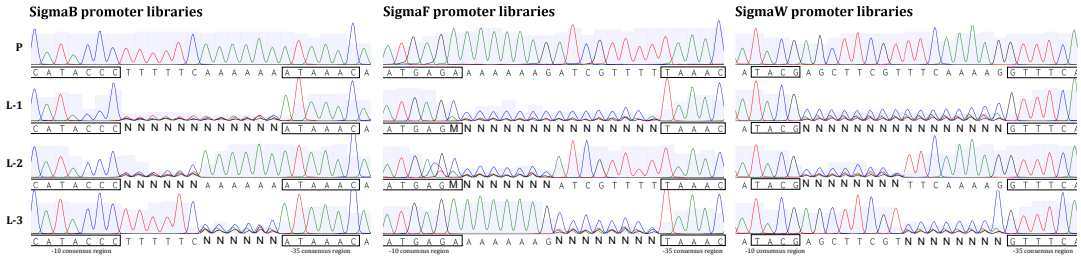


Figure S4: **A.** Flow cytometry data was gated with the Bioconductor flowPeaks package in R (parameters: tol = 0.1, h0 = 3, h = 3). Clustering is based on the transformed (inverse hyperbolic sine function) sfGFP expression and the log transformed forward scatter. The green cluster is situated in the auto-fluorescence region and the red cluster is the retained data. **B.** The clustered data (Figure S3) is subjected to outlier removal with the Bioconductor flowPeaks package (parameters: tol = 0.01, fc = 0.6). Black dots represent excluded data points. Additionally, the percentage of the total sample clustered in group 1 (C1) (*#cluster1 / #sampleTotal * 100*) and the fraction of the outliers in cluster 1 (O) was calculated (*#outliersCluster1 / #cluster1 * 100*).

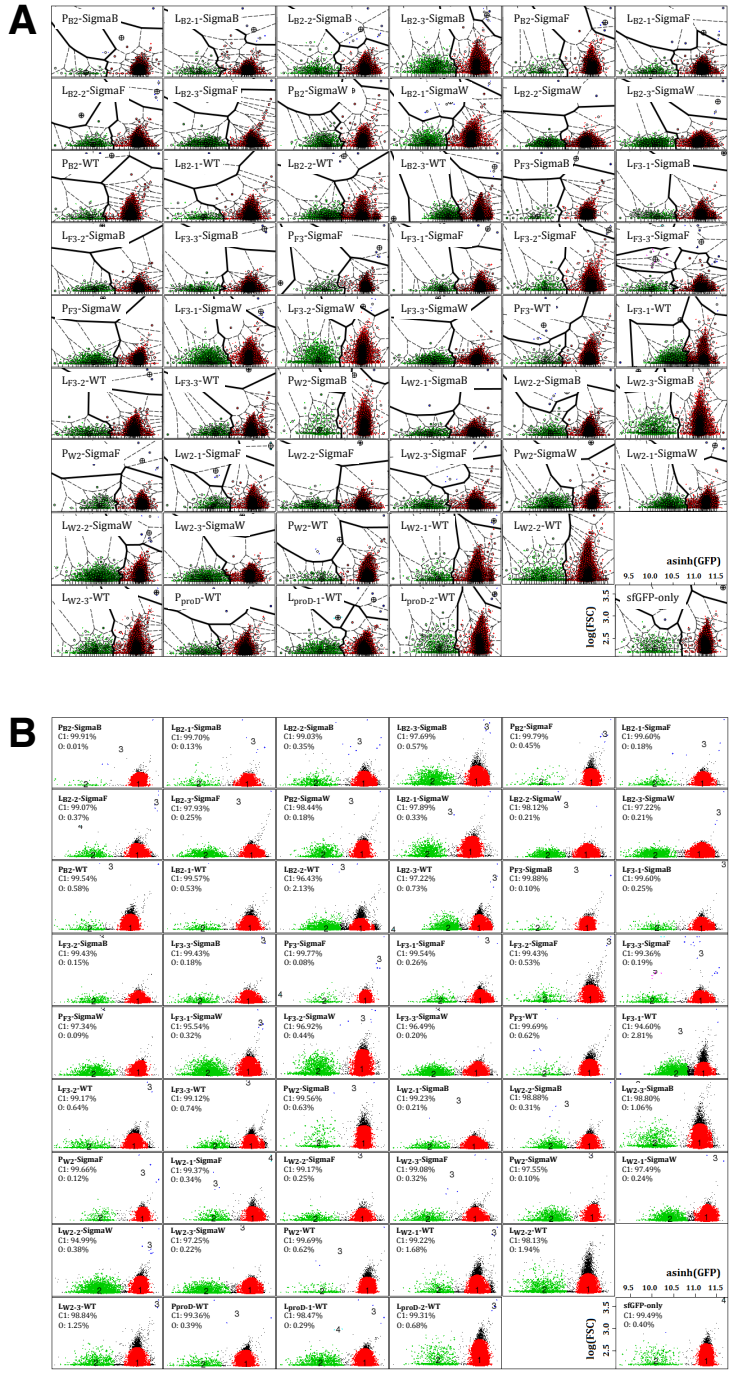


Figure S5: Change in activity of the promoter libraries displayed as the fraction of the distribution situated above the negative control based threshold compared to the respective original promoter. Randomizing the promoter sequences causes a subset of the libraries to become inactive in presence of their cognate sigma factor resulting in a negative shift. The same applies to non-cognate partners, which indicates a gain of orthogonality. A positive shift indicates a portion of the library promoters gained in promoter activity.