

# Supporting Information

## Novel genetic modules encoding high-level antibiotic-free protein

### expression in probiotic lactobacilli

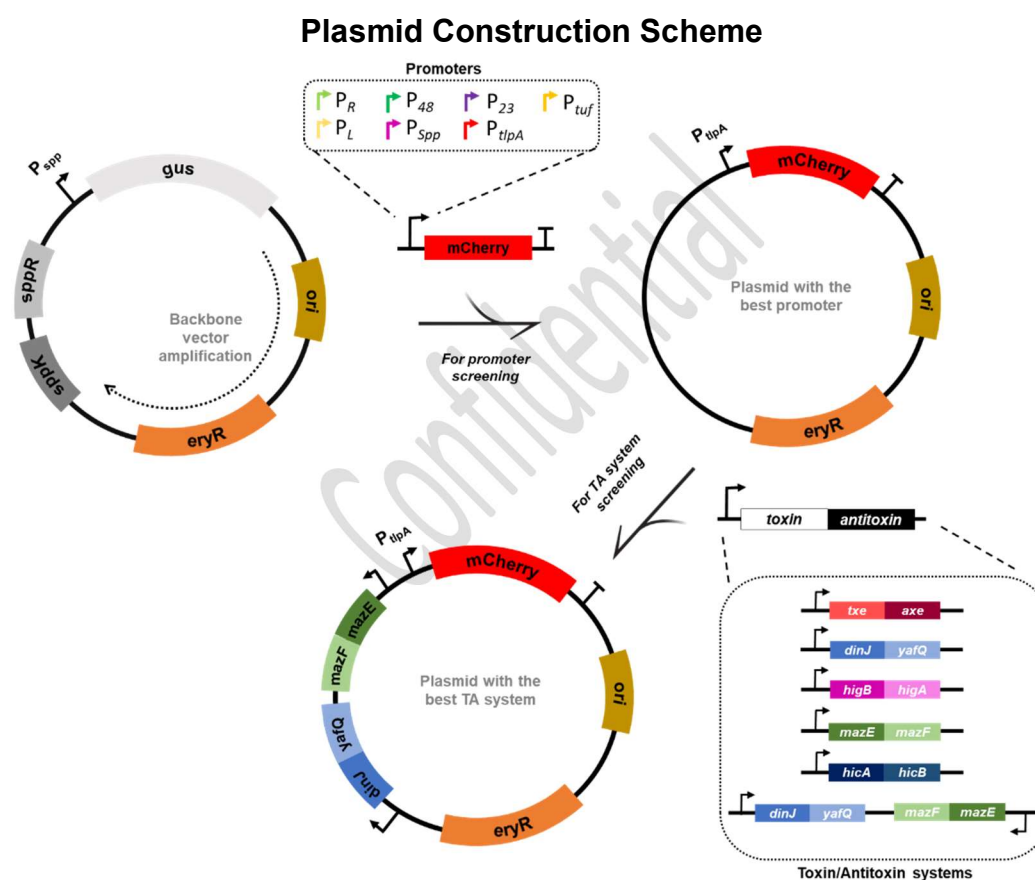
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**Supplementary Figure S1** Construction of plasmid variants using the direct cloning approach developed in this study. The p256 origin of replication and the erythromycin resistance marker has been amplified from the pSIP403 vector backbone. The interchangeable promoter sequences were upstream of the mCherry reporter gene with  $P_{tipA}$  promoter showing the best performance (highlighted). The activity of the toxin/antitoxin modules were checked in the pTIpA mcherry plasmid with the combo TA system showing the best plasmid retention (highlighted).

**Supplementary Table S1.** Nucleotide Sequences of the common genetic parts and interchangeable promoter sequences tested in this study

NAME	GENETIC PART	COMMON FEATURE	ORIENTATION	SEQUENCE
Ori p256	Origin of replication	Yes	→	<p>ttgagatccttttttctgcgcgtaatctgctgctgcaa                      acaaaaaaacaccgctaccagcgggtgttgttggc                      ggatcaagagctaccaactcttttccgaaggtaactg                      gcttcagcagagcgcagataccaaatactgttctta                      gtgtagccgtagttaggccaccactcaagaactctgt                      agcaccgcctacatacctcgtctgctaactcgtttacc                      agtggctgctgccagtggcgataagtcgtgtcttaccg                      ggttgactcaagacgatagttaccggataaggcgca                      gcggtcgggctgaacggggggttcgtgcacacagccc                      agcttgagcgaacgacctacaccgaactgagatacc                      tacagcgtgagctatgagaaagccacgcttcccga                      gggagaaaggcggacaggtatccgtaagcggcagg                      gtcggaacaggagagcgcacgaggagcttcagggg                      ggaaacgctgtatctttatagctctgctgggttcgc                      cacctctgactgagcgtgattttgtgatgctcgtca                      gggggcggagcctatggaaa</p>
eryR	Antibiotic resistance	Yes	→	<p>ttgaacaaaaatataaaatattctcaaaacttttaac                      gagtgaaaaagtactcaacaaataataaaacaatt                      gaatttaaaagaaaccgataccgtttacgaaattgga                      acaggtaaagggcatttaacgacgaaactggctaaa                      ataagtaaacagtaacgtctattgaattagacagtca                      tctattcaacttatcgtcagaaaaattaaactgaata                      ctctgtcactttaattcaccaagatattctacagttca                      attccctaacaacagaggataaaattgttgggaata                      ttccttacaatttaagcacacaattattaaaaaagtg                      gttttgaaagccgtgctgtgacatctatctgactgtg                      aagaaggattctacaagcgtacctggatattcaccga                      acaactagggtgctcttgcaactcaagtctgattcag                      caattgcttaagctgccagcggaatgctttcatcctaa                      accaaaagtaaacagtgtcttaataaaacttaccgc                      cataccacagatgtccagataaatattggaagtata                      taagtactttgtttcaaaatgggtcaatcgagaatatcg                      tcaactgtttactaaaaatcagttcgtcaagcaatga                      aacacgccaagtaacaatttaagtaccattacttat                      gagcaagtattgtctattttaatagttatctattatta                      acgggaggaaataa</p>
mCherry	Reporter gene	No	→	<p>atggttcaaagggtgaagaagataacatggctatca                      tcaaggaattcatgctttcaaggttcatggaagggt                      tcagttaacggtcacgaattcgaatcgaagggaag                      gtgaaggctcctacgaaggtactcaaaactgctaa                      gttaaaggttactaagggtggtccattaccattcgctg                      ggatatcttatcaccacaattcatgacggttcaaaggc                      ttacgttaagcaccagctgatatccagattacttaaa</p>

				ggtatcattcccagaaggtttcaagtgggaacgtgta tgaacttcgaagatgggtggtggttactgttactcaag attcatcattacaagatgggtgaattcatctacaaggtt aagttacgtggtactaactcccatcagatgggtccagtt atgcaaaagaagactatgggtgggaagttcatcag aacgtatgtaccagaagatgggtccttaaagggtga aatcaagcaacgtttaaagttaaaggatgggtggtcac tacgatgctgaagttaagactacttaaggctaaga agccagtcaattaccaggtgcttacaacgttaacatc aagttagatatcacttcacacaacgaagattactat cgttgaacaatacgaacgtgctgaaggtcgtcactcaa ctgggtgatggatgaattatacaagtaa
RBS	Ribosome-binding site	No	→	ttgtttaactttaagaaggaga
<i>P<sub>tIpA</sub></i>	Promoter	No	→	tttaattgtttgtagttagtttattgttggttgtttgt gttataatat
<i>P<sub>spp</sub></i>	Promoter	No	→	gccatattaacgtttaaccgataaagttgaacgttaa tattttttt
<i>P<sub>48</sub></i>	Promoter	No	→	tcgtaagttgtgacatggaacgaggaatgtgataatc tgtgagt
<i>P<sub>23</sub></i>	Promoter	No	→	ctgatgacaaaaagagaaaaatttgataaaatagtct tagaattaaataaaaa
<i>P<sub>Tuf</sub></i>	Promoter	No	→	tctgtttacaaatcagattaggctatataatatttaa gga
<i>P<sub>L</sub></i>	Promoter	No	→	ttgacataaataccactggcggtgatact
<i>P<sub>R</sub></i>	Promoter	No	→	ttgactattttacctctggcggtgataa
<i>PHAMP</i>	Promoter	No	→	caaaatgctgtgacgtttatgttggttatttacgtaata aatcacgac
T7 ter	Terminator	No	→	ctagcataacccttggggccttaaacgggtcctgagg ggtttttg

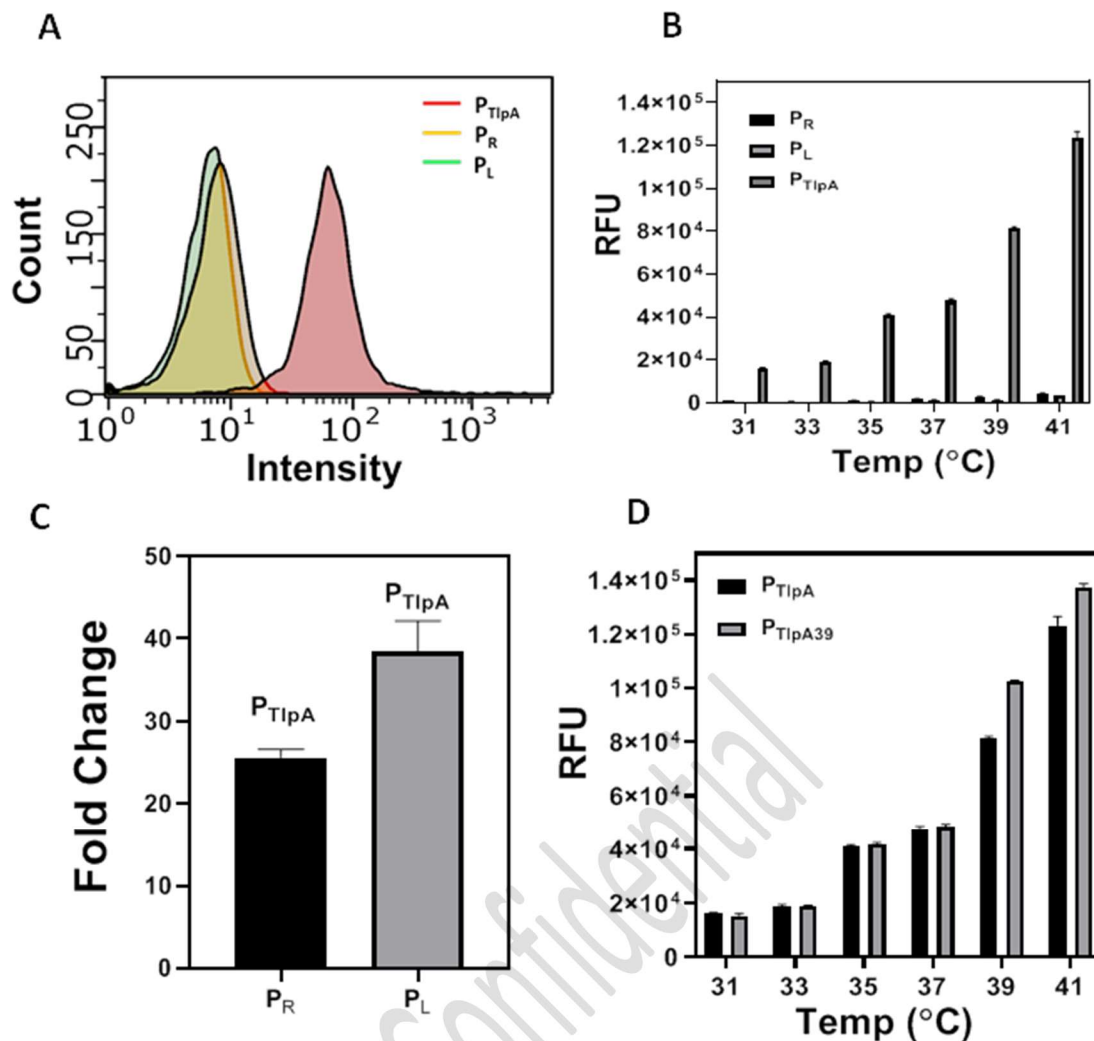
**Supplementary Table S2.** Nucleotide Sequences of the toxin/antitoxin modules tested in this study

NAME	GENETIC PART	COMMON FEATURE	ORIENTATION	SEQUENCE
YafQ/DinJ	Toxin-Antitoxin operon	No	→	<p>agatggcagttacgcttccatttgcgaagaaaaagcca  gaaatccggttgaattcatctcacatgatgttatcccactgt  gtttacattgggataattcgtgataataatagggttagat  aggaaggagtggttagcaatggcagccacaaagaaaga  aactcgttgaatattcgtgttgatccggaattaaaaagt  gctgctcaaatcgtagcaaatgatatggcatcgacttga  ccgacgtgttactatgttcatgacgaaaatggtgaaaga  tcacgccctccggttaccacaagaagctaccagttgaaa  ccttacaggcgtgaaagaagcaagcaccagagctgc  tcaaaaaatacagcagcctgatgacatgtggagagact  tgaatgtatgctggttccgacgcctacatttaagcgcga  tctaaaacgactctcaagaagcattggccgatggacga  actaaagacggctgtaatctcctagctgctggtacaat  gctgaactattaagcaaaaagtatgcagatcatgcctgt  cttcaagcagcagtggaaggatctggaactacatg  ttgacggcctcgtggcactggttgcatactataaaatt  aagcagcaagatctcattttgacctgggttag  aactggatctcatcaaacctttgggtaaatagaactacg  aaaggccgtcaaaaaaggcggcttttgtgtgtggtcaa  ccagcagattcattcgtcaatatacacgtgacttaacgcca  atcttcaatcagagattagaaccggaactcgtcggccc  agtaaaaatgttcagctatcgagatgagttgggtgatctg  gggaagtgagcatcaataagatccaatctttatcatgca  gggttggaaacgaaaatthaaccaatg</p>
MazF/MazE	Toxin-Antitoxin operon	No	←	<p>gaaggattacggtcaacgagtgctttcggacacgtccgt  tgatattgttatttgactcgtggttactccaatcaattat  actgacaagcatcgaaatataaacgccctacctaac  ctgagtaagttacagggcgaagtagtctgattagatgg  aaagtcgaagttgtaatacaccgtttgcgcgacgtgataa  aaatcggcaactgcgatggtttcgatgtatgtgatactccg  tcctgctctggggctgcatcaaacgaataaatttgggca  gcagcaatttgcctgggtattgtaaccattaagactaa  agtagccgggcaaatcagcattgtagacgtaattggtg  aaacaattacaaaccggttctgagtgtagagattact  actaagaactacagctggccgacgtttctttatcgcggc  cacgttgggtcaaatcaatccagatgatgtctttgt  ttaggtaataagtcattactcgacctcattccagtcgac  atcggaccatgcttcagtcattggccgtttcggcttgagc  cggcataaggttctcgatacgtggtacgagtgatatga  ctcaccatcacttgatgggatgagtaaccatcgttgcctt  tcttaaacgaccgtctttaggtagactaatccaatga  attaccagactaactgtctttacctcaatcaatcaaggc  ctcctcgattaataaacacactatttatgaacgagat  aacagctattacgaaataagacagctaaagcaatcgcg</p>

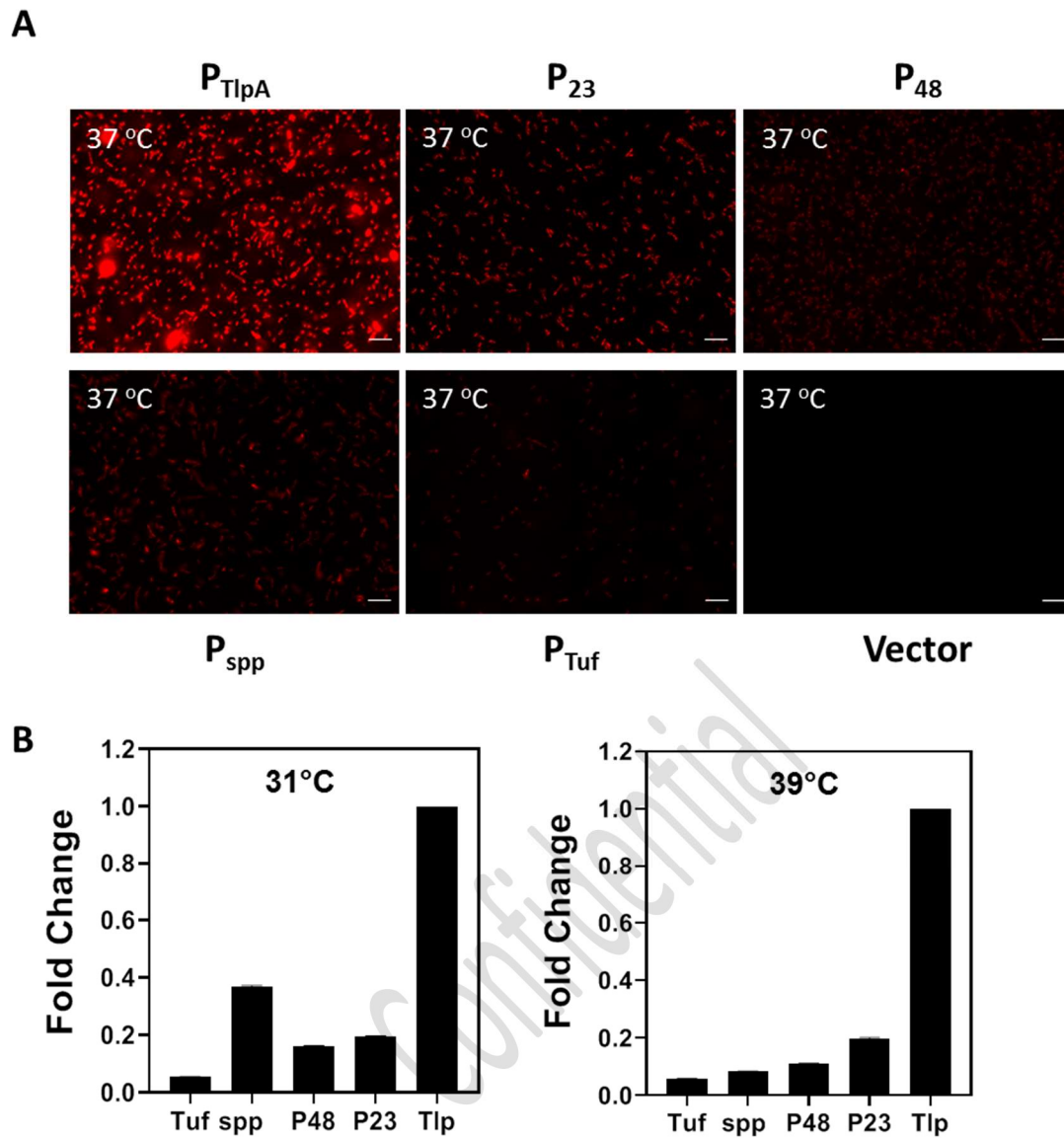
				<p>ataactaattgat  aagcacacggtttttagtaattcatcaataaaggaagag  actaattaatgtcgccgtaactcagtggtatcaagttggat  ggcccgcttagtattttccaccttagcctctt</p>
HicA/HicB	Toxin-Antitoxin operon	No	→	<p>tactgatgatgaacatgccccagcctgtttagctctttgc  gcaataaatcatttatttctgctggattgaatagagcttga  gcaaaatctttggtaaaatcattcatgaaggagtcttctt  tctgtatgtgtttttgttcaaaccaatcatacagagaaag  gaactccttttctaagttaaagaaataaatttaagga  atcattcatccttacacaaactattttacagctcattctt  cataaacacatcctagaaaagaactattaccatataca  atcaaacaaattcaagataaaatcaaagagatgaata  aagatgaaaaaactaatcaatatatgaagtataagg  ggtatgaaggttcaattgaatatactttggaggataaga  ttcttttggtaagggtcaggggattaagagcttatttctt  acgaaggtaatacaatagatgaattggagaaggatttc  aaggagcattgatgattatctaagctgtaaggaag  atggagtataccagagaaaccttttaaggttaatttaa  tgtgcgcatgactcattacatgaaaaattggcta  tatgctgcaacgaagcaccaatcattgaatgctagtgta  gaagaagctataaaaagattttggcttaggtgaaga  cgcttgaattttcaagcgtttttcatgaaaacaattg  ctaaattcattgaatgagtgactattttagtataacaat  agttgaacgggtgatgtcggcgatatagg</p>
HigB/HigA	Toxin-Antitoxin operon	No	→	<p>tactgatgatgaacatgcccactctggttggctctagcta  aaataggggtcgtactagtcgggtaacagatgaattgg  atattgggtgtggtcagtaataataggagcaagagtc  ggatggattgagatatacagtgatacgggtataatcga  ttaagaattttgagataaggaaactcacaaggttacc  aacaaaaattcttaacaaatgcccaccaaccattcagc  aattagcattgcgaaaactaactgattgatcatgcgga  acaattaatgattgagcctaccgctccaatcactg  gaaaaattaagtcatgatgcgaagggaatataagcatc  aggattaataatcagatcggatagttttgcaattcgaa  atggtaatgagttttatgatgttgaatagtcgattatcat  catggttaggagacaaaaatgaatgaaatccaaca  cctaaaattagtgaaattcttgaagaagagtttatggctc  ccttgcatatcctgcatatttttggctcaacagattgggg  taccacgtcaggttcaagacttactacatgatcgtcga  caggtgacggtgatacatcgtcggctaggacgattctt  tggagtgcggatcgttatttcttggaaactcaaatgata  ttgaaattcgtaatttgaacagatacatggtgctgaata  tgcaagataaaaaagatcaagtcagttaaataagga  cgtagaccgtagttgcttggtagcgtactacggttttgca  aatataacctaaactgataagcagcagatctaaatttta  aggttatcaacaggtggataaagcagtggtgctttgtatta  tccactgttgataatcattgagttggttaattgtactttca  ctactgggttaactggtcgtgagaaaaatggtaagcatt  agtttcgtggtgatgtcggcgatatagg</p>

Txe/Axe	Toxin-Antitoxin operon	No	←	<p>acgcgtaacaacacattcattaataacaatcaacacta  agctatattagcttttaaatgactagggtaaataaaata  aggatcaaaaactttaagtttctgacctttccttacttcc  gattggttaatagtgatcttttcagaataaataaatc  gtttcattttcaactctatataatcagctctatgttcatctgta  attcttctggaccattttccagataaatcatgctttaatgg  ctcaggttttccaatccagcaaagggggaacgatcgat  atcttttataacttgtaatctttttatattgctttgtttc  cttgctcatgccaataaagataaatcatccaagcatcatc  agaccaagccttaatcatcagattcaacctcgattaagt  catgtgttttaaatgcacctttggagaattgttcatctctc  gacgaatttttccatgacgtaattattagaaagtgttctc  aacgtttcttgatagaatcataatctttttgataaac  aacaactgtatcttcatcttactgttacaataagtg  ttcagcatcctcattaactgtttcatataactacgtaaa  tttggcggaaattgaataagctactgcttcattccttt  cacccctatttatttctataaaacaattgtacattatatt  gtacaattaagcaaatgattaatattctaacttctataa  ttaaagtgctatcacttttagtttttaggataaaagtg  acgtcacttttgagtgtggtacctcaaatcctgcttt</p>
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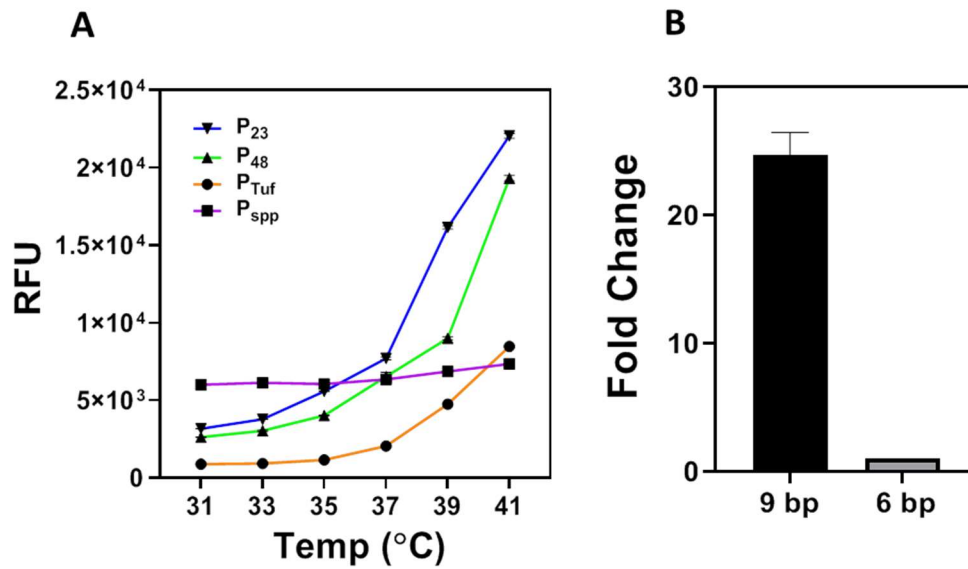


**Supplementary Figure S2.** (A) Flow Cytometry analysis of  $P_R$ ,  $P_L$  and  $P_{TlpA}$  driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C. (B) Fluorescence Spectroscopy analysis of the  $P_R$ ,  $P_L$  and  $P_{TlpA}$  driven mCherry expression after 18 h incubation in the Thermocycler setup at thermal gradients ranging from 31°C to 41°C (See Materials and Methods section “Microplate reader Setup for Thermal Gradient Analysis”). (C) Fold Change of  $P_{TlpA}$  driven mCherry expression in comparison to  $P_R$  and  $P_L$  promoters at 37°C (D) RFU Plot of mcherry production by plasmids pTlpA and pTlpA39 after 18 h incubation at thermal gradients ranging from 31°C to 41°C. The pTlpA39 plasmid encodes for an additional  $P_{48}$  promoter-driven codon-optimized TlpA39 repressor. The data in B, C and D corresponds to three independently conducted biological replicates with column heights representing means and whiskers representing SD.



**Supplementary Figure S3.** (A) Fluorescence microscopy images of  $P_{tlpA}$ ,  $P_{23}$ ,  $P_{48}$ ,  $P_{spp}$ ,  $P_{tuf}$  driven mCherry expression and Empty Vector constructs in *L. plantarum* WCFS1 cultivated at 37°C for 18 h (Scale = 10 $\mu$ m). (B) Fold Change of the  $P_{tuf}$ ,  $P_{23}$ ,  $P_{48}$ ,  $P_{spp}$  driven mCherry expression normalized to the expression level of the  $P_{tlpA}$  promoter at 31°C and 39°C respectively. The data corresponds to three independently conducted biological replicates with whiskers as SD.

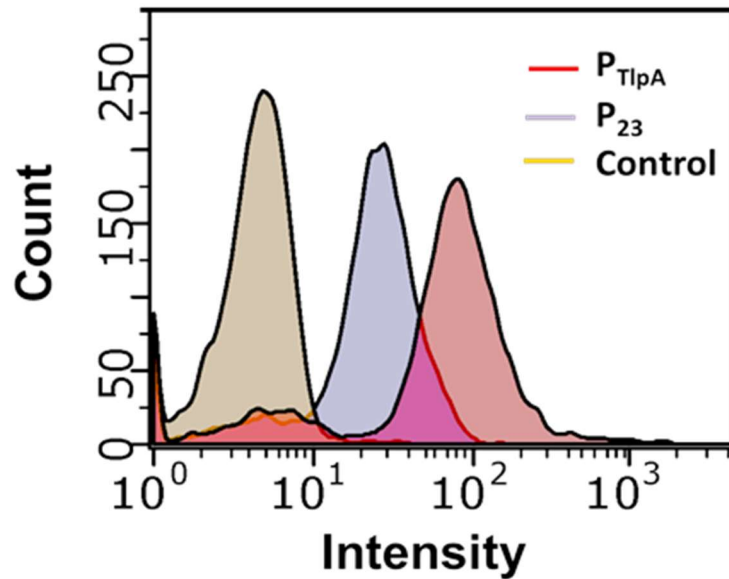




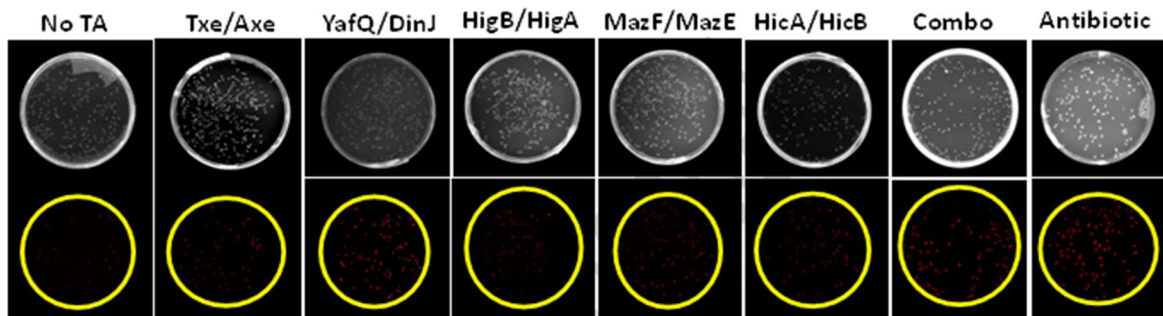
**Supplementary Figure S4.** (A) RFU Plot of P<sub>23</sub>, P<sub>48</sub> and P<sub>tuf</sub> driven mCherry expression showing enhanced fluorescence expression in contrast to P<sub>spp</sub> within the thermal gradients ranging from 31°C to 41°C. The data corresponds to three independently conducted biological replicates with whiskers as SD. (B) Fold Change of the P<sub>tuf</sub> promoter driven mCherry expression with 9 bp spacer (between the RBS and the start codon) in comparison to the P<sub>tuf</sub>-6 bp spacer construct.

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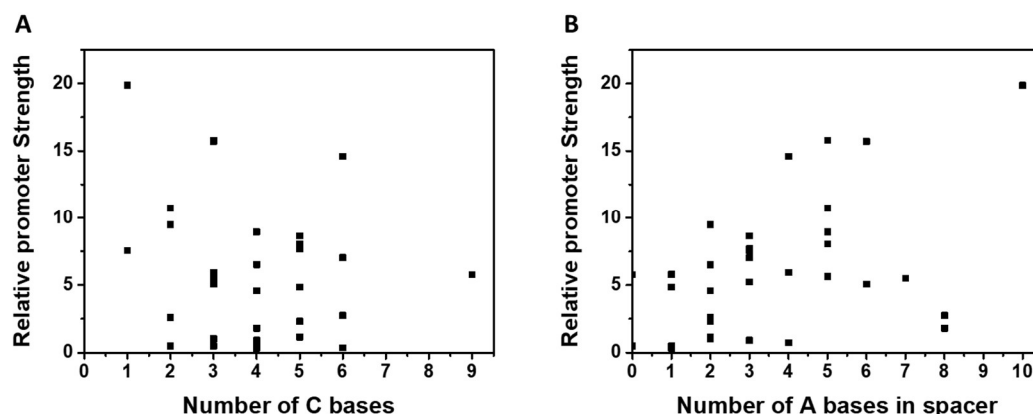
A



B



**Supplementary Figure S5.** (A) Flow cytometry plots showing that strains with  $P_{23}$ -driven mCherry expression produces relatively low intensities with part of the population overlapping with the signal gained from bacteria that do not express any fluorescent proteins (control). In comparison, the signal from  $P_{TlpA}$  is clearly demarcated from that of control. (B) Agar-plate based analysis of plasmid retention provided by the different TA systems. The total number of colonies can be determined from the brightfield images and the red colonies visible in the fluorescent images represent the plasmid-retaining bacteria.



**Supplementary Figure S6.** Plots representing relative promoter strengths of 34 promoters in Table S3 vs (A) number of C bases in the -35 to -10 region and (B) number of A bases in the spacer between the -35 and -10 region (non-bold region in Table S3).

**Supplementary Table S3:** Promoter sequences included in Figure S7 and their relative strengths. Sequences and strengths derived from reference “Rud, I., et al., *A synthetic promoter library for constitutive gene expression in Lactobacillus plantarum*. Microbiology, 2006. **152**(4): p. 1011-1019.” The relative strengths of P<sub>23</sub> and P<sub>Tuf</sub> from this study were estimated based on expression levels we determined in relation to P<sub>48</sub>.

Promoter	Sequence	Relative strength
P <sub>20</sub>	-35 <b>TTGACA</b> ACCTGTGGGCGGTTT <b>GATT</b> <b>TGTT</b> -10	0.33
P <sub>17</sub>	-35 <b>TTGACA</b> CTGATCCCGGCTGGTGG <b>TAAATT</b> -10	0.35
P <sub>13</sub>	-35 <b>TTGACAG</b> CGTGGGTTGGT <b>GCTGGTAAATTT</b> -10	0.48
P <sub>41</sub>	-35 <b>-TGACAG</b> GGGCTGTGATGGT <b>TGTTATTGT</b> -10	0.50
P <sub>43</sub>	-35 <b>TTGACAG</b> GATAAAGGTCGCCTGG <b>TATGGT</b> -10	0.74
P <sub>40</sub>	-35 <b>TCAACA</b> TACATGGATCG--- <b>TGGTATGTT</b> -10	0.91
P <sub>44</sub>	-35 <b>TTGACA</b> CTTGGAGGGTTT <b>GATGGTAAATCT</b> -10	1.02
P <sub>10</sub>	-35 <b>TTGACA</b> TTGTTAATGGCCCT <b>GATATATT</b> -10	1.15
P <sub>34</sub>	-35 <b>TTGACAG</b> ACAAACATAAGGAT <b>GATATGCT</b> -10	1.80
P <sub>9</sub>	-35 <b>TTGACAG</b> GGGCTGAGCTGACCTGG <b>TATGGT</b> -10	2.33
P <sub>42</sub>	-35 <b>TTGACA</b> GAGTTGTCTGGTATT <b>GATATTGT</b> -10	2.60
P <sub>5</sub>	-35 <b>TTGACA</b> CACAAAACCAGACATGG <b>TATTAT</b> -10	2.75
P <sub>33</sub>	-35 <b>TTGACA</b> TGTGACCCGTTTT <b>TATGGTATTAT</b> -10	4.58
P <sub>21</sub>	-35 <b>TTGACA</b> TTCCGGGCATCTCGTGG <b>TATAAT</b> -10	4.84
P <sub>26</sub>	-35 <b>TGGACA</b> AGTGATAAAACGGGT <b>GATATGAT</b> -10	5.06
P <sub>22</sub>	-35 <b>TTGACAG</b> ATTAGGGCGGTCATGG <b>TAAAAT</b> -10	5.23
P <sub>18</sub>	-35 <b>TTGACAG</b> GGGAGGCTTCGTT <b>TGTGATAAGAT</b> -10	5.79
P <sub>47</sub>	-35 <b>TTGACAG</b> ACGCGGGAGAATATGG <b>TAAAGT</b> -10	5.65
P <sub>16</sub>	-35 <b>TTGACA</b> TCCCCCTCTCTT <b>TCTGGTATAAT</b> -10	5.76
P <sub>3</sub>	-35 <b>TTGACA</b> AAGAAGCCGGGTTTT <b>TGGTATAAT</b> -10	5.93
P <sub>29</sub>	-35 <b>TTGACAG</b> TTCTGGCTGGATATGG <b>TAAACT</b> -10	6.51

P <sub>6</sub>	-35	<b>TTGACAGGCAGCCATCTCTATGGTAAAAT</b>	-10	7.04
P <sub>38</sub>	-35	<b>TTGACAGAATGTTTTTGTAGTGGTATAAT</b>	-10	7.56
P <sub>30</sub>	-35	<b>TTGACAGCCGAGGTACCATGTGGTATAAT</b>	-10	7.69
P <sub>31</sub>	-35	<b>TTGACAAAAGTCCCAGGGTATGATATACT</b>	-10	8.04
P <sub>8</sub>	-35	<b>TTGACAAATTCCGGTGTCTATGGTATTCT</b>	-10	8.64
P <sub>25</sub>	-35	<b>TTGACATTAAGGCACATCATTGATATGGT</b>	-10	8.94
P <sub>35</sub>	-35	<b>TTGACATGCAGGGAGTTTGTGGTATAAT</b>	-10	9.49
P <sub>1</sub>	-35	<b>TTGACAGGGATATTAGAGTATGGTATGCT</b>	-10	10.74
P <sub>4</sub>	-35	<b>TTGACACGCGCAGCAAGGCATGATATAAT</b>	-10	14.60
P <sub>11</sub>	-35	<b>TTGACAGAATGGACATACTATGATATATT</b>	-10	15.70
P <sub>48</sub>	-35	<b>TTGACATGGAACGAGGAATGTGATAATCT</b>	-10	15.79
P <sub>23</sub>	-35	<b>ATGACAAAAGAGAAAATTTTGATAAAAAT</b>	-10	19.86
P <sub>Tuf</sub>	-35	<b>TTTACAAATCAGATTAGGCTATATATAAT</b>	-10	5.49

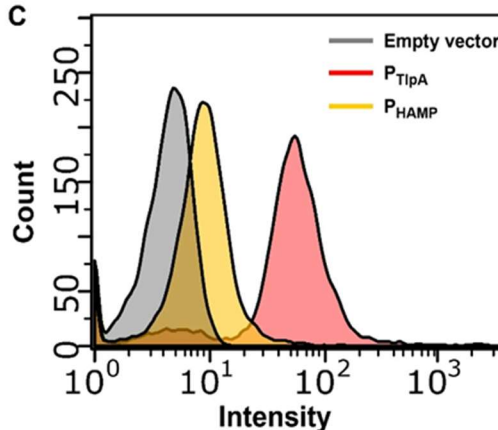
### A

Hit	Upstream a gene	Position	Sequence	Sequence length	Identity Matrix
Hit 1	Yes	257,256..257,284	GTTTATGTTGGTTATTTACGTAATAAAAAT	29	82.76
Hit 2	No	1,292,083..1,292,110	ATTTTTAAACAACATAACAAAAATGAAC	28	69.23
Hit 3	No	1,541,627..1,541,653	GTTATTTTTAGTTTGTGAAATTT	27	77.78
Hit 4	No	2,772,508..2,772,536	ATACTTAAACACAACAAAAACACTTAAC	29	32.82
Hit 5	No	3,129,614..3,129,645	ATATGGTCAGTCAATGAAATCAACAAATAAAC	32	60.00
Hit 6	No	3,209,426..3,209,455	ATAGTAACCACCAGCACACCACAAGTAAAC	30	71.43

### B

P<sub>HAMP</sub> GTTTA--TGTTGG-TTATTTACGTAATAAAAAT  
 P<sub>TipA</sub> -TTTATTTGTTGGTTTGTGTTATAATAT  
 \*\*\*\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

### C



**Supplementary Figure S7.** (A) Results obtained after screening the entire genome of *L. plantarum* WCFS1 for a P<sub>tipA</sub>-like sequence. (B) Sequence alignment of P<sub>HAMP</sub> and P<sub>tipA</sub> promoters. (C) Flow Cytometry analysis of P<sub>tipA</sub> and P<sub>HAMP</sub> driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C.