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# Supplementary Materials for

## High-throughput genetic engineering of nonmodel and undomesticated bacteria via iterative site-specific genome integration

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**The PDF file includes:**



**Other Supplementary Material for this manuscript includes the following:**

Supplementary Data D1
Figure Source Data
Supplementary Files F1 to F5
Supplementary File P1
Supplementary Files T1 and T2

## Supplemental Materials

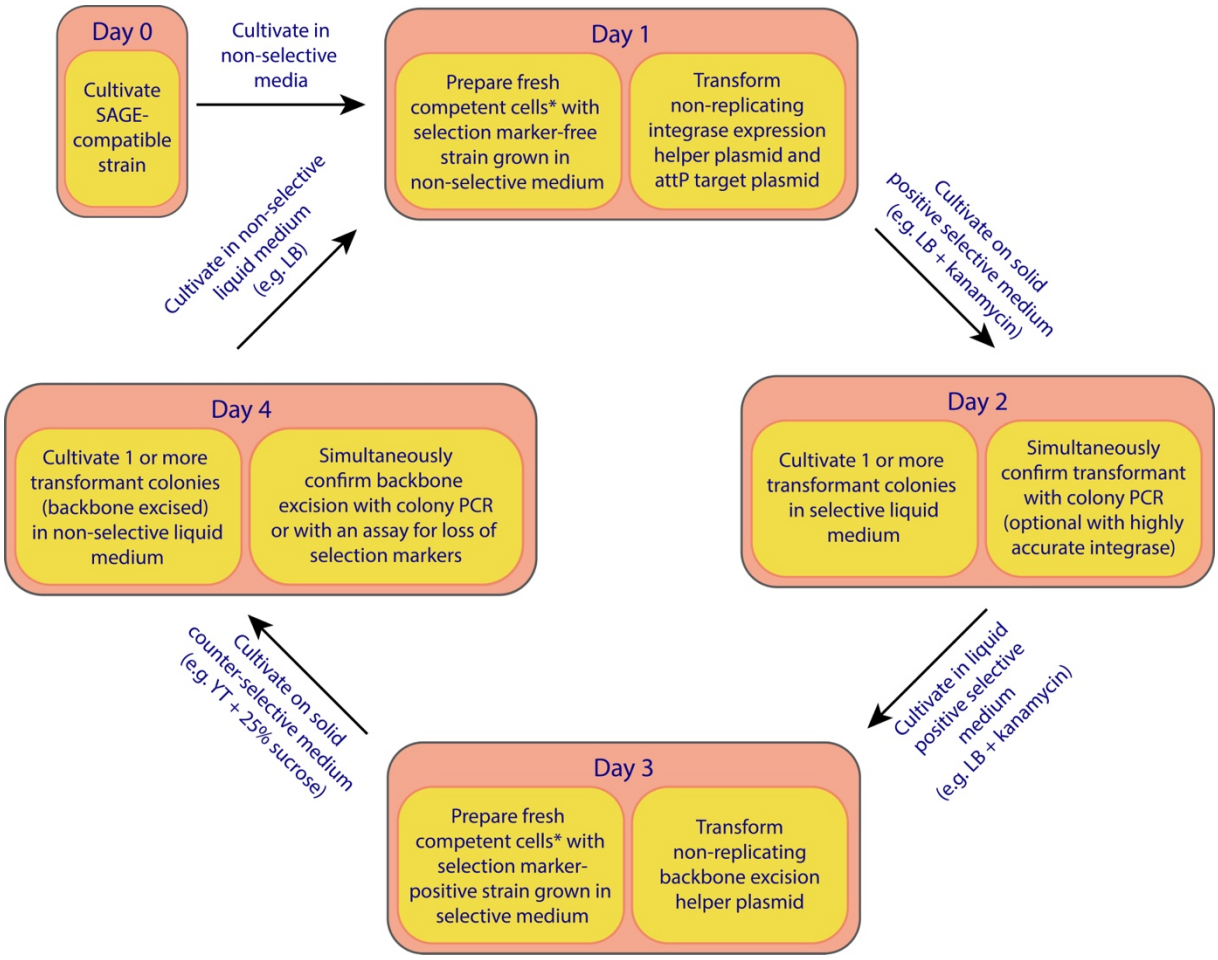



**Fig. S1. SAGE cycle workflow and timeline.** Example SAGE cycle workflow for an organism that forms visible colonies overnight. The same steps are taken for slower growing organisms, but with extended time for cultivation. *Competent cell preparation methods will vary from organism to organism.

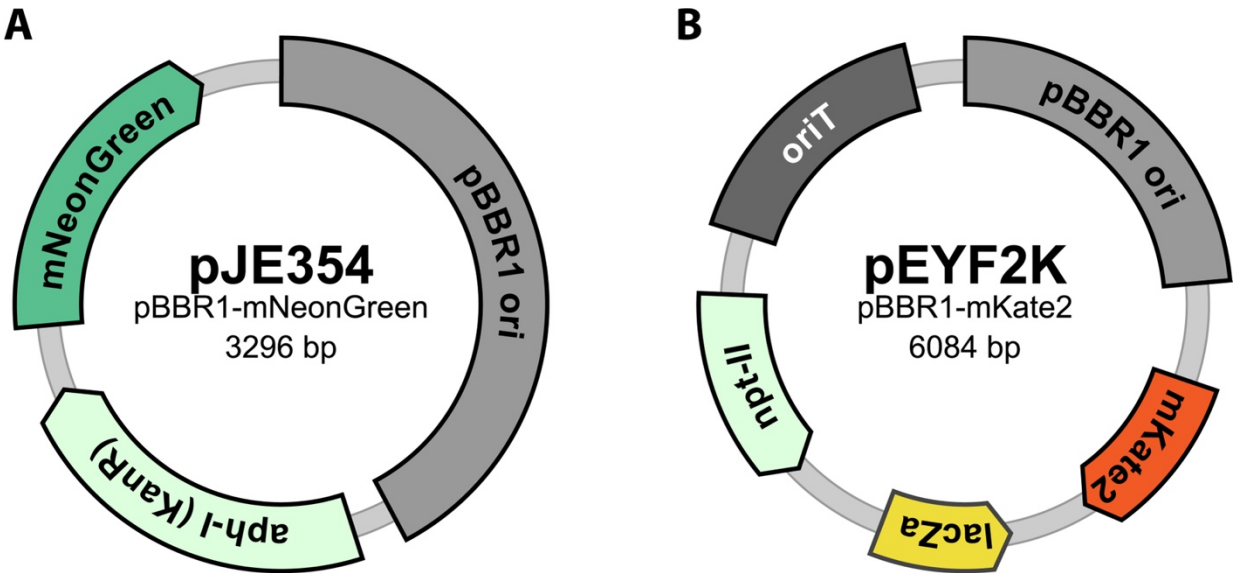



**Fig. S2. Plasmid maps of replicating plasmids used in this study.** Both (A) pJE354 and (B) pEYF2K contain the widely utilized broad host-range pBBR1 origin that enables autonomous replication in many Gram-negative bacterial hosts. (A) pJE354 is a derivative of pBTL-2(*80*) that contains a constitutive $P_{tac}$:mNeonGreen fluorescent protein reporter cassette, as well as the kanamycin resistance selection marker *aph-I*. (B) pEYF2K is a derivative of pBBR-MCS-2, contains a different kanamycin resistance marker than pJE354 (*npt-II* – of note, this is the same marker used in all KanR SAGE plasmids), a constitutive $P_{J23150}$:mKate2 fluorescent reporter cassette, an origin of transfer that enables conjugative transfer with the RK2/RP4 mobilization systems, and a lacZ$\alpha$ fragment for blue-white screening when cloning.

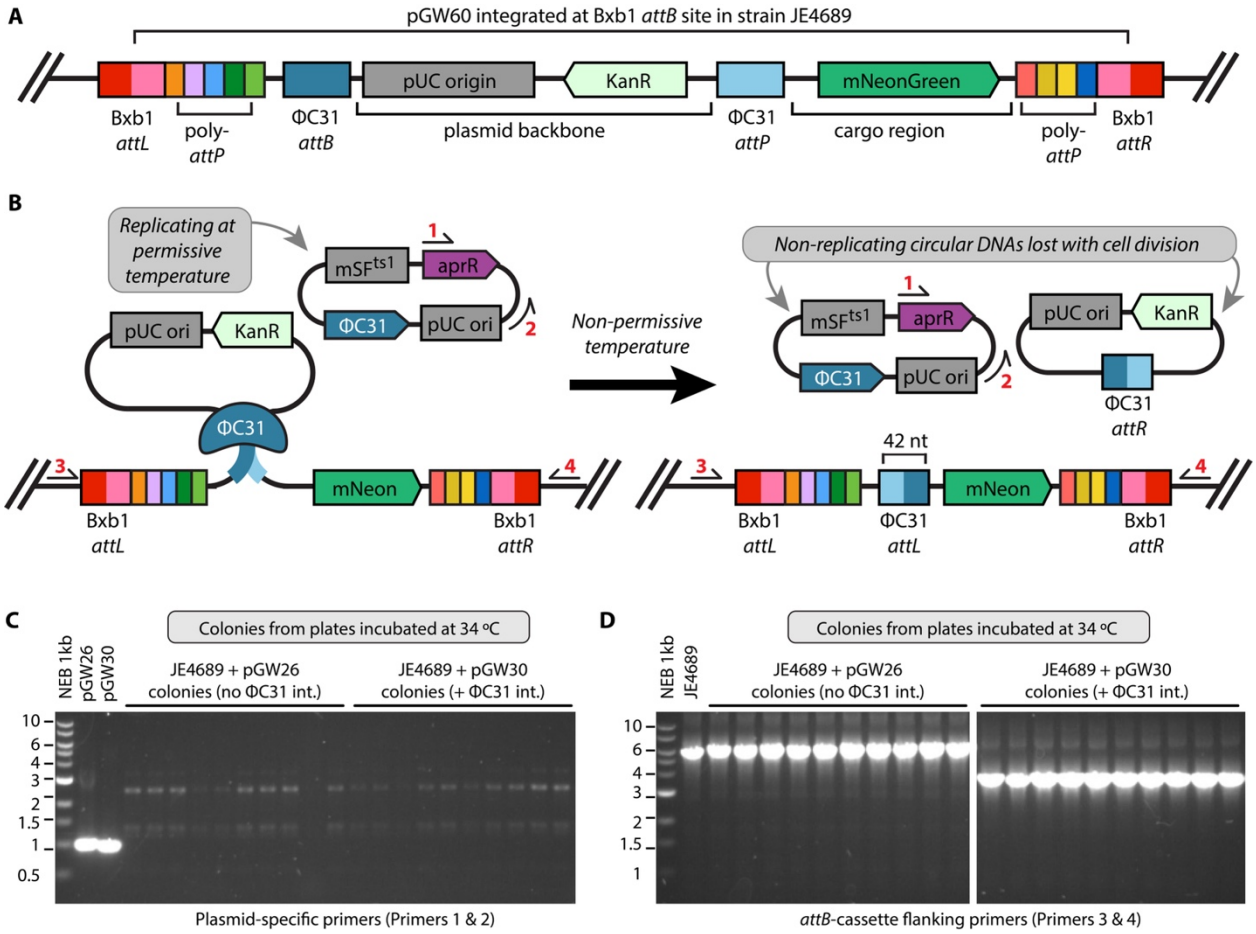



**Fig. S3. ΦC31-integrase mediated excision of 'target' plasmid backbones enables selection marker recycling.** (A) Diagram of the genome of JE4621 with pGW60 integrated at the Bxb1 *attB* site. (B) Diagram of pGW60 plasmid backbone excision using a temperature sensitive (ts) helper plasmid to deliver ΦC31-integrase. Colony PCR primers are indicated by arrows and red numbers. (C-D) Colony PCR validation of ts plasmid loss (C) and pGW60 plasmid backbone excision (D) following overnight cultivation of apramycin-resistant colonies at 34 °C.

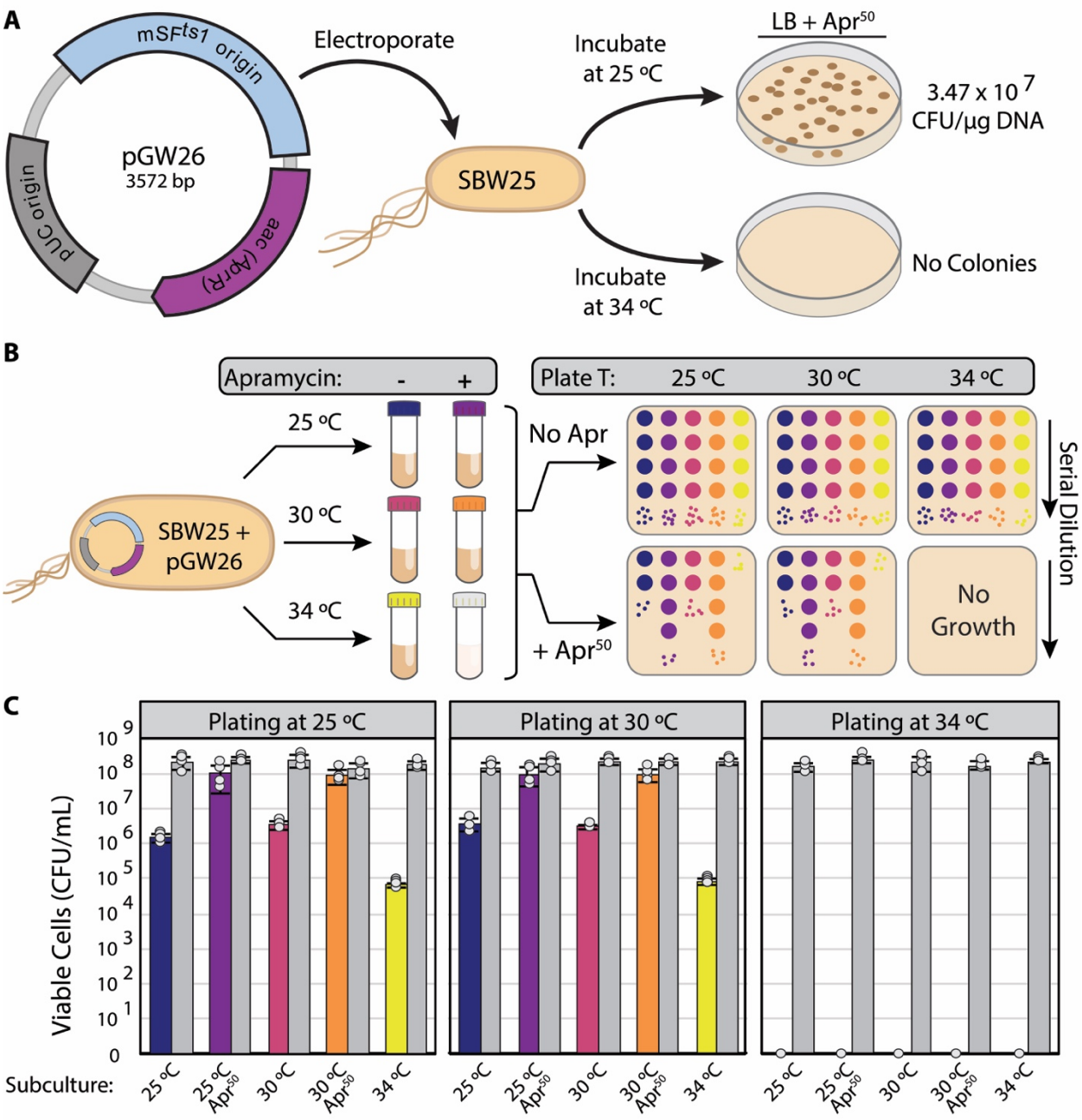
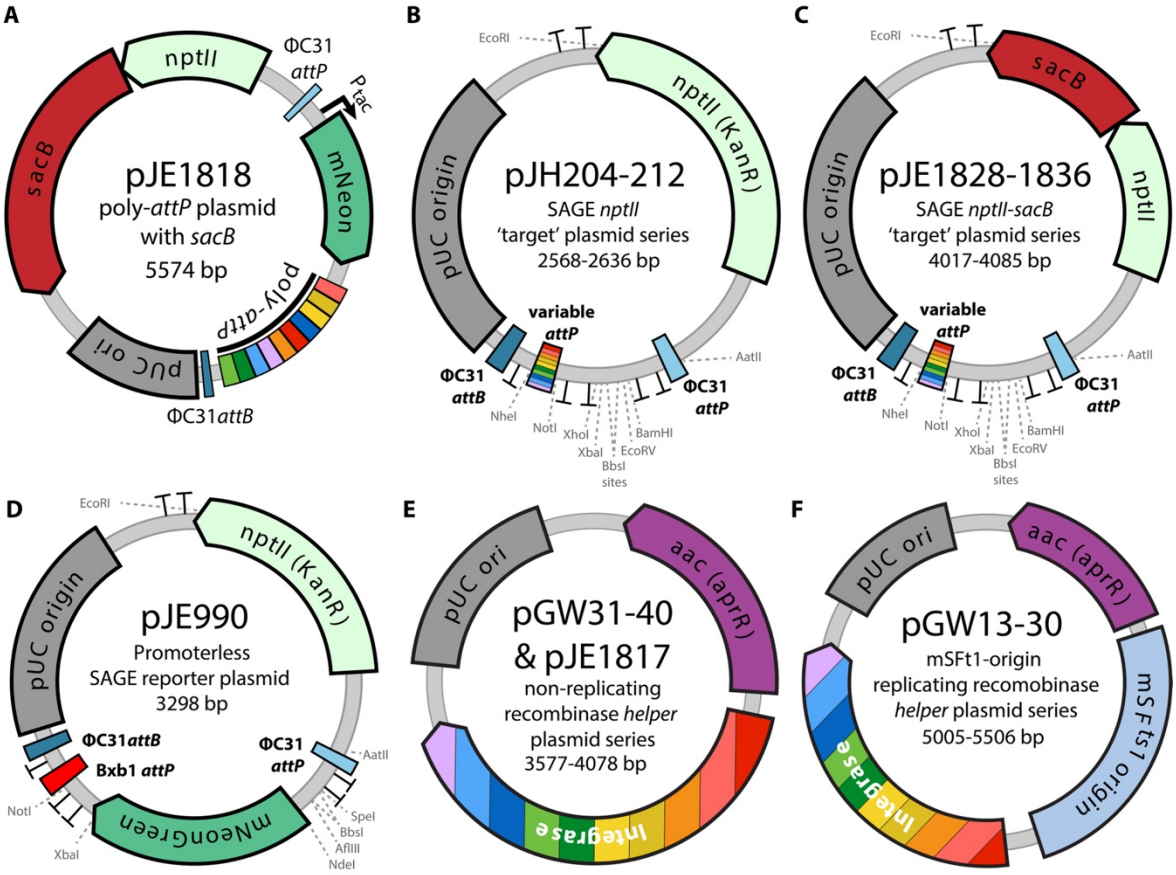



**Fig. S4. Development of a conditionally-replicating plasmid for broad use in Pseudomonads.** (A) Graphic diagram of the temperature-sensitive pGW26 plasmid and an initial assay for determining its sensitivity to replication at 34 °C in *Pseudomonas fluorescens* SBW25. pGW26 contains a pUC origin for high copy replication in *E. coli*, an *aac* gene for selection with apramycin in *E. coli* and Pseudomonads, and a temperature-sensitive Pseudomonad-specific origin of replication mSF$^{ts1}$. (B) Graphic diagram of a plasmid stability assay to determine the stability of pGW26 under growth without selection at temperatures that are 'permissive' and 'non-permissive' for plasmid replication. SBW25 containing pGW26 was cultivated with apramycin selection at 25 °C, and subsequently subcultured in selective or non-selective liquid medium at three temperatures. Each resulting culture was evaluated for total culture viability (cell viability with no apramycin) or plasmid maintenance (cell viability with apramycin) at each of the three temperatures. No growth was observed in subculture in selective medium at 34 °C so it is not included in the subsequent plasmid maintenance assay. (C) Charts displaying results of plasmid stability assay. Gray bars indicate viable cells per mL in each culture, and colored bars indicate viable cells with apramycin selection (i.e., cells hosting plasmid). Dots represent individual samples. Error bars represent the two-sided standard deviation in four biological replicates.




**G**

| Integrase | *nptII attP* target plasmid | *nptII-sacB attP* target plasmid | Non-replicating recombinase *helper* plasmid | mSF$^{ts1}$ ori recombinase *helper* plasmid |
|---|---|---|---|---|
| Bxb1 | pJH204 | pJE1828 | pGW31 | pGW13 |
| RV | pJH205 | pJE1829 | pGW32 | pGW21 |
| Φ370 | pJH206 | pJE1830 | pGW37 | pGW20 |
| R4 | pJH207 | pJE1831 | pGW39 | pGW23 |
| ΦBT1 | pJH208 | pJE1832 | pGW33 | pGW14 |
| MR11 | pJH209 | pJE1833 | pGW36 | pGW18 |
| TG1 | pJH210 | pJE1834 | pGW38 | pGW22 |
| BL3 | pJH211 | pJE1835 | pGW40 | pGW24 |
| A118 | pJH212 | pJE1836 | pGW35 | pGW17 |
| ΦC1 | --- | --- | pGW34 | pGW16 |
| ΦC31 | --- | --- | pJE1817 | pGW30 |

**H**

| Poly-*attP* Plasmid | Reporter gene | Selection Marker |
|---|---|---|
| pGW28 | --- | *nptII* |
| pGW60 | mNeon | *nptII* |
| pGW63 | --- | *aacC1 (gentR)* |
| pJE1866 | mNeon | *aacC1 (gentR)* |
| pJE1818 | --- | *nptII / sacB* |

**I**

| Plasmid | Reporter gene | Promoter |
|---|---|---|
| pJE990 | mNeon | --- |
| pJE1045 | mNeon | tac |
| pJE1040 | mNeon | T7 |
| pJE1118 | mNeon | T7_C4 |
| pJE1119 | mNeon | T7_H10 |
| pJE1120 | mNeon | T7_H9 |
| pJE1121 | mNeon | T7_G6 |
| pGW55 | mKate2 | tac |

**Fig. S5. The SAGE toolkit plasmid set.** We have developed a series of modular plasmids that can either be used directly to perform SAGE or that can be readily modified to address host-specific needs. Shown above are maps of these plasmids with the exception of poly-*attP* plasmids other than (A) pJE1818 (e.g., pGW60 in Fig 2c). This toolkit includes a modular collection of single *attP* 'target' plasmids, each which contains either the *nptII* (kanamycin resistance) marker (B) alone (pJH204-212) or (C) in combination with *sacB* for sucrose counter-selection (pJE1828-1836). Each component of the *attP* plasmids (e.g., selection marker, *attP* site, *E. coli* origin, cargo insertion site) is designed to be modular and is flanked by unique restriction sites to allow easy replacement of components, or for addition of genetic elements (e.g., oriT sequences for conjugation). The 'cargo' region in each plasmid is flanked by a pair of double rho-independent terminators (indicated by 'T' symbols) and contains several unique restriction sites – including a pair of BbsI recognition sites that are compatible with Golden Gate cloning. Additional terminator sequences insulate neighboring genomic DNA from plasmid cargo expression. Each *attP* plasmid contains a pair of ΦC31 *attP* / *attB* sites (TT att site variants) for plasmid backbone excision. (D) Plasmid pJE990 is a related plasmid that is designed to be used as a fluorescent reporter plasmid, with sites for modular integration and replacement of promoter (multiple), ribosomal binding site (NdeI/AflII), terminator sequences (XbaI/NotI), or reporter gene with any gene of interest (NdeI/XbaI)(*68*). Recombinase expression plasmids are available as both (E) suicide (non-replicating) and (F) mSF$^{ts1}$-based (temperature-sensitive Pseudomonas origin) plasmids. In addition to the reporter plasmids generated in this study, additional pJE990-based reporter plasmids containing the commonly used tac and T7 promoter (variants)(*67*), or the far-red fluorescent protein mKate2 are also currently available (*31, 68*). (G-I) Tables of plasmids described above. We anticipate that these tools and future expansions of the SAGE genetic toolkit will be broadly useful for genetic manipulation across the entire bacterial domain.

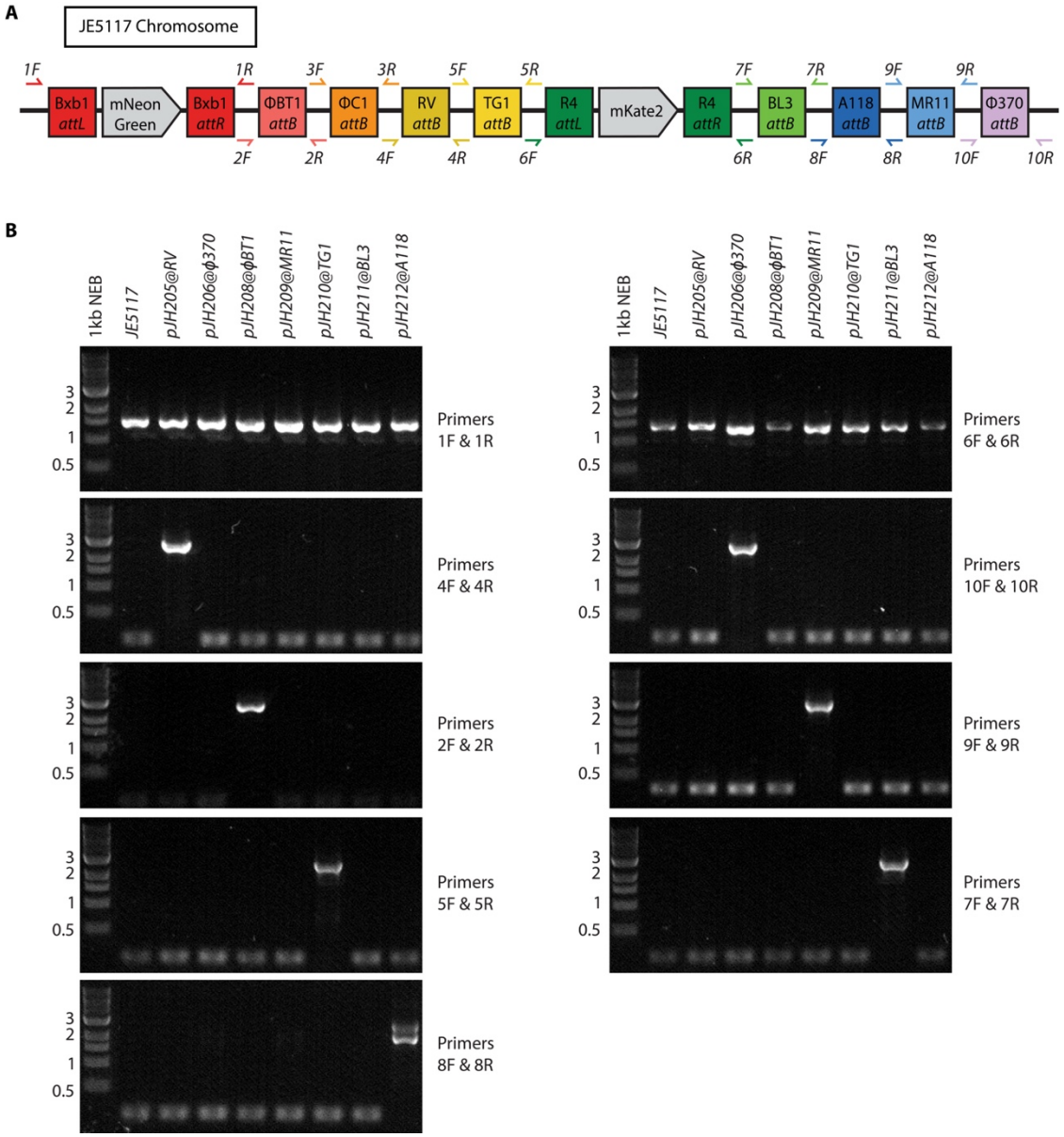



**Fig. S6. PCR validation of *P. fluorescens* strains generated by performing 3 consecutive SAGE cycles.** (A) Map of the poly-*attB* cassette in *P. fluorescens* JE5117 – a derivative of *P. fluorescens* JE4621 generated by performing two cycles of plasmid integration and backbone excision with plasmids pJE1286 (mNeonGreen) and pJE1992 (mKate2). The binding location of primers for screening integration into each *attB* site are indicated by colored arrows and bind in 40 nt randomly generated DNA sequences that intersperse each of the *attB* sites. (B) Gel electrophoresis analysis of PCR products generated by amplification of 9 *attB* sites in both JE5117 and 7 of its derivatives. Each derivative was generated by integration of an additional *attP* target plasmid at one of the remaining unused *attB* sites in JE5117. Primer sets 1F/1R and 6F/6R were used to screen for the mNeonGreen and mKate2 expression constructs found in the JE5117 chromosome at the Bxb1 and R4 *attB* sites, respectively. The sizes (kb) of select bands from the NEB 1 kb DNA ladder are indicated next to each image.

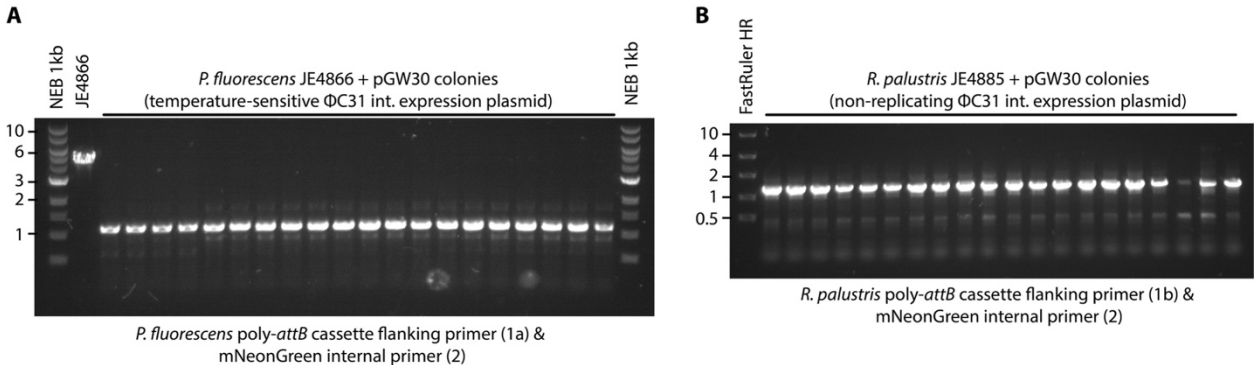



**Fig. S7. Transient expression of ϕC31 integrase with pGW30 excises the *attP*-plasmid backbone and enables selection marker recycling.** (A-B) Colony PCR validation of plasmid backbone excision in sucrose-resistant (A) *P. fluorescens* JE4621 and (B) *R. palustris* JE4632-based strains following incubation on sucrose-containing medium. Primer binding sites indicated in Fig 3. Expected band sizes are as follows: JE4866 (4741 bp), JE4866 with backbone excision (1152 bp), JE4855 with backbone excision (1375 bp). Control PCR with JE4855 parent strain can be observed in Figure 3C, which was performed concurrently with these colony PCR reactions.

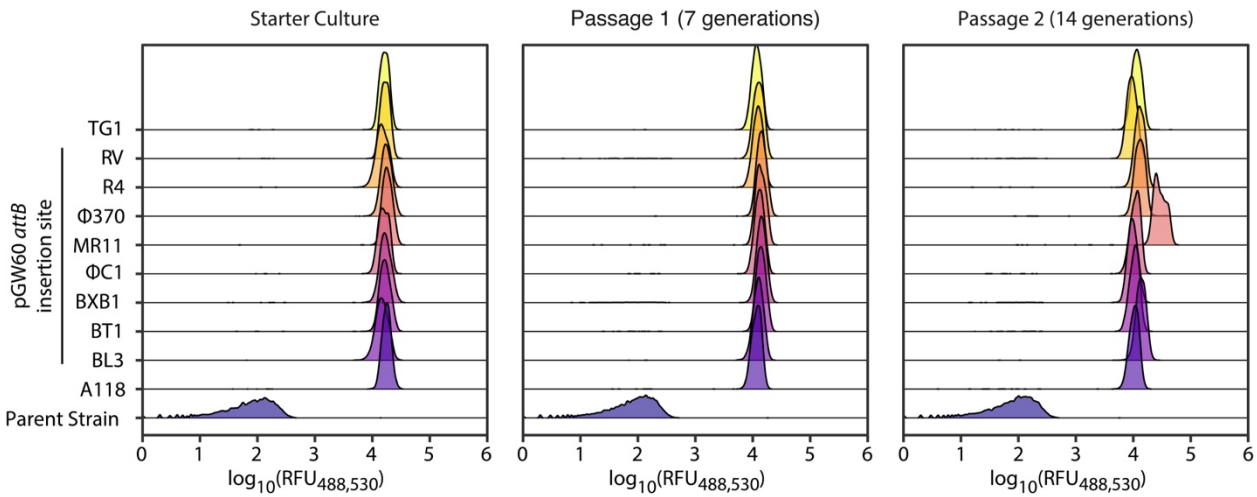



**Fig. S8. SAGE enables stable expression of heterologous genes in the absence of selection.** Flow cytometry measurements of fluorescence in populations of *R. palustris* CGA009 containing either no heterologous expression construct (Parent Strain) or a non-replicating mNeonGreen expression vector that has been chromosomally integrated at the indicated *attB* site by the corresponding recombinase. Starter cultures except for the Parent Strain were cultivated in medium containing 200 µg/mL kanamycin sulfate. Passages were cultivated without antibiotic, each inoculated with a 128-fold dilution of its precursor culture (Starter -> Passage 1 -> Passage 2) to enable 7 generations of exponential growth per passage. The x-axis indicates the relative fluorescence of each cell. The y-axis represents the abundance of cells in the population with a given relative fluorescence value (RFU).

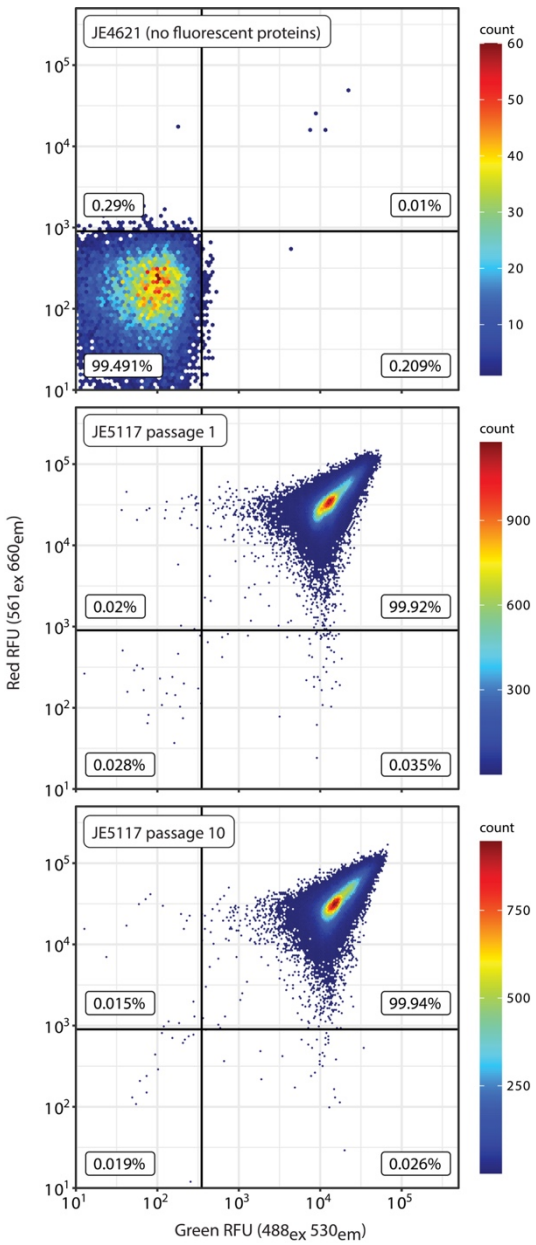



**Fig. S9. Stable maintenance of multi-cycle SAGE constructs containing two backbone excision sites.** Flow cytometry measurements of mNeonGreen (x-axis) and mKate2 (y-axis) fluorescence in populations of *P. fluorescens* SBW25 containing either no heterologous expression construct (JE4621*)* or a dual-fluorescent mNeonGreen and mKate2 expression strain (JE5117) generated by successive SAGE cycles using recombinases Bxb1 and R4, respectively. JE5117 fluorescence shown at passage 1 (10 generations) and passage 10 (100 generations). Full multi-passage fluorescence results for JE5117 shown in Table S4.

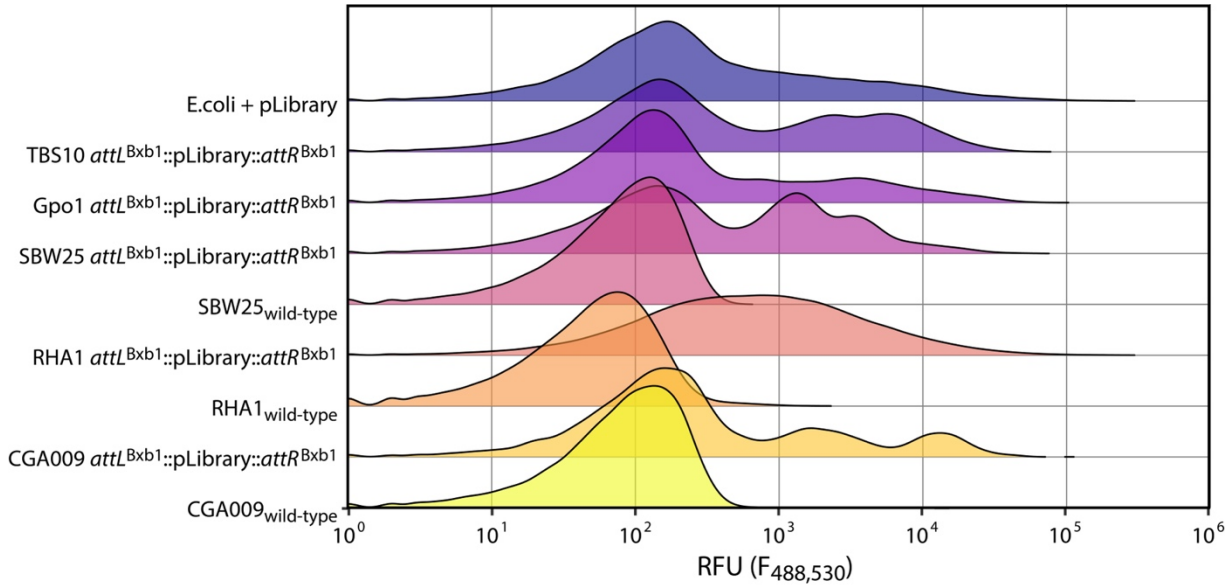



**Fig. S10. Flow cytometry evaluation of promoter strength distribution for pLibrary replicating in *E. coli* and for the five SAGE integrated promoter libraries.** Background autofluorescence of wild-type *R. palustris* (CGA009), *P. fluorescens* (SBW25), and *R. jostii* (RHA1) are shown for reference. The y-axis represents the abundance of cells in the population with a given relative fluorescence value (RFU).

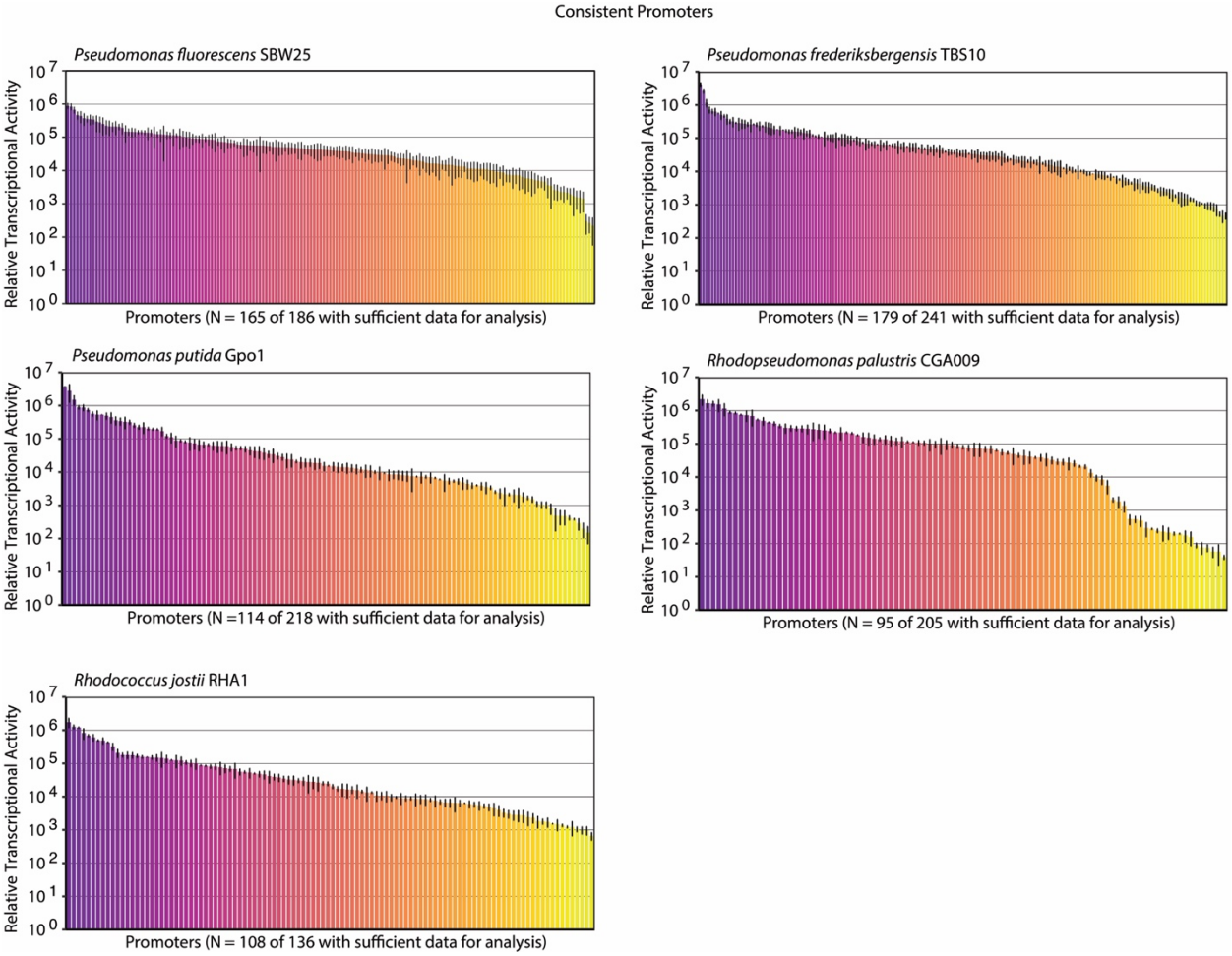



**Fig. S11. Consistent promoters.** Charts displaying the mean relative transcriptional activity for consistent (5'UTR and condition insensitive) promoters in each SAGE bacterial host. Error bars represent two-sided standard deviation of between 36-80 (SBW25), 27-60 (TBS10, Gpo1, RHA1), or 18-40 (CGA009) samples (see source data file and Data 1 for exact numbers of replicates for each promoter).

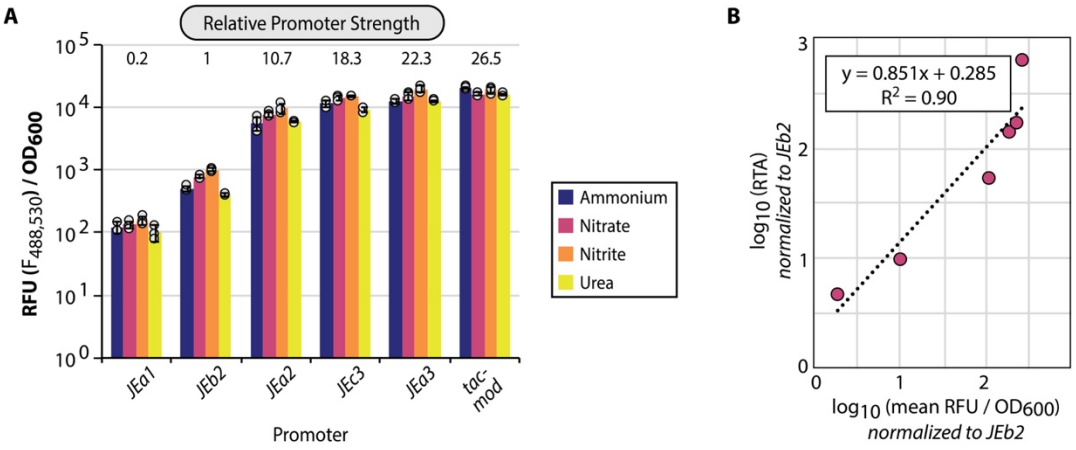



**Fig. S12. Evaluating consistency between microtiter plate reader experiments and RTP for *Pseudomonas fluorescens* SBW25.** (A) Promoter performance for a subset of pLibrary promoters in microtiter plate growth assays during growth from various nitrogen sources. Error bars represent two-sided standard deviations in three replicates. Relative promoter strength is calculated by comparing mean RFU/OD600 values across all nitrogen sources, normalized to JEb2. (B) Correlation between relative expression levels determined by RTP (y-axis) and fluorescent protein reporter assay (x-axis) for the set of promoters used in panel a. Linear equation and coefficient of determination (box inset) as determined by Pearson correlation.

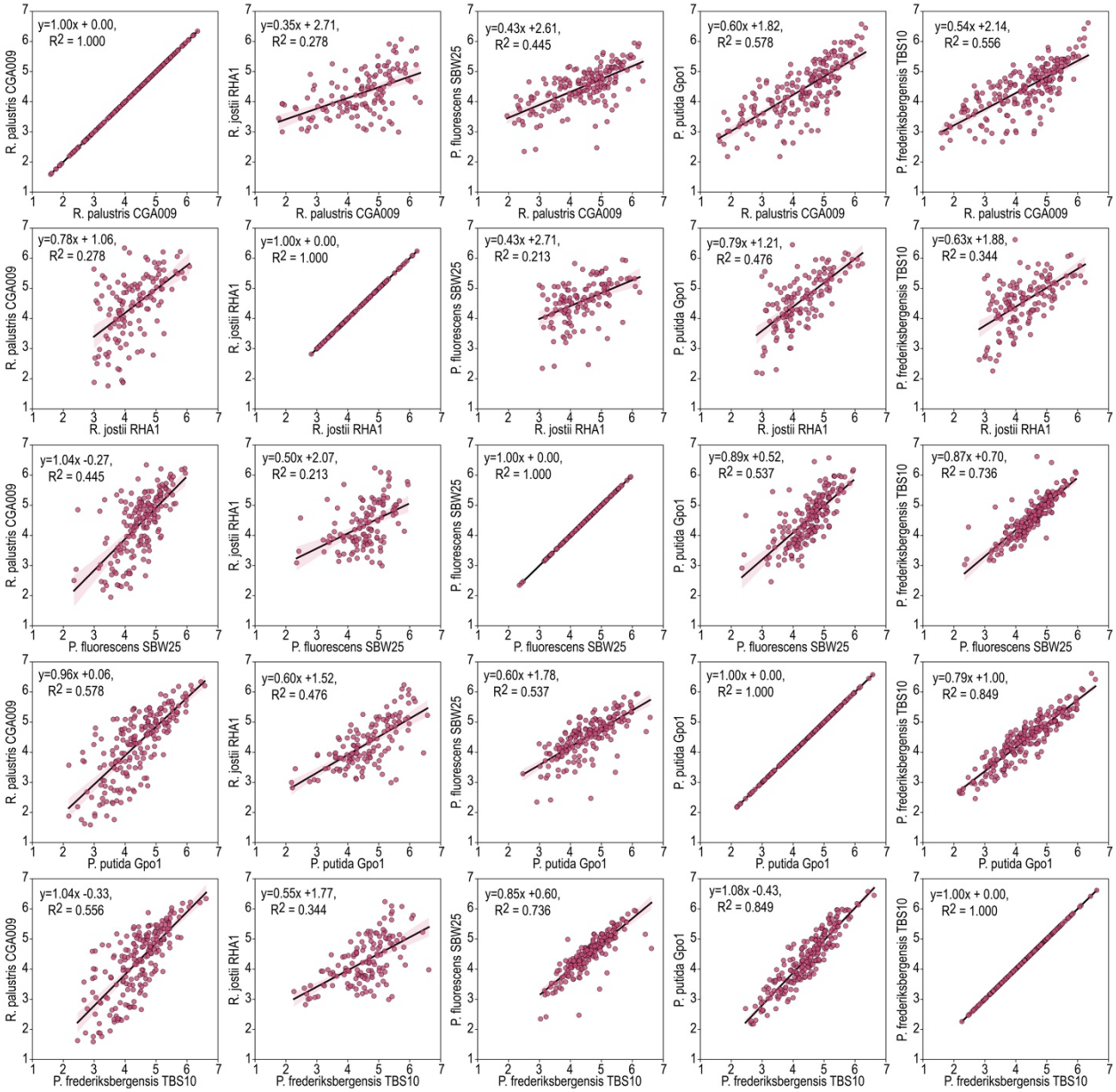



**Fig. S13. Scatter plots comparing relative transcriptional activity of promoters between organisms.** Scatter plot of data used to perform Pearson correlation and the linear equation and coefficient of determination generated using Pearson correlation. All charts generated using data set that includes all promoters regardless of their consistency. Data used to generate this figure is located in data file D1.

**Table S1. Strains and Plasmids used in this study.**

| Name | Relevant Genotype | Source |
|---|---|---|
| *Strains* | | |
| NEB 5-alpha F'Iq | *Escherichia coli* F´ *proA⁺B⁺ lacI^q Δ(lacZ)M15 zzf::Tn10* (Tet^R) / *fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | New England Biolabs |
| Epi400 | *Escherichia coli* F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG trfA tonA pcnB4 dhfr* | Lucigen |
| QP15 | *Escherichia coli proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10 (TetR) / mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (StrR) nupG trfA tonA pcnB4 dhfr* | *(68)* |
| SBW25 | *Pseudomonas fluorescens SBW25* | *(50)* |
| CGA009 | *Rhodopseudomonas palustris CGA009* | *(51)* |
| TBS10 | *Pseudomonas frederiksbergensis* TBS10 | this work |
| Gpo1 | *Pseudomonas putida (oleovorans*) Gpo1 | *(52)* |
| RHA1 | *Rhodococcus jostii* RHA1 | *(53)* |
| JE4621 | *P. fluorescens* SBW25 *ampC:10x poly-attB* | this work |
| JE4632 | *R. palustris* CGA009 ΔRPA1300:*10x poly-attB* | this work |
| JE5029 | *P. putida* Gpo1 *att*$^{Tn5}$:*nptII*:10x poly-*attB*:*att*$^{Tn5}$ | this work |
| JE5035 | *P. putida* Gpo1 *att*$^{Tn5}$:10x poly-*attB*:*att*$^{Tn5}$ | this work |
| JE5031 | *P. frederiksbergensis* TBS10 *att*$^{Tn5}$:*nptII*:10x poly-*attB*:*att*$^{Tn5}$ | this work |
| JE5041 | *P. frederiksbergensis* TBS10 *att*$^{Tn5}$:10x poly-*attB*:*att*$^{Tn5}$ | this work |
| JE4689 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pGW60: *attR*$^{Bxb1}$ | this work |
| JE4866 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1818: *attR*$^{Bxb1}$ | this work |
| JE4885 | *R. palustris* JE4632 *attL*$^{Bxb1}$:pJE1818: *attR*$^{Bxb1}$ | this work |
| JE4763 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1045: *attR*$^{Bxb1}$ | this work |
| JE4768 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1281: *attR*$^{Bxb1}$ | this work |
| JE4770 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1283: *attR*$^{Bxb1}$ | this work |
| JE4771 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1284:*attR*$^{Bxb1}$ | this work |
| JE4772 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1285:*attR*$^{Bxb1}$ | this work |
| JE4773 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:pJE1286:*attR*$^{Bxb1}$ | this work |
| JE5117 | *P. fluorescens* JE4621 *attL*$^{Bxb1}$:P$_{JEa3}$:*mNeonGreen*:*attR*$^{Bxb1}$ *attL*$^{R4}$:P$_{tac}$:*mKate2*:*attR*$^{R4}$ | this work |
| JE4670 | *E. coli* QP15 + pLibrary | this work |
| AG5879 | *R. jostii RHA1_RS20555*::10x poly-*attB* | this work |
| *Plasmids* | | |
| pK18sB | pUC origin, *nptII*, *sacB* | (*81*) |
| pJQ200SK | p15a origin, *sacB*, gentR, *mob* | (*61*) |
| pJE354 | pBBR1 origin, *aphI*, P$_{tac}$:*mNeonGreen* | this work |
| pEYF2K | pBBR1 origin, *nptII*, *mKate2*, *mob*, *lacZalpha* | this work |
| pJE1610 | pJQ200SK ΔRPA1300::*10x poly-attB* | this work |

| | | |
|---|---|---|
| pJE1700 | pK18sB *ampC*(PFLU3467):*10x poly-attB* | this work |
| pK18mobsacB | pUC origin, *nptII*, *sacB*, *oriT* | (*30*) |
| pGW26 | pUC origin, mSF$^{ts1}$, *aac* (AprR) | this work |
| pGW13 | pGW26 $P_{tac}$:*Bxb1 integrase* | this work |
| pGW14 | pGW26 $P_{tac}$:ϕ*BT1 integrase* | this work |
| pGW16 | pGW26 $P_{tac}$:ϕC*1 integrase* | this work |
| pGW17 | pGW26 $P_{tac}$:A118 *integrase* | this work |
| pGW18 | pGW26 $P_{tac}$:MR11 *integrase* | this work |
| pGW20 | pGW26 $P_{tac}$:ϕ370 *integrase* | this work |
| pGW21 | pGW26 $P_{tac}$:RV *integrase* | this work |
| pGW22 | pGW26 $P_{tac}$:TG*1 integrase* | this work |
| pGW23 | pGW26 $P_{tac}$:R4 *integrase* | this work |
| pGW24 | pGW26 $P_{tac}$:*BL3 integrase* | this work |
| pGW30 | pGW26 $P_{tac}$:ϕC31 *integrase* | this work |
| pGW31 | pGW26 $P_{tac}$:*Bxb1 integrase* ΔmSF$^{ts1}$ | this work |
| pGW32 | pGW26 $P_{tac}$:RV *integrase* ΔmSF$^{ts1}$ | this work |
| pGW33 | pGW26 $P_{tac}$:ϕ*BT1 integrase* ΔmSF$^{ts1}$ | this work |
| pGW34 | pGW26 $P_{tac}$:ϕC*1 integrase* ΔmSF$^{ts1}$ | this work |
| pGW35 | pGW26 $P_{tac}$:A118 *integrase* ΔmSF$^{ts1}$ | this work |
| pGW36 | pGW26 $P_{tac}$:MR11 *integrase* ΔmSF$^{ts1}$ | this work |
| pGW37 | pGW26 $P_{tac}$:ϕ370 *integrase* ΔmSF$^{ts1}$ | this work |
| pGW38 | pGW26 $P_{tac}$:TG*1 integrase* ΔmSF$^{ts1}$ | this work |
| pGW39 | pGW26 $P_{tac}$:R4 *integrase* ΔmSF$^{ts1}$ | this work |
| pGW40 | pGW26 $P_{tac}$:*BL3 integrase* ΔmSF$^{ts1}$ | this work |
| pJE1817 | pGW26 $P_{tac}$:ϕC31 *integrase* ΔmSF$^{ts1}$ | this work |
| pJE990 | pUC origin, *nptII*, *mNeonGreen* (promoterless), Bxb1 *attP* | (*31*) |
| pLibrary | pJE990 $P_{library}$:*mNeonGreen* | this work |
| pGW60 | pJE990 $P_{tac\text{-}mod}$:*mNeonGreen,* 10x poly-*attP* cassette | this work |
| pJH204 | pUC origin, *nptII*, Bxb1 *attP* | this work |
| pJH205 | pUC origin, *nptII*, RV *attP* | this work |
| pJH206 | pUC origin, *nptII*, ϕ370 *attP* | this work |
| pJH207 | pUC origin, *nptII*, R4 *attP* | this work |
| pJH208 | pUC origin, *nptII*, ϕBT1 *attP* | this work |
| pJH209 | pUC origin, *nptII*, MR11 *attP* | this work |
| pJH210 | pUC origin, *nptII*, TG1 *attP* | this work |
| pJH211 | pUC origin, *nptII*, BL3 *attP* | this work |
| pJH212 | pUC origin, *nptII*, A118 *attP* | this work |
| pJE1828 | pUC origin, *nptII-sacB*, Bxb1 *attP* | this work |
| pJE1829 | pUC origin, *nptII-sacB*, RV *attP* | this work |
| pJE1830 | pUC origin, *nptII-sacB*, ϕ370 *attP* | this work |
| pJE1831 | pUC origin, *nptII-sacB*, R4 *attP* | this work |
| pJE1832 | pUC origin, *nptII-sacB*, ϕBT1 *attP* | this work |
| pJE1833 | pUC origin, *nptII-sacB*, MR11 *attP* | this work |

| | | |
|---|---|---|
| pJE1834 | pUC origin, *nptII-sacB*, TG1 *attP* | this work |
| pJE1835 | pUC origin, *nptII-sacB*, BL3 *attP* | this work |
| pJE1836 | pUC origin, *nptII-sacB*, A118 *attP* | this work |
| pJE1045 | pJE990 $P_{tac\text{-}mod}$:*mNeonGreen* | (*68*) |
| pJE1281 | pJE990 $P_{JEa1}$:*mNeonGreen* | this work |
| pJE1283 | pJE990 $P_{JEb2}$:*mNeonGreen* | this work |
| pJE1284 | pJE990 $P_{JEa2}$:*mNeonGreen* | this work |
| pJE1285 | pJE990 $P_{JEc3}$:*mNeonGreen* | this work |
| pJE1286 | pJE990 $P_{JEa3}$:*mNeonGreen* | this work |
| pJE1818 | pJE990 $P_{tac\text{-}mod}$:*mNeonGreen,* 10x poly-*attP* cassette, *sacB* | this work |
| pGW28 | pUC origin, *nptII*, 10x poly-*attP* cassette | this work |
| pGW63 | pUC origin, *aacC1* (gentR), 10x poly-*attP* cassette | this work |
| pJE1866 | pUC origin, aacC1 (gentR), 10x poly-attP cassette $P_{JEa3}$:*mNeonGreen* | this work |
| pJE1854 | pTwist_Amp_HC with EZ-Tn5-*aac-attB* cassette | this work |
| pJE1855 | pTwist_Amp_HC with EZ-Tn5-*nptII-attB* cassette | this work |
| pQP425 | pK18mobsacB *RHA1_RS20555*::*10x poly-attB* | this work |
| pJE1992 | pJH207 $P_{tac}$:mKate2 | this work |

**Table S2. Phage integrase attachment (*att*) sequences.**

| Integrase | Attachment sites |
|---|---|
| **phiC31** | |
| ***attB-TT*** | tgcgggtgccagggcgtgccc*tt*gggctccccgggcgcgtactcc |
| ***attP-TT*** | gtgccccaactggggtaacct*tt*gagttctctcagttggggg |
| ***attB-CA*** | tgcgggtgccagggcgtgccc*ca*gggctccccgggcgcgtactcc |
| ***attP-CA*** | gtgccccaactggggtaacct*ca*gagttctctcagttggggg |
| **phiBT1** | |
| ***attB*** | gtccttgaccaggtttttgacgaaagtgatccagatgatccagctccacaccccgaacgc |
| ***attP*** | ggtgctgggttgttgtctctggacagtgatccatgggaaactactcagcaccaccaatgttcc |
| **R4** | |
| ***attB*** | gcgcccaagttgcccatgaccatgccgaagcagtggtagaagggcaccggcagacac |
| ***attP*** | aggcatgttccccaaagcgataccacttgaagcagtggtactgcttgtgggtacactctgcgggtgatga |
| **BxB1** | |
| ***attB*** | tcggccggcttgtcgacgacggcggtctccgtcgtcaggatcatccgggc |
| ***attP*** | gtcgtggtttgtctggtcaaccaccgcggtctcagtggtgtacggtacaaaccccgac |
| **RV** | |
| ***attB*** | tctcgtggtggtggaaggtgttggtgcggggttggccgtggtcgaggtggggtggtggtagccattcg |
| ***attP*** | gcacaggtgtagtgtatctcacaggtccacggttggccgtggactgctgaagaacattccacgccagga |
| **TG1** | |
| ***attB*** | gatcagctccgcgggcaagaccttctccttcacggggtggaaggtc |
| ***attP*** | tcaaccccgttccagcccaacagtgttagtctttgctcttacccagttgggcgggatagcctgcccg |
| **phiC1** | |
| ***attB*** | atatttaaccgcttcccgaaaaatttcgcgtggatgagcaatactttgattcagtgaacctttgaaaatcgttttctgttggataa |
| ***attP*** | attaaatataaattttagtacatagtgttatatacactaataaacaaaatcatatacctaaaatattacatt |
| **MR11** | |
| ***attB*** | acaggtcaacacatcgcagttatcgaacaatcttcgaaaatgtatggaggcacttgtatcaatataggatgtataccttcgaagacacttgtacat gatggattagaaggcaaatccttt |
| ***attP*** | caaaataaaaaacattgatttttattaacttcttttgtgcggaactacgaacagttcattaatacgaagtgtacaaacttccatacaaaaataacc acgacaattaagacgtggtttcta |
| **phi370** | |
| ***attB*** | tgtaaaggagactgataatggcatgtacaactatactcgtcggtaaaaaggca |
| ***attP*** | taaaaaaatacagcgtttttcatgtacaactatactagttgtagtgcctaaa |
| **A118** | |
| ***attB*** | tgtaacttttcggatcaagctatgaaggacgcaaagagggaactaaacacttaatt |
| ***attP*** | ttgtttagttcctcgttttctctcgttggaagaagaagaaacgagaaactaaaatta |
| **BL3** | |
| ***attB*** | caacctgttgacatgtttccacagacaactcacgtggaggtagtcacggcttttacgttagtt |
| ***attP*** | gagaatactgttgaacaatgaaaaactaggcatgtagaagttgtttgtgcactaactttaa |

**Table S3. Oligos used in study.**

| **Oligo Name** | **Sequence** (5'-3') | **Purpose** |
|---|---|---|
| *Promoter library amplification* | | |
| oPNL824-prom_lib_fwd2 | aacgtaccgagGAAGACaaGTCTgactgctgcgagtc | Forward primer for amplification of promoter library from twist biosciences for cloning into pJE990 to construct pLibrary |
| oPNL825-prom_lib_rev2 | ttcttgcttGAAGACcataagCTTAcGAGACCggaccac | Reverse primer for amplification of promoter library from twist biosciences for cloning into pJE990 to construct pLibrary |
| *Illumina sequencing library prep* | | |
| oPNL887-mNeon-RT | TGCAGTTCATGCGTAGCTGGCAAC | Reverse transcription from mNeonGreen |
| oPNL888-3'-adaptor | /5Phos/ NNATGTACTCTGCGTTGATACCACTGCTT /3SpC3/ | Adaptor for 3'-cDNA end ligation |
| oPNL889-DNA-amp1-F | GAGTTCAGACGTGTGCTCTTCCGATCTGTCTGACTGCTGCGAGTC | Fwd Primer for 1st amplification of DNA |
| oPNL890-RNA-amp1-F | GAGTTCAGACGTGTGCTCTTCCGATCTAAGCAGTGGTATCAACGC | Fwd Primer for 1st amplification of RNA |
| oPNL891-Amp1-R-N3 | CCTACACGACGCTCTTCCGATCT NNN CCTGTGTGAGTTAATCTTAAGCT | Rev primer for 1st amplification of DNA and RNA |
| oPNL892-Amp1-R-N4 | CCTACACGACGCTCTTCCGATCT NNNN CCTGTGTGAGTTAATCTTAAGCT | Rev primer for 1st amplification of DNA and RNA |
| oPNL893-Amp1-R-N5 | CCTACACGACGCTCTTCCGATCT NNNNN CCTGTGTGAGTTAATCTTAAGCT | Rev primer for 1st amplification of DNA and RNA |
| oPNL894-Amp1-R-N6 | CCTACACGACGCTCTTCCGATCT NNNNNN CCTGTGTGAGTTAATCTTAAGCT | Rev primer for 1st amplification of DNA and RNA |
| oPNL895-Amp2-F-IT058 | CAAGCAGAAGACGGCATACGAGAT ACAAAC GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL896-Amp2-F-IT008 | CAAGCAGAAGACGGCATACGAGAT ACTTGA GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL897-Amp2-F-IT030 | CAAGCAGAAGACGGCATACGAGAT CACCGG GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL898-Amp2-F-IT020 | CAAGCAGAAGACGGCATACGAGAT GTGGCC GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL899-Amp2-F-IT091 | CAAGCAGAAGACGGCATACGAGAT TGCCAT GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL926-Amp2-F-IT010 | CAAGCAGAAGACGGCATACGAGAT TAGCTT GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL927-Amp2-F-IT076 | CAAGCAGAAGACGGCATACGAGAT CGAGAA GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL928-Amp2-F-IT021 | CAAGCAGAAGACGGCATACGAGAT GTTTCG GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL929-Amp2-F-IT028 | CAAGCAGAAGACGGCATACGAGAT CAAAAG GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL930-Amp2-F-IT066 | CAAGCAGAAGACGGCATACGAGAT AGCATC GTGACTGGAGTTCAGACGTGTGCTCTTC | Fwd primer for 2nd amplification (P7) |
| oPNL900-Amp2-R-IT055 | AATGATACGGCGACCACCGAGATCTACAC AAGCGA ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL901-Amp2-R-IT022 | AATGATACGGCGACCACCGAGATCTACAC CGTACG ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL902-Amp2-R-IT084 | AATGATACGGCGACCACCGAGATCTACAC GCACTT ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL903-Amp2-R-IT017 | AATGATACGGCGACCACCGAGATCTACAC GTAGAG ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |

| | | |
|---|---|---|
| oPNL904-Amp2-R-IT095 | AATGATACGGCGACCACCGAGATCTACAC TTCTCC ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL931-Amp2-R-IT042 | AATGATACGGCGACCACCGAGATCTACAC TAATCG ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL932-Amp2-R-IT027 | AATGATACGGCGACCACCGAGATCTACAC ATTCCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL933-Amp2-R-IT037 | AATGATACGGCGACCACCGAGATCTACAC CGGAAT ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL934-Amp2-R-IT045 | AATGATACGGCGACCACCGAGATCTACAC TCATTC ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |
| oPNL935-Amp2-R-IT080 | AATGATACGGCGACCACCGAGATCTACAC GACGGA ACACTCTTTCCCTACACGACGCTCTTCCGATCT | Rev primer for 2nd amplification (P5) |

*P. fluorescens poly-attB strain construction screening primers*

| | | |
|---|---|---|
| oPNL611-3'ampC_fl_F | CTCGGTGAGCAAAACCTTC | flanking primers to screen for insertion of DNA downstream of ampC locus |
| oPNL612-3'ampC_fl_R | TGCTGATGATCGCGATCTA | flanking primers to screen for insertion of DNA downstream of ampC locus |

*R. palustris poly-attB strain construction screening primers*

| | | |
|---|---|---|
| oPNL413-RPA1300_fl_F | CTTGATGCCCGAAGCCTTCT | primers flanking RPA1300 to screen for its deletion and replacement with poly-attB cassette in R. palustris CGA009 |
| oPNL414-RPA1300_fl_R | TTCGTCGTCACCTTCGGTTC | primers flanking RPA1300 to screen for its deletion and replacement with poly-attB cassette in R. palustris CGA009 |
| oPNL415-RPA1300_int_F | CAGTGGGCTGTCGATGTCTT | primers internal to RPA1300 to screen for its deletion in R. palustris CGA009 |
| oPNL416-RPA1300_int_R | TGAGTTTCGTTTGCACTGCG | primers internal to RPA1300 to screen for its deletion in R. palustris CGA009 |

*R. jostii poly-attB strain construction screening primers*

| | | |
|---|---|---|
| oQP2195 | GTCGGCGATCTCTCGACGTG | Fwd Primer for verifying attB insertion flanking the attB site in R. jostii RHA1 |
| oQP2196 | TTAACGAGTCTCTGCACTCGTCAAC | Rev Primer for verifying attB insertion flanking the attB site in R. jostii RHA1 |
| oQP1722 | CTAAATCCGCGTGATAGGGGATTTC | Fwd primer internal to the attB insertion |
| oQP1521 | GTTCTGCAGCTGCGGGAAC | Rev primer internal to the attB insertion |

*Primers for screening ts plasmid loss and attP plasmid backbone excision*

| | | |
|---|---|---|
| oPNL556 | AGCGAGTCAGTGAGCGA | screening for pGW26/30 plasmid loss |
| oPNL879 | TTCTTAAGATTAACTCACACAGGAGA | screening for pGW26/30 plasmid loss |
| oPNL621 | CATCGGCGTGGTGATATTGG | screening for pGW60 and pJE1817 backbone excision by phiC31 integrase |
| oPNL622 | TGCCGTGGCTGATGATCC | screening for pGW60 and pJE1817 backbone excision by phiC31 integrase |

*Primers for generating DNA sequences in EZ-Tn5 transposomes*

| | | |
|---|---|---|
| oPNL1254 | [pho]CTGTCTCTTATACACATCTattaatgcagctggcacgac | amplification of Mini-Tn5 transposons designed to integrate poly-*attB* cassettes with EZ-Tn5 transposases |
| oPNL1255 | [pho]CTGTCTCTTATACACATCTagctagcttatcgccattcg | amplification of Mini-Tn5 transposons designed to integrate poly-*attB* cassettes with EZ-Tn5 transposases |

*Primers for screening excision of nptII / aac from Tn5 inserted poly-attB cassettes*

| | | |
|---|---|---|
| oPNL911 | ATTAATGCAGCTGGCACGAC | Forward primer that binds 5' end of the antibiotic marker / poly-*attB* cassette, pair with oPNL912 to amplify entire cassette. |
| oPNL912 | AGCTAGCTTATCGCCATTCG | Reverse primer that binds 3' end of the antibiotic marker / poly-*attB* cassette, pair with oPNL911 to amplify entire cassette. |
| oPNL623 | CGTCCAAGCGGATGCAATGC | partner with oPNL1011 primer to amplify fragment of 10x poly-*attB* cassette. |
| oPNL1011 | AAGTACTGACGAGCTGCAAGA | partner with oPNL623 primer to amplify fragment of 10x poly-*attB* cassette. |
| oPNL1147 | CTTCCTCGTGCTTTACGGTATC | alternate screening primer for use with oPNL912 to screen for presence of *nptII*-10x poly-*attB* cassette. Binds within *nptII*. |
| *Primers for screening attP plasmid insertion in P. fluorescens* | | |
| oPNL622 | TGCCGTGGCTGATGATCC | screening for *attP* plasmid insertion downstream of *ampC* in JE4621 |
| oPNL629 | AAAACCGCCCAGTCTAGCTATCG | screening of genomic integration of *attP* plasmids |
| *Primers for screening attP plasmid insertion in R. palustris* | | |
| oPNL819 | GTGGCAGAAAGCTTTCACGG | forward primer for screening attR side of pGW60 into ΔRPA_1300::12x poly-*attB* cassette, binds in ColE1/pUC origin |
| oPNL817 | GAGGGGCAGGCAGAACATAG | reverse primer for screening attR side of pGW60 into ΔRPA_1300::10x poly-*attB* cassette, binds in R. palustris CGA009 genome |
| *Primers for screening attP plasmid insertion in P. frederiksbergensis, P. putida, R. jostii* | | |
| oPNL1010 | TTGATCGAATTCTTTCATTTAAGACC | Forward primer for screening insertion of *attP* plasmids into the 10x poly-*attB* cassette. Binds just inside the upstream terminator. |
| oPNL816 | AGCTCTTGATCCGGCAAACA | Reverse primer for screening pGW60 insertion into *attB* sites. Binds in ColE1/pUC origin of pGW60. |
| *Primers for screening attP plasmid insertion into individual attB sites in the poly-attB cassette* | | |
| Bxb1_F | TGATCGAATTCTTTCATTTAAGACCCT | Forward primer that binds in the spacer upstream of the Bxb1 attB site in the poly-attB cassette and is used to specifically screen for integration into the Bxb1 attB site. (Primer 1F in Fig. S6) |
| Bxb1_R | GGCAGAATTTTGGGAGTGGCAT | Reverse primer that binds in the spacer upstream of the Bxb1 attB site in the poly-attB cassette and is used to specifically screen for integration into the Bxb1 attB site. (Primer 1R in Fig. S6) |
| BT1_F | TCCCAAAATTCTGCCTAGAAAGTC | Forward primer that binds in the spacer upstream of the BT1 attB site in the poly-attB cassette and is used to specifically screen for integration into the BT1 attB site. (Primer 2F in Fig. S6) |
| BT1_R | TAGTGGAGCGAACTTAAACAGC | Reverse primer that binds in the spacer upstream of the BT1 attB site in the poly-attB cassette and is used to specifically screen for integration into the BT1 attB site. (Primer 2R in Fig. S6) |
| phiC1_F | GACTACGAGCGCCGGATCA | Forward primer that binds in the spacer upstream of the phiC1 attB site in the poly-attB cassette and is used to specifically screen for integration into the phiC1 attB site. (Primer 3F in Fig. S6) |
| phiC1_R | TCCTCTTGAGGTAGAAACGGGG | Reverse primer that binds in the spacer upstream of the phiC1 attB site in the poly-attB cassette and is used to specifically screen for integration into the phiC1 attB site. (Primer 3R in Fig. S6) |
| RV_F | GAATTCCGACATGGCAATAACCC | Forward primer that binds in the spacer upstream of the RV attB site in the poly-attB cassette and is used to specifically screen for integration into the RV attB site. (Primer 4F in Fig. S6) |

| | | |
|---|---|---|
| RV_R | GCCGACAGCAGTCATGTTAATAC | Reverse primer that binds in the spacer upstream of the RV attB site in the poly-attB cassette and is used to specifically screen for integration into the RV attB site. (Primer 4R in Fig. S6) |
| TG1_F | AAGTATTAACATGACTGCTGTCGG | Forward primer that binds in the spacer upstream of the TG1 attB site in the poly-attB cassette and is used to specifically screen for integration into the TG1 attB site. (Primer 5F in Fig. S6) |
| TG1_R | GGAGGATCGCATTGCATCCG | Reverse primer that binds in the spacer upstream of the TG1 attB site in the poly-attB cassette and is used to specifically screen for integration into the TG1 attB site. (Primer 5R in Fig. S6) |
| R4_F | CGTCCAAGCGGATGCAATG | Forward primer that binds in the spacer upstream of the R4 attB site in the poly-attB cassette and is used to specifically screen for integration into the R4 attB site. (Primer 6F in Fig. S6) |
| R4_R | GAAGTGTTCAACGCGTACGC | Reverse primer that binds in the spacer upstream of the R4 attB site in the poly-attB cassette and is used to specifically screen for integration into the R4 attB site. (Primer 6R in Fig. S6) |
| BL3_F | TGGCGTACGCGTTGAACAC | Forward primer that binds in the spacer upstream of the BL3 attB site in the poly-attB cassette and is used to specifically screen for integration into the BL3 attB site. (Primer 7F in Fig. S6) |
| BL3_R | CACCCGCGCTTATAGCTTCC | Reverse primer that binds in the spacer upstream of the BL3 attB site in the poly-attB cassette and is used to specifically screen for integration into the BL3 attB site. (Primer 7R in Fig. S6) |
| A118_F | TAAGCGCGGGTGAGAGGGTA | Forward primer that binds in the spacer upstream of the A118 attB site in the poly-attB cassette and is used to specifically screen for integration into the A118 attB site. (Primer 8F in Fig. S6) |
| A118_R | CCGTGCAACATCAGATGCTCAC | Reverse primer that binds in the spacer upstream of the A118 attB site in the poly-attB cassette and is used to specifically screen for integration into the A118 attB site. (Primer 8R in Fig. S6) |
| MR11_F | TGAGCATCTGATGTTGCACGG | Forward primer that binds in the spacer upstream of the MR11 attB site in the poly-attB cassette and is used to specifically screen for integration into the MR11 attB site. (Primer 9F in Fig. S6) |
| MR11_R | GAGTCGTGAGTACTGATAGTAGTGAG | Reverse primer that binds in the spacer upstream of the MR11 attB site in the poly-attB cassette and is used to specifically screen for integration into the MR11 attB site. (Primer 9R in Fig. S6) |
| phi370_F | CGTTCAGGTAATGAGTGCAGC | Forward primer that binds in the spacer upstream of the phi370 attB site in the poly-attB cassette and is used to specifically screen for integration into the phi370 attB site. (Primer 10F in Fig. S6) |
| phi370_R | AGCTGCAAGAACGAAATGCG | Reverse primer that binds in the spacer upstream of the phi370 attB site in the poly-attB cassette and is used to specifically screen for integration into the phi370 attB site. (Primer 10R in Fig. S6) |

**Table S4. Genetic stability of *P. fluorescens* with multiple heterologous DNA insertions**

Percentage of flow cytometry events (percentage of population)

| Lineage 1 | JE4621* | JE5517 Starter | JE5117 Passage | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| both colors | 0.01% | 99.58% | 99.92% | 99.94% | 99.94% | 99.91% | 99.93% | 99.92% | 99.96% | 99.92% | 99.93% | 99.94% |
| green | 0.209% | 0.347% | 0.035% | 0.022% | 0.019% | 0.025% | 0.024% | 0.032% | 0.016% | 0.035% | 0.032% | 0.026% |
| red | 0.29% | 0.038% | 0.02% | 0.013% | 0.013% | 0.029% | 0.015% | 0.023% | 0.019% | 0.018% | 0.02% | 0.015% |
| no color | 99.49% | 0.033% | 0.028% | 0.025% | 0.026% | 0.04% | 0.034% | 0.022% | 0.009% | 0.022% | 0.021% | 0.019% |

Percentage of flow cytometry events (percentage of population)

| Lineage 2 | JE4621* | JE5517 Starter | JE5117 Passage | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| both colors | 0.01% | 99.64% | 99.91% | 99.93% | 99.93% | 99.89% | 99.85% | 99.94% | 99.92% | 99.86% | 99.87% | 99.9% |
| green | 0.209% | 0.265% | 0.034% | 0.022% | 0.026% | 0.023% | 0.026% | 0.014% | 0.02% | 0.028% | 0.033% | 0.04% |
| red | 0.29% | 0.045% | 0.026% | 0.036% | 0.03% | 0.042% | 0.068% | 0.044% | 0.045% | 0.081% | 0.077% | 0.027% |
| no color | 99.49% | 0.049% | 0.026% | 0.017% | 0.018% | 0.044% | 0.056% | 0.0078% | 0.01% | 0.03% | 0.02% | 0.04% |

*data from a single non-fluorescent parent strain culture included in each table

**Table S5. Overall promoter assay statistics.**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | expression range | Problematic percent | Number of promoters w prob bc | % barcodes that are outliers. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RHA1 | 713 | 190 | 136 | 568 | 112 | 118 | 102 | 3 | 4 | 2574 | 23.76% | 93 | 20.34% |
| Pflu | 930 | 221 | 186 | 797 | 156 | 184 | 154 | 3 | 4 | 4054 | 22.81% | 128 | 14.30% |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 203 | 169 | 3 | 4 | 8930 | 24.91% | 173 | 11.04% |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 148 | 103 | 3 | 4 | 18413 | 30.04% | 167 | 14.25% |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 159 | 85 | 3 | 4 | 55797 | 50.92% | 185 | 17.62% |

num bc pass = number of barcodes that have the minimal amount of data for analysis.

num prom in bc pass = number of promoters represented in the group of barcodes with the minimum amount of data (within an individual barcode promoter variant's data) for use in analysis

num prom suff data = number of promoters with sufficient barcode variants for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num bc pass outlier = number of barcodes that pass the barcode outlier test

num UTR insensitive = number of promoters that are insensitive to 5' UTR sequence

num Cond insensitive = number of promoters that are insensitive to tested conditions

num consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions

UTR cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences

Cond cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions

expression range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

Problematic percent = % of promoter barcode variants whose expression would have been outside of the UTR cutoff range had they been the sole promoter variant tested. This includes all promoters, including all promoters represented in num bc pass.

Number of promoters w prob bc = number of promoters that had at least one problematic barcode variant.

% barcodes that are outliers = % of barcodes whose expression was statistically distinct from the other barcode variants for the given promoter

**Table S6. Expression stats for SBW25 with different sensitivity cutoffs.**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | Expression range |
|---|---|---|---|---|---|---|---|---|---|---|
| SBW25 | 930 | 221 | 186 | 797 | 112 | 137 | 81 | 2 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 169 | 101 | 2 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 176 | 105 | 2 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 183 | 109 | 2 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 184 | 110 | 2 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 184 | 110 | 2 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 185 | 111 | 2 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 185 | 111 | 2 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 185 | 111 | 2 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 112 | 186 | 112 | 2 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 137 | 100 | 2.5 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 169 | 126 | 2.5 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 176 | 132 | 2.5 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 183 | 137 | 2.5 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 184 | 138 | 2.5 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 184 | 138 | 2.5 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 185 | 139 | 2.5 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 185 | 139 | 2.5 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 185 | 139 | 2.5 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 140 | 186 | 140 | 2.5 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 137 | 113 | 3 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 169 | 141 | 3 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 176 | 147 | 3 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 183 | 153 | 3 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 184 | 154 | 3 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 184 | 154 | 3 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 185 | 155 | 3 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 185 | 155 | 3 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 185 | 155 | 3 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 156 | 186 | 156 | 3 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 137 | 123 | 3.5 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 169 | 152 | 3.5 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 176 | 158 | 3.5 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 183 | 164 | 3.5 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 184 | 165 | 3.5 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 184 | 165 | 3.5 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 185 | 166 | 3.5 | 6 | 4054.09 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SBW25 | 930 | 221 | 186 | 797 | 167 | 185 | 166 | 3.5 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 185 | 166 | 3.5 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 167 | 186 | 167 | 3.5 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 137 | 130 | 4 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 169 | 159 | 4 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 176 | 165 | 4 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 183 | 172 | 4 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 184 | 173 | 4 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 184 | 173 | 4 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 185 | 174 | 4 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 185 | 174 | 4 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 185 | 174 | 4 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 175 | 186 | 175 | 4 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 137 | 133 | 5 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 169 | 164 | 5 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 176 | 170 | 5 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 183 | 177 | 5 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 184 | 178 | 5 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 184 | 178 | 5 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 185 | 179 | 5 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 185 | 179 | 5 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 185 | 179 | 5 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 180 | 186 | 180 | 5 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 137 | 136 | 6 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 169 | 167 | 6 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 176 | 173 | 6 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 183 | 180 | 6 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 184 | 181 | 6 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 184 | 181 | 6 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 6 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 6 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 6 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 186 | 183 | 6 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 137 | 136 | 7 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 169 | 167 | 7 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 176 | 173 | 7 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 183 | 180 | 7 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 184 | 181 | 7 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 184 | 181 | 7 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 7 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 7 | 7 | 4054.09 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SBW25 | 930 | 221 | 186 | 797 | 183 | 185 | 182 | 7 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 183 | 186 | 183 | 7 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 137 | 137 | 8 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 169 | 168 | 8 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 176 | 174 | 8 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 183 | 181 | 8 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 184 | 182 | 8 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 184 | 182 | 8 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 185 | 183 | 8 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 185 | 183 | 8 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 185 | 183 | 8 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 184 | 186 | 184 | 8 | 10 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 137 | 137 | 10 | 2 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 169 | 168 | 10 | 2.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 176 | 175 | 10 | 3 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 183 | 182 | 10 | 3.5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 184 | 183 | 10 | 4 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 184 | 183 | 10 | 5 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 185 | 184 | 10 | 6 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 185 | 184 | 10 | 7 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 185 | 184 | 10 | 8 | 4054.09 |
| SBW25 | 930 | 221 | 186 | 797 | 185 | 186 | 185 | 10 | 10 | 4054.09 |

num_bc_pass = number of barcodes that have the minimal amount of data for analysis.

num_prom_in_bc_pass = number of promoters represented in the group of barcodes with the minimum amount of data for analysis

num_prom_suff_data = number of promoters with sufficient data for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num_bc_pass_outlier = number of barcodes that pass the barcode outlier test

num_UTR_insensitive = number of promoters that are insensitive to 5' UTR sequence

num_Cond_insensitive = number of promoters that are insensitive to tested conditions

num_consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions
UTR_cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences

Cond_cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions

expression_range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

**Table S7. Expression stats for TBS10 with different sensitivity cutoffs.**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | expression range |
|---|---|---|---|---|---|---|---|---|---|---|
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 115 | 63 | 2 | 2 | 4491.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 155 | 82 | 2 | 2.5 | 4491.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 180 | 98 | 2 | 3 | 4491.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 196 | 111 | 2 | 3.5 | 7977.70 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 203 | 113 | 2 | 4 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 214 | 116 | 2 | 5 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 220 | 118 | 2 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 227 | 119 | 2 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 230 | 120 | 2 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 123 | 232 | 122 | 2 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 115 | 85 | 2.5 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 155 | 114 | 2.5 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 180 | 135 | 2.5 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 196 | 149 | 2.5 | 3.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 203 | 152 | 2.5 | 4 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 214 | 156 | 2.5 | 5 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 220 | 159 | 2.5 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 227 | 160 | 2.5 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 230 | 161 | 2.5 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 165 | 232 | 163 | 2.5 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 115 | 96 | 3 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 155 | 129 | 3 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 180 | 152 | 3 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 196 | 166 | 3 | 3.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 203 | 169 | 3 | 4 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 214 | 173 | 3 | 5 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 220 | 176 | 3 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 227 | 178 | 3 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 230 | 179 | 3 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 184 | 232 | 181 | 3 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 115 | 102 | 3.5 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 155 | 136 | 3.5 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 180 | 161 | 3.5 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 196 | 175 | 3.5 | 3.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 203 | 179 | 3.5 | 4 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 214 | 185 | 3.5 | 5 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 220 | 188 | 3.5 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 227 | 191 | 3.5 | 7 | 23096.74 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 230 | 193 | 3.5 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 199 | 232 | 195 | 3.5 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 115 | 107 | 4 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 155 | 141 | 4 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 180 | 166 | 4 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 196 | 180 | 4 | 3.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 203 | 185 | 4 | 4 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 214 | 192 | 4 | 5 | 8929.99 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 220 | 195 | 4 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 227 | 199 | 4 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 230 | 201 | 4 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 208 | 232 | 203 | 4 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 115 | 112 | 5 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 155 | 148 | 5 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 180 | 173 | 5 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 196 | 189 | 5 | 3.5 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 203 | 194 | 5 | 4 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 214 | 202 | 5 | 5 | 14357.24 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 220 | 205 | 5 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 227 | 209 | 5 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 230 | 211 | 5 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 219 | 232 | 213 | 5 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 115 | 112 | 6 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 155 | 151 | 6 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 180 | 176 | 6 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 196 | 192 | 6 | 3.5 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 203 | 197 | 6 | 4 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 214 | 206 | 6 | 5 | 14357.24 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 220 | 209 | 6 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 227 | 213 | 6 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 230 | 215 | 6 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 223 | 232 | 217 | 6 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 115 | 112 | 7 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 155 | 151 | 7 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 180 | 176 | 7 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 196 | 192 | 7 | 3.5 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 203 | 198 | 7 | 4 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 214 | 207 | 7 | 5 | 14357.24 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 220 | 210 | 7 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 227 | 214 | 7 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 230 | 216 | 7 | 8 | 23096.74 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TBS10 | 1205 | 260 | 241 | 1072 | 224 | 232 | 218 | 7 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 115 | 112 | 8 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 155 | 151 | 8 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 180 | 176 | 8 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 196 | 192 | 8 | 3.5 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 203 | 198 | 8 | 4 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 214 | 207 | 8 | 5 | 14357.24 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 220 | 211 | 8 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 227 | 216 | 8 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 230 | 218 | 8 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 227 | 232 | 220 | 8 | 10 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 115 | 115 | 10 | 2 | 5572.04 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 155 | 155 | 10 | 2.5 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 180 | 180 | 10 | 3 | 8828.35 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 196 | 196 | 10 | 3.5 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 203 | 203 | 10 | 4 | 9412.80 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 214 | 213 | 10 | 5 | 14357.24 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 220 | 217 | 10 | 6 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 227 | 222 | 10 | 7 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 230 | 224 | 10 | 8 | 23096.74 |
| TBS10 | 1205 | 260 | 241 | 1072 | 234 | 232 | 226 | 10 | 10 | 23096.74 |

num_bc_pass = number of barcodes that have the minimal amount of data for analysis.

num_prom_in_bc_pass = number of promoters represented in the group of barcodes with the minimum amount of data for analysis

num_prom_suff_data = number of promoters with sufficient data for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num_bc_pass_outlier = number of barcodes that pass the barcode outlier test

num_UTR_insensitive = number of promoters that are insensitive to 5' UTR sequence

num_Cond_insensitive = number of promoters that are insensitive to tested conditions

num_consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions
UTR_cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences
Cond_cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions
expression_range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

**Table S8. Expression stats for Gpo1 with different sensitivity cutoffs.**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | expression range |
|---|---|---|---|---|---|---|---|---|---|---|
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 70 | 27 | 2 | 2 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 97 | 43 | 2 | 2.5 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 112 | 51 | 2 | 3 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 132 | 54 | 2 | 3.5 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 148 | 60 | 2 | 4 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 161 | 64 | 2 | 5 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 172 | 69 | 2 | 6 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 179 | 72 | 2 | 7 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 188 | 78 | 2 | 8 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 97 | 191 | 81 | 2 | 10 | 9733.06 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 70 | 42 | 2.5 | 2 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 97 | 60 | 2.5 | 2.5 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 112 | 71 | 2.5 | 3 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 132 | 78 | 2.5 | 3.5 | 3927.49 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 148 | 87 | 2.5 | 4 | 12813.96 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 161 | 94 | 2.5 | 5 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 172 | 101 | 2.5 | 6 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 179 | 105 | 2.5 | 7 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 188 | 113 | 2.5 | 8 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 138 | 191 | 116 | 2.5 | 10 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 70 | 50 | 3 | 2 | 7429.86 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 97 | 71 | 3 | 2.5 | 7429.86 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 112 | 83 | 3 | 3 | 7429.86 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 132 | 94 | 3 | 3.5 | 7429.86 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 148 | 103 | 3 | 4 | 18412.57 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 161 | 112 | 3 | 5 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 172 | 120 | 3 | 6 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 179 | 124 | 3 | 7 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 188 | 132 | 3 | 8 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 159 | 191 | 135 | 3 | 10 | 19793.07 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 70 | 56 | 3.5 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 97 | 78 | 3.5 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 112 | 91 | 3.5 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 132 | 105 | 3.5 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 148 | 114 | 3.5 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 161 | 124 | 3.5 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 172 | 132 | 3.5 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 179 | 137 | 3.5 | 7 | 24286.63 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 188 | 145 | 3.5 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 172 | 191 | 148 | 3.5 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 70 | 58 | 4 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 97 | 82 | 4 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 112 | 95 | 4 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 132 | 111 | 4 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 148 | 121 | 4 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 161 | 131 | 4 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 172 | 139 | 4 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 179 | 144 | 4 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 188 | 152 | 4 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 180 | 191 | 155 | 4 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 70 | 63 | 5 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 97 | 88 | 5 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 112 | 102 | 5 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 132 | 120 | 5 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 148 | 132 | 5 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 161 | 143 | 5 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 172 | 151 | 5 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 179 | 157 | 5 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 188 | 165 | 5 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 193 | 191 | 168 | 5 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 70 | 65 | 6 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 97 | 90 | 6 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 112 | 104 | 6 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 132 | 124 | 6 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 148 | 136 | 6 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 161 | 148 | 6 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 172 | 157 | 6 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 179 | 164 | 6 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 188 | 172 | 6 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 201 | 191 | 175 | 6 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 70 | 67 | 7 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 97 | 92 | 7 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 112 | 106 | 7 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 132 | 126 | 7 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 148 | 140 | 7 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 161 | 152 | 7 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 172 | 163 | 7 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 179 | 170 | 7 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 188 | 179 | 7 | 8 | 24286.63 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gpo1 | 1116 | 250 | 218 | 957 | 209 | 191 | 182 | 7 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 70 | 67 | 8 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 97 | 92 | 8 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 112 | 106 | 8 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 132 | 126 | 8 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 148 | 140 | 8 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 161 | 153 | 8 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 172 | 164 | 8 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 179 | 171 | 8 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 188 | 180 | 8 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 210 | 191 | 183 | 8 | 10 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 70 | 68 | 10 | 2 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 97 | 93 | 10 | 2.5 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 112 | 108 | 10 | 3 | 13942.65 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 132 | 128 | 10 | 3.5 | 18390.70 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 148 | 143 | 10 | 4 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 161 | 156 | 10 | 5 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 172 | 167 | 10 | 6 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 179 | 174 | 10 | 7 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 188 | 183 | 10 | 8 | 24286.63 |
| Gpo1 | 1116 | 250 | 218 | 957 | 213 | 191 | 186 | 10 | 10 | 24286.63 |

num_bc_pass = number of barcodes that have the minimal amount of data for analysis.

num_prom_in_bc_pass = number of promoters represented in the group of barcodes with the minimum amount of data for analysis

num_prom_suff_data = number of promoters with sufficient data for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num_bc_pass_outlier = number of barcodes that pass the barcode outlier test

num_UTR_insensitive = number of promoters that are insensitive to 5' UTR sequence

num_Cond_insensitive = number of promoters that are insensitive to tested conditions

num_consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions
UTR_cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences

Cond_cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions

expression_range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

**Table S9. Expression stats for CGA009 with different sensitivity cutoffs**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | expression range |
|---|---|---|---|---|---|---|---|---|---|---|
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 109 | 44 | 2 | 2 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 124 | 49 | 2 | 2.5 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 142 | 53 | 2 | 3 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 153 | 54 | 2 | 3.5 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 159 | 54 | 2 | 4 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 169 | 56 | 2 | 5 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 172 | 56 | 2 | 6 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 172 | 56 | 2 | 7 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 177 | 57 | 2 | 8 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 60 | 180 | 57 | 2 | 10 | 41576.41 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 109 | 56 | 2.5 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 124 | 62 | 2.5 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 142 | 69 | 2.5 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 153 | 72 | 2.5 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 159 | 72 | 2.5 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 169 | 74 | 2.5 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 172 | 74 | 2.5 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 172 | 74 | 2.5 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 177 | 76 | 2.5 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 79 | 180 | 76 | 2.5 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 109 | 65 | 3 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 124 | 72 | 3 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 142 | 79 | 3 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 153 | 84 | 3 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 159 | 85 | 3 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 169 | 87 | 3 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 172 | 87 | 3 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 172 | 87 | 3 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 177 | 89 | 3 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 92 | 180 | 89 | 3 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 109 | 71 | 3.5 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 124 | 80 | 3.5 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 142 | 88 | 3.5 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 153 | 94 | 3.5 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 159 | 95 | 3.5 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 169 | 97 | 3.5 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 172 | 97 | 3.5 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 172 | 97 | 3.5 | 7 | 55796.73 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 177 | 99 | 3.5 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 102 | 180 | 99 | 3.5 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 109 | 75 | 4 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 124 | 86 | 4 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 142 | 94 | 4 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 153 | 101 | 4 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 159 | 102 | 4 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 169 | 104 | 4 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 172 | 104 | 4 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 172 | 104 | 4 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 177 | 106 | 4 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 109 | 180 | 106 | 4 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 109 | 83 | 5 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 124 | 95 | 5 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 142 | 104 | 5 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 153 | 111 | 5 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 159 | 112 | 5 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 169 | 114 | 5 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 172 | 114 | 5 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 172 | 114 | 5 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 177 | 116 | 5 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 119 | 180 | 116 | 5 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 109 | 85 | 6 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 124 | 98 | 6 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 142 | 107 | 6 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 153 | 115 | 6 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 159 | 117 | 6 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 169 | 120 | 6 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 172 | 120 | 6 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 172 | 120 | 6 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 177 | 122 | 6 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 126 | 180 | 122 | 6 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 109 | 86 | 7 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 124 | 99 | 7 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 142 | 110 | 7 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 153 | 120 | 7 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 159 | 122 | 7 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 169 | 125 | 7 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 172 | 125 | 7 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 172 | 125 | 7 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 177 | 127 | 7 | 8 | 55796.73 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CGA009 | 1061 | 254 | 205 | 874 | 132 | 180 | 128 | 7 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 109 | 92 | 8 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 124 | 106 | 8 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 142 | 118 | 8 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 153 | 129 | 8 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 159 | 131 | 8 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 169 | 135 | 8 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 172 | 135 | 8 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 172 | 135 | 8 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 177 | 137 | 8 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 142 | 180 | 138 | 8 | 10 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 109 | 92 | 10 | 2 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 124 | 107 | 10 | 2.5 | 41905.69 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 142 | 119 | 10 | 3 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 153 | 130 | 10 | 3.5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 159 | 132 | 10 | 4 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 169 | 137 | 10 | 5 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 172 | 137 | 10 | 6 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 172 | 137 | 10 | 7 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 177 | 139 | 10 | 8 | 55796.73 |
| CGA009 | 1061 | 254 | 205 | 874 | 144 | 180 | 140 | 10 | 10 | 55796.73 |

num_bc_pass = number of barcodes that have the minimal amount of data for analysis.

num_prom_in_bc_pass = number of promoters represented in the group of barcodes with the minimum amount of data for analysis

num_prom_suff_data = number of promoters with sufficient data for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num_bc_pass_outlier = number of barcodes that pass the barcode outlier test

num_UTR_insensitive = number of promoters that are insensitive to 5' UTR sequence

num_Cond_insensitive = number of promoters that are insensitive to tested conditions

num_consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions
UTR_cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences

Cond_cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions

expression_range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

**Table S10. Expression stats for RHA1 with different sensitivity cutoffs**

| Organism | num bc pass | num prom in bc pass | num prom suff data | num bc pass outlier | num UTR insensitive | num Cond insensitive | num consistent | UTR cutoff | Cond cutoff | expression range |
|---|---|---|---|---|---|---|---|---|---|---|
| RHA1 | 713 | 190 | 136 | 568 | 73 | 50 | 36 | 2 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 78 | 51 | 2 | 2.5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 95 | 59 | 2 | 3 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 110 | 66 | 2 | 3.5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 118 | 68 | 2 | 4 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 128 | 70 | 2 | 5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 128 | 70 | 2 | 6 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 130 | 70 | 2 | 7 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 133 | 71 | 2 | 8 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 73 | 134 | 71 | 2 | 10 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 50 | 41 | 2.5 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 78 | 58 | 2.5 | 2.5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 95 | 73 | 2.5 | 3 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 110 | 85 | 2.5 | 3.5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 118 | 88 | 2.5 | 4 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 128 | 91 | 2.5 | 5 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 128 | 91 | 2.5 | 6 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 130 | 92 | 2.5 | 7 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 133 | 93 | 2.5 | 8 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 96 | 134 | 94 | 2.5 | 10 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 50 | 48 | 3 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 78 | 70 | 3 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 95 | 85 | 3 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 110 | 97 | 3 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 118 | 102 | 3 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 128 | 106 | 3 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 128 | 106 | 3 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 130 | 107 | 3 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 133 | 109 | 3 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 112 | 134 | 110 | 3 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 50 | 49 | 3.5 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 78 | 74 | 3.5 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 95 | 89 | 3.5 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 110 | 102 | 3.5 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 118 | 108 | 3.5 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 128 | 114 | 3.5 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 128 | 114 | 3.5 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 130 | 115 | 3.5 | 7 | 2574.18 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| RHA1 | 713 | 190 | 136 | 568 | 120 | 133 | 117 | 3.5 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 120 | 134 | 118 | 3.5 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 50 | 49 | 4 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 78 | 76 | 4 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 95 | 91 | 4 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 110 | 104 | 4 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 118 | 111 | 4 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 128 | 117 | 4 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 128 | 117 | 4 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 130 | 118 | 4 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 133 | 120 | 4 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 123 | 134 | 121 | 4 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 50 | 49 | 5 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 78 | 76 | 5 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 95 | 92 | 5 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 110 | 105 | 5 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 118 | 112 | 5 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 128 | 120 | 5 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 128 | 120 | 5 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 130 | 122 | 5 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 133 | 125 | 5 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 128 | 134 | 126 | 5 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 50 | 50 | 6 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 78 | 78 | 6 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 95 | 94 | 6 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 110 | 108 | 6 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 118 | 116 | 6 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 6 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 6 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 130 | 127 | 6 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 133 | 130 | 6 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 134 | 131 | 6 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 50 | 50 | 7 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 78 | 78 | 7 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 95 | 94 | 7 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 110 | 108 | 7 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 118 | 116 | 7 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 7 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 7 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 130 | 127 | 7 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 133 | 130 | 7 | 8 | 2574.18 |

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| RHA1 | 713 | 190 | 136 | 568 | 133 | 134 | 131 | 7 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 50 | 50 | 8 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 78 | 78 | 8 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 95 | 94 | 8 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 110 | 108 | 8 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 118 | 116 | 8 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 8 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 128 | 125 | 8 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 130 | 127 | 8 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 133 | 130 | 8 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 133 | 134 | 131 | 8 | 10 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 50 | 50 | 10 | 2 | 1858.27 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 78 | 78 | 10 | 2.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 95 | 94 | 10 | 3 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 110 | 109 | 10 | 3.5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 118 | 117 | 10 | 4 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 128 | 126 | 10 | 5 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 128 | 126 | 10 | 6 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 130 | 128 | 10 | 7 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 133 | 131 | 10 | 8 | 2574.18 |
| RHA1 | 713 | 190 | 136 | 568 | 134 | 134 | 132 | 10 | 10 | 2574.18 |

num_bc_pass = number of barcodes that have the minimal amount of data for analysis.

num_prom_in_bc_pass = number of promoters represented in the group of barcodes with the minimum amount of data for analysis

num_prom_suff_data = number of promoters with sufficient data for analysis. This involves having at least 3 barcode variants with sufficient data for analysis for a given promoter

num_bc_pass_outlier = number of barcodes that pass the barcode outlier test

num_UTR_insensitive = number of promoters that are insensitive to 5' UTR sequence

num_Cond_insensitive = number of promoters that are insensitive to tested conditions

num_consistent = number of promoters that are both insensitive to 5' UTR sequence and tested conditions
UTR_cutoff = fold expression difference between barcode variants that must be passed for a promoter to be considered insensitive to 5' UTR sequences

Cond_cutoff = fold expression difference between conditions that must be passed for a promoter to be considered insensitive to conditions

expression_range = range of expression generated by dividing RTA for strongest and weakest consistent promoters in the given organism

**Additional Supplementary Materials**

**Supplementary Data D1:** Promoter source & DNA sequence with relative transcriptional profiling scores for gene expression library.

**Figure Source Data:** Measurement data used to visualize figure panels with N < 20, with individual Excel tabs for each relevant figure panel.

**Supplementary File F1:** Relative transcriptional activity plot for all promoters, conditions, and associated barcodes in *Pseudomonas fluorescens* SBW25.

**Supplementary File F2:** Relative transcriptional activity plot for all promoters, conditions, and associated barcodes in *Pseudomonas frederiksbergensis* TBS10.

**Supplementary File F3:** Relative transcriptional activity plot for all promoters, conditions, and associated barcodes in *Pseudomonas putida* Gpo1.

**Supplementary File F4:** Relative transcriptional activity plot for all promoters, conditions, and associated barcodes in *Rhodopseudomonas palustris* CGA009.

**Supplementary File F5:** Relative transcriptional activity plot for all promoters, conditions, and associated barcodes in *Rhodococcus jostii* RHA1.

**Supplementary File P1:** Sequence maps for all plasmids constructed for this study.

**Supplementary File T1:** Source and DNA sequence information for 287 natural and synthetic transcriptional promoters for bacteria included in the SAGE expression library.

**Supplementary File T2:** Relative transcriptional activity (RTA) scores across strains, conditions, and barcodes.

**REFERENCES**


1. V. M. Isabella, B. N. Ha, M. J. Castillo, D. J. Lubkowicz, S. E. Rowe, Y. A. Millet, C. L. Anderson, N. Li, A. B. Fisher, K. A. West, P. J. Reeder, M. M. Momin, C. G. Bergeron, S. E. Guilmain, P. F. Miller, C. B. Kurtz, D. Falb, Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat. Biotechnol.* **36**, 857–864 (2018).

2. V. Bahuguna, G. Bhatt, R. Maikhuri, D. Chandra, in *Microbial Metatranscriptomics Belowground*, M. Nath, D. Bhatt, P. Bhargava, D. K. Choudhary, Eds. (Springer Singapore, 2021), pp. 109–122.

3. R. K. Goyal, M. A. Schmidt, M. F. Hynes, Molecular biology in the improvement of biological nitrogen fixation by rhizobia and extending the scope to cereals. *Microorganisms* **9**, 125 (2021).

4. A. Z. Werner, R. Clare, T. D. Mand, I. Pardo, K. J. Ramirez, S. J. Haugen, F. Bratti, G. N. Dexter, J. R. Elmore, J. D. Huenemann, G. L. Peabody V, C. W. Johnson, N. A. Rorrer, D. Salvachúa, A. M. Guss, G. T. Beckham, Tandem chemical deconstruction and biological upcycling of poly(ethylene terephthalate) to β-ketoadipic acid by Pseudomonas putida KT2440. *Metab. Eng.* **67**, 250–261 (2021).

5. A. Banner, H. S. Toogood, N. S. Scrutton, Consolidated bioprocessing: Synthetic biology routes to fuels and fine chemicals. *Microorganisms* **9**, 1079 (2021).

6. J. Kim, S. Hwang, S. M. Lee, Metabolic engineering for the utilization of carbohydrate portions of lignocellulosic biomass. *Metab. Eng.* **71**, 2–12 (2022).

7. F. Käß, S. Junne, P. Neubauer, W. Wiechert, M. Oldiges, Process inhomogeneity leads to rapid side product turnover in cultivation of Corynebacterium glutamicum. *Microb. Cell Fact.* **13**, 6 (2014).

8. M. Pigou, J. Morchain, Investigating the interactions between physical and biological heterogeneities in bioreactors using compartment, population balance and metabolic models. *Chem. Eng. Sci.* **126**, 267–282 (2015).

9. L. A. Riley, A. M. Guss, Approaches to genetic tool development for rapid domestication of non-model microorganisms. *Biotechnol. Biofuels* **14**, 30 (2021).

10. M. E. Kovach, P. H. Elzer, D. Steven Hill, G. T. Robertson, M. A. Farris, R.M. Roop II, K. M. Peterson, Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176 (1995).

11. T. S. Lee, R. A. Krupa, F. Zhang, M. Hajimorad, W. J. Holtz, N. Prasad, S. K. Lee, J. D. Keasling, BglBrick vectors and datasheets: A synthetic biology platform for gene expression. *J. Biol. Eng.* **5**, 12 (2011).

12. W. Pansegrau, E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, C. M. Thomas, Complete nucleotide sequence of Birmingham IncPα plasmids. *J. Mol. Biol.* **239**, 623–663 (1994).

13. R. H. Durland, A. Toukdarian, F. Fang, D. R. Helinski, Mutations in the trfA replication gene of the broad-host-range plasmid RK2 result in elevated plasmid copy numbers. *J. Bacteriol.* **172**, 3859–3867 (1990).

14. J. Frey, M. M. Bagdasarian, M. Bagdasarian, Replication and copy number control of the broad-host-range plasmid RSF1010. *Gene* **113**, 101–106 (1992).

15. E. De Rossi, A. Milano, P. Brigidi, F. Bini, G. Riccardi, Structural organization of pBC1, a cryptic plasmid from Bacillus coagulans. *J. Bacteriol.* **174**, 638–642 (1992).

16. L. De Gelder, J. J. Williams, J. M. Ponciano, M. Sota, E. M. Top, Adaptive plasmid evolution results in host-range expansion of a broad-host-range plasmid. *Genetics* **178**, 2179–2190 (2008).

17. W. P. Gill, N. S. Harik, M. R. Whiddon, R. P. Liao, J. E. Mittler, D. R. Sherman, A replication clock for Mycobacterium tuberculosis. *Nat. Med.* **15**, 211–214 (2009).

18. C. A. Mason, J. E. Bailey, Effects of plasmid presence on growth and enzyme activity of *Escherichia coli* DH5α. *Appl. Microbiol. Biotechnol.* **32**, 54–60 (1989).

19. J. Mi, A. Sydow, F. Schempp, D. Becher, H. Schewe, J. Schrader, M. Buchhaupt, Investigation of plasmid-induced growth defect in Pseudomonas putida. *J. Biotechnol.* **231**, 167–173 (2016).

20. A. San Millan, M. Toll-Riera, Q. Qi, A. Betts, R. J. Hopkinson, J. McCullagh, R. C. MacLean, Integrative analysis of fitness and metabolic effects of plasmids in Pseudomonas aeruginosa PAO1. *ISME J.* **12**, 3014–3024 (2018).

21. M. Sota, H. Yano, J. M Hughes, G. W. Daughdrill, Z. Abdo, L. J. Forney, E. M. Top, Shifts in the host range of a promiscuous plasmid through parallel evolution of its replication initiation protein. *ISME J.* **4**, 1568–1580 (2010).

22. L. Yin, H. Ma, E. S. Nakayasu, S. H. Payne, D. R. Morris, C. S. Harwood, Bacterial longevity requires protein synthesis and a stringent response. *MBio* **10**, e02189-19 (2019).

23. S. Lin-Chao, H. Bremer, Effect of the bacterial growth rate on replication control of plasmid pBR322 in Escherichia coli. *Mol. Gen. Genet.* **203**, 143–149 (1986).

24. J. Paulsson, M. Ehrenberg, Noise in a minimal regulatory network: Plasmid copy number control. *Q. Rev. Biophys.* **34**, 1–59 (2001).

25. A. Pena-Gonzalez, L. M. Rodriguez-R, C. K. Marston, J. E. Gee, C. A. Gulvik, C. B. Kolton, E. Saile, M. Frace, A. R. Hoffmaster, K. T. Konstantinidis, Genomic characterization and copy number variation of *bacillus anthracis* plasmids pXO1 and pXO2 in a historical collection of 412 strains. **3**, e00065-18 (2018).

26. T. B. Cook, J. M. Rand, W. Nurani, D. K. Courtney, S. A. Liu, B. F. Pfleger, Genetic tools for reliable gene expression and recombineering in Pseudomonas putida. *J. Ind. Microbiol. Biotechnol.* **45**, 517–527 (2018).

27. S. C. Troeschel, S. Thies, O. Link, C. I. Real, K. Knops, S. Wilhelm, F. Rosenau, K. E. Jaeger, Novel broad host range shuttle vectors for expression in Escherichia coli, Bacillus subtilis and Pseudomonas putida. *J. Biotechnol.* **161**, 71–79 (2012).

28. L. Li, X. Liu, K. Wei, Y. Lu, W. Jiang, Synthetic biology approaches for chromosomal integration of genes and pathways in industrial microbial systems. *Biotechnol. Adv.* **37**, 730–745 (2019).

29. R. D. Arroyo-Olarte, R. B. Rodriguez, E. Morales-Rios, Genome editing in bacteria: CRISPR-cas and beyond. *Microorganisms* **9**, 844 (2021).

30. R. D. Arroyo-Olarte, R. B. Rodríguez, E. Morales-Ríos, Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: Selection of defined deletions in the chromosome of Corynebacterium glutamicum. *Gene* **145**, 69–73 (1994).

31. J. R. Elmore, A. Furches, G. N. Wolff, K. Gorday, A. M. Guss, Development of a high efficiency integration system and promoter library for rapid modification of Pseudomonas putida KT2440. *Metab. Eng. Commun.* **5**, 1–8 (2017).

32. E. M. Lammens, P. I. Nikel, R. Lavigne, Exploring the synthetic biology potential of bacteriophages for engineering non-model bacteria. *Nat. Commun.* **11**, 5294 (2020).

33. J. A. N. Brophy, A. J. Triassi, B. L. Adams, R. L. Renberg, D. N. Stratis-Cullum, A. D. Grossman, C. A. Voigt, Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. *Nat. Microbiol.* **3**, 1043–1053 (2018).

34. M. A. Gregory, R. Till, M. C. Smith, Integration site for Streptomyces phage phiBT1 and development of site-specific integrating vectors. *J. Bacteriol.* **185**, 5320–5323 (2003).

35. A. M. Guss, M. Rother, J. K. Zhang, G. Kulkarni, W. W. Metcalf, New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for Methanosarcina species. *Archaea* **2**, 193–203 (2008).

36. A. Keravala, A. C. Groth, S. Jarrahian, B. Thyagarajan, J. J. Hoyt, P. J. Kirby, M. P. Calos, A diversity of serine phage integrases mediate site-specific recombination in mammalian cells. *Mol. Genet. Genomics* **276**, 135–146 (2006).

37. L. Li, K. Wei, X. Liu, Y. Wu, G. Zheng, S. Chen, W. Jiang, Y. Lu, aMSGE: Advanced multiplex site-specific genome engineering with orthogonal modular recombinases in actinomycetes. *Metab. Eng.* **52**, 153–167 (2019).

38. C. A. Merrick, J. Zhao, S. J. Rosser, Serine integrases: Advancing synthetic biology. *ACS Synth. Biol.* **7**, 299–310 (2018).

39. J. G. Thomson, R. Chan, J. Smith, R. Thilmony, Y. Y. Yau, Y. J. Wang, D. W. Ow, The Bxb1 recombination system demonstrates heritable transmission of site-specific excision in Arabidopsis. *BMC Biotechnol.* **12**, 9 (2012).

40. Z. Xu, W. R. Brown, Comparison and optimization of ten phage encoded serine integrases for genome engineering in Saccharomyces cerevisiae. *BMC Biotechnol.* **16**, 13 (2016).

41. G. Wang, Z. Zhao, J. Ke, Y. Engel, Y.M. Shi, D. Robinson, K. Bingol, Z. Zhang, B. Bowen, K. Louie, B. Wang, R. Evans, Y. Miyamoto, K. Cheng, S. Kosina, M. de Raad, L. Silva, A. Luhrs, A. Lubbe, D. W. Hoyt, C. Francavilla, H. Otani, S. Deutsch, N. M. Washton, E. M. Rubin, N. J. Mouncey, A. Visel, T. Northen, J.F. Cheng, H. B. Bode, Y. Yoshikuni, CRAGE enables rapid activation of biosynthetic gene clusters in undomesticated bacteria. *Nat. Microbiol.* **4**, 2498–2510 (2019).

42. W. R. A. Brown, N. C. O. Lee, Z. Y. Xu, M. C. M. Smith, Serine recombinases as tools for genome engineering. *Methods* **53**, 372–379 (2011).

43. P. C. Fogg, S. Colloms, S. Rosser, M. Stark, M. C. Smith, New applications for phage integrases. *J. Mol. Biol.* **426**, 2703–2716 (2014).

44. T. E. Saleski, M. T. Chung, D. N. Carruthers, A. Khasbaatar, K. Kurabayashi, X. N. Lin, Optimized gene expression from bacterial chromosome by high-throughput integration and screening. *Sci. Adv.* **7**, eabe1767 (2021).

45. B. Wang, Z. Zhao, L. K. Jabusch, D. M. Chiniquy, K. Ono, J. M. Conway, Z. Zhang, G. Wang, D. Robinson, J.F. Cheng, J. L. Dangl, T. R. Northen, Y. Yoshikuni, CRAGE-duet facilitates modular assembly of biological systems for studying plant-microbe interactions. *ACS Synth. Biol.* **9**, 2610–2615 (2020).

46. M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. Nagaraja Rao, B. E. Schoner, Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. *Gene* **116**, 43–49 (1992).

47. Y. Jiang, B. Chen, C. Duan, B. Sun, J. Yang, S. Yang, Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system. *Appl. Environ. Microb.* **81**, 2506–2514 (2015).

48. C. W. Song, J. Lee, S. Y. Lee, Genome engineering and gene expression control for bacterial strain development. *Biotechnol. J.* **10**, 56–68 (2015).

49. P. L. H. Vo, C. Ronda, S. E. Klompe, E. E. Chen, C. Acree, H. H. Wang, S. H. Sternberg, CRISPR RNA-guided integrases for high-efficiency, multiplexed bacterial genome engineering. *Nat. Biotechnol.* **39**, 480–489 (2021).

50. M. J. Bailey, A. K. Lilley, I. P. Thompson, P. B. Rainey, R. J. Ellis, Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet; Stability and potential for marker gene transfer. *Mol. Ecol.* **4**, 755–764 (1995).

51. F. W. Larimer, P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. do, M. L. Land, D. A. Pelletier, J. T. Beatty, A. S. Lang, F. R. Tabita, J. L. Gibson, T. E. Hanson, C. Bobst, J. L. T. Torres, C. Peres, F. H. Harrison, J. Gibson, C. S. Harwood, Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. *Nat. Biotechnol.* **22**, 55–61 (2004).

52. E. J. McKenna, M. J. Coon, Enzymatic omega-oxidation. IV. Purification and properties of the omega-hydroxylase of Pseudomonas oleovorans. *J. Biol. Chem.* **245**, 3882–3889 (1970).

53. M. Seto, K. Kimbara, M. Shimura, T. Hatta, M. Fukuda, K. Yano, A novel transformation of polychlorinated biphenyls by rhodococcus sp. strain RHA1. *Appl. Environ. Microbiol.* **61**, 3353–3358 (1995).

54. Y. J. Chen, P. Liu, A. A. K. Nielsen, J. A. N. Brophy, K. Clancy, T. Peterson, C. A. Voigt, Characterization of 582 natural and synthetic terminators and quantification of their design constraints. *Nat. Methods* **10**, 659–664 (2013).

55. N. C. Shaner, G. G. Lambert, A. Chammas, Y. Ni, P. J. Cranfill, M. A. Baird, B. R. Sell, J. R. Allen, R. N. Day, M. Israelsson, M. W. Davidson, J. Wang, A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. *Nat. Methods* **10**, 407–409 (2013).

56. D. Shcherbo, C. S. Murphy, G. V. Ermakova, E. A. Solovieva, T. V. Chepurnykh, A. S. Shcheglov, V. V. Verkhusha, V. Z. Pletnev, K. L. Hazelwood, P. M. Roche, S. Lukyanov, A. G. Zaraisky, M. W. Davidson, D. M. Chudakov, Far-red fluorescent tags for protein imaging in living tissues. *Biochem. J.* **418**, 567–574 (2009).

57. Y. Hong, M. K. Hondalus, Site-specific integration of *Streptomyces* ΦC31 integrase-based vectors in the chromosome of *Rhodococcus* equi. *FEMS Microbiol. Lett.* **287**, 63–68 (2008).

58. J. P. du Toit, D. J. Lea-Smith, A. Git, J. R. D. Hervey, C. J. Howe, R. W. M. Pott, Expression of alternative nitrogenases in *Rhodopseudomonas palustris* is enhanced using an optimized genetic toolset for rapid, markerless modifications. *ACS Synth.Biol.* **10**, 2167–2178 (2021).

59. A. C. Groth, M. P. Calos, Phage integrases: Biology and applications. *J. Mol. Biol.* **335**, 667–678 (2004).

60. S. D. Colloms, C. A. Merrick, F. J. Olorunniji, W. M. Stark, M. C. M. Smith, A. Osbourn, J. D. Keasling, S. J. Rosser, Rapid metabolic pathway assembly and modification using serine integrase site-specific recombination. *Nucleic Acids Res.* **42**, e23 (2014).

61. L. A. Silo-Suh, B. Elmore, D. E. Ohman, S. J. Suh, Isolation, characterization, and utilization of a temperature-sensitive allele of a Pseudomonas replicon. *J. Microbiol. Meth.* **78**, 319–324 (2009).

62. J. Quandt, M. F. Hynes, Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**, 15–21 (1993).

63. H. Alper, C. Fischer, E. Nevoigt, G. Stephanopoulos, Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12678–12683 (2005).

64. N. I. Johns, A. L. C. Gomes, S. S. Yim, A. Yang, T. Blazejewski, C. S. Smillie, M. B. Smith, E. J. Alm, S. Kosuri, H. H. Wang, Metagenomic mining of regulatory elements enables programmable species-selective gene expression. *Nat. Methods* **15**, 323–329 (2018).

65. N. Xu, L. Wei, J. Liu, Recent advances in the applications of promoter engineering for the optimization of metabolite biosynthesis. *World J. Microbiol. Biotechnol.* **35**, 33 (2019).

66. M. D. Engstrom, B. F. Pfleger, Transcription control engineering and applications in synthetic biology. *Synth. Syst. Biotechnol.* **2**, 176–191 (2017).

67. C. Engler, R. Kandzia, S. Marillonnet, A one pot, one step, precision cloning method with high throughput capability. *PLOS ONE* **3**, e3647 (2008).

68. J. A. Jones, V. R. Vernacchio, D. M. Lachance, M. Lebovich, L. Fu, A. N. Shirke, V. L. Schultz, B. Cress, R. J. Linhardt, M. A. G. Koffas, ePathOptimize: A combinatorial approach for transcriptional balancing of metabolic pathways. *Sci. Rep.* **5**, 11301 (2015).

69. J. R. Elmore, G. N. Dexter, D. Salvachúa, J. Martinez-Baird, E. A. Hatmaker, J. D. Huenemann, D. M. Klingeman, G. L. Peabody V, D. J. Peterson, C. Singer, G. T. Beckham, A. M. Guss, Production of itaconic acid from alkali pretreated lignin by dynamic two stage bioconversion. *Nat. Commun.* **12**, 2261 (2021).

70. M. G. Durrant, A. Fanton, J. Tycko, M. Hinks, S. S. Chandrasekaran, N. T. Perry, J. Schaepe, P. P. du, P. Lotfy, M. C. Bassik, L. Bintu, A. S. Bhatt, P. D. Hsu, Systematic discovery of recombinases for efficient integration of large DNA sequences into the human genome. *Nat. Biotechnol.* (2022). 10.1038/s41587-022-01494-w

71. L. Yang, A. A. K. Nielsen, J. Fernandez-Rodriguez, C. J. McClune, M. T. Laub, T. K. Lu, C. A. Voigt, Permanent genetic memory with >1-byte capacity. *Nat. Methods* **11**, 1261–1266 (2014).

72. P. Ghosh, L. R. Wasil, G. F. Hatfull, Control of phage Bxb1 excision by a novel recombination directionality factor. *PLOS Biol.* **4**, e186 (2006).

73. T. Khaleel, E. Younger, A. R. McEwan, A. S. Varghese, M. C. Smith, A phage protein that binds φC31 integrase to switch its directionality. *Mol. Microbiol.* **80**, 1450–1463 (2011).

74. J. R. Elmore, G. N. Dexter, D. Salvachúa, M. O'Brien, D. M. Klingeman, K. Gorday, J. K. Michener, D. J. Peterson, G. T. Beckham, A. M. Guss, Engineered Pseudomonas putida simultaneously catabolizes five major components of corn stover lignocellulose: Glucose, xylose, arabinose, p-coumaric acid, and acetic acid. *Metab. Eng.* **62**, 62–71 (2020).

75. L. Yin, C. S. Harwood, Charging state analysis of transfer RNA from an alpha-proteobacterium. *Bio Protoc.* **10**, e3834 (2020).

76. C. Gao, L. Montoya, L. Xu, M. Madera, J. Hollingsworth, E. Purdom, V. Singan, J. Vogel, R. B. Hutmacher, J. A. Dahlberg, D. Coleman-Derr, P. G. Lemaux, J. W. Taylor, Fungal community assembly in drought-stressed sorghum shows stochasticity, selection, and universal ecological dynamics. *Nat. Commun.* **11**, 34 (2020).

77. L. Xu, D. Naylor, Z. Dong, T. Simmons, G. Pierroz, K. K. Hixson, Y.M. Kim, E. M. Zink, K. M. Engbrecht, Y. Wang, C. Gao, S. DeGraaf, M. A. Madera, J. A. Sievert, J. Hollingsworth, D. Birdseye, H. V. Scheller, R. Hutmacher, J. Dahlberg, C. Jansson, J. W. Taylor, P. G. Lemaux, D. Coleman-Derr, Drought delays development of the sorghum root microbiome and enriches for monoderm bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E4284–E4293 (2018).

78. G. L. Peabody, J. R. Elmore, J. Martinez-Baird, A. M. Guss, Engineered Pseudomonas putida KT2440 co-utilizes galactose and glucose. *Biotechnol. Biofuels* **12**, 295 (2019).

79. C. W. Johnson, D. Salvachúa, N. A. Rorrer, B. A. Black, D. R. Vardon, P. C. St. John, N. S. Cleveland, G. Dominick, J. R. Elmore, N. Grundl, P. Khanna, C. R. Martinez, W. E. Michener, D. J. Peterson, K. J. Ramirez, P. Singh, T. A. VanderWall, A. N. Wilson, X. Yi, M. J. Biddy, Y. J. Bomble, A. M. Guss, G. T. Beckham, Innovative chemicals and materials from bacterial aromatic catabolic pathways. *Joule* **3**, 1523–1537 (2019).

80. S. S. Yim, N. I. Johns, J. Park, A. L.C. Gomes, R. M. McBee, M. Richardson, C. Ronda, S. P. Chen, D. Garenne, V. Noireaux, H. H. Wang, Multiplex transcriptional characterizations across diverse bacterial species using cell-free systems. *Mol. Syst. Biol.* **15**, e8875 (2019).

81. L. N. Jayakody, C. W. Johnson, J. M. Whitham, R. J. Giannone, B. A. Black, N. S. Cleveland, D. M. Klingeman, W. E. Michener, J. L. Olstad, D. R. Vardon, R. C. Brown, S. D. Brown, R. L. Hettich, A. M. Guss, G. T. Beckham, Thermochemical wastewater valorization via enhanced microbial toxicity tolerance. *Energ. Environ. Sci.* **11**, 1625–1638 (2018).