Portable bacterial CRISPR transcriptional activation enables metabolic engineering in *Pseudomonas putida*

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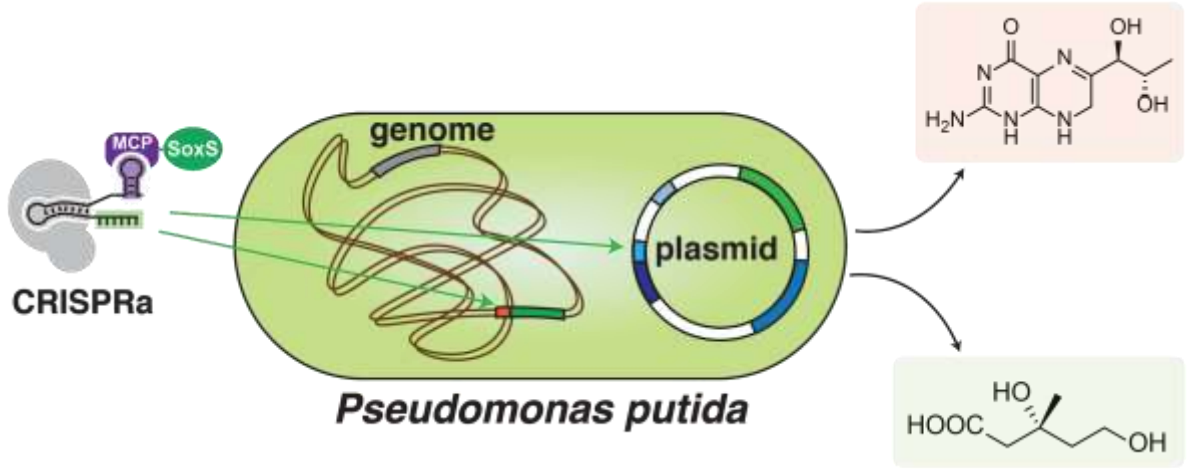
Abstract

CRISPR-Cas transcriptional programming in bacteria is an emerging tool to regulate gene expression for metabolic pathway engineering. Here we implement CRISPR-Cas transcriptional activation (CRISPRa) in *P. putida* using a system previously developed in *E. coli*. We provide a methodology to transfer CRISPRa to a new host by first optimizing expression levels for the CRISPRa system components, and then applying rules for effective CRISPRa based on a systematic characterization of promoter features. Using this optimized system, we regulate biosynthesis in the biopterin and mevalonate pathways. We demonstrate that multiple genes can be activated simultaneously by targeting multiple promoters or by targeting a single promoter in a multi-gene operon. This work will enable new metabolic engineering strategies in *P. putida* and pave the way for CRISPR-Cas transcriptional programming in other bacterial species.

Keywords

- CRISPR activation
- *Pseudomonas putida*
- Biopterin
- Mevalonate

Graphical Abstracts



Highlights

- Bacterial CRISPR activation (CRISPRa) was successfully ported to *P. putida*
- Multi-gene programs with simultaneous CRISPRa and CRISPRi can be implemented
- Systematic analysis shows that rules for effective *E. coli* CRISPRa apply in *P. putida*
- CRISPRa can activate endogenous promoters
- Inducible CRISPRa can be obtained by expressing dCas9 from inducible promoters

1. Introduction

The development of microbial platforms for industrial chemical production frequently requires optimizing the expression levels of multiple genes (Lee et al., 2019; Nielsen and Keasling, 2016). The advent of CRISPR-Cas tools that can be used to rapidly program gene expression promises to accelerate pathway engineering for the efficient production of high-value compounds (Fontana et al., 2020b). The application of CRISPR-Cas tools for transcriptional repression (CRISPRi) in bacterial metabolic engineering is well-established (Banerjee et al., 2020; Kim et al., 2019; Qi et al., 2013; Tian et al., 2019; Zheng et al., 2019). By comparison, the development of CRISPR-Cas tools for programmable transcriptional activation (CRISPRa) has lagged due to the paucity of effective transcriptional activators (Dong et al., 2018), and the complexity of the rules governing CRISPRa-directed transcription from bacterial promoters (Fontana et al., 2020a). Despite these challenges, the potential for using CRISPRa to program gene expression has been demonstrated through the successful implementation in *E. coli*, *M. xanthus*, *K. oxytoca*, and *S. enterica* (Bikard et al., 2013; Dong et al., 2018; Ho et al., 2020; Liu et al., 2019; Peng et al., 2018). Determining how to strategically port CRISPRa systems into other microbes could significantly improve our metabolic engineering capabilities.

Pseudomonas putida is a gram-negative soil bacterium that has recently received attention as a potential chassis for bioproduction due to desirable metabolic capabilities and the capacity to survive harsh bioprocessing conditions (Nikel et al., 2016; Nikel and de Lorenzo, 2018) *P. putida* has high reducing power (Chavarría et al., 2013) and the ability to metabolize a broad range of feedstocks, from glucose to the toxic products of

aromatic lignin degradation (Elmore et al., 2020; Johnson and Beckham, 2015; Kim et al., 2000; Loeschcke and Thies, 2015). The successful implementation of CRISPR genome editing and CRISPRi in *P. putida* (Aparicio et al., 2018; Banerjee et al., 2020; Kim et al., 2019; Tan et al., 2018; Wirth et al., 2019), shows that CRISPR gene targeting can be effective in *P. putida* and provides a starting point to assess whether gene activation with a CRISPRa system can be achieved.

CRISPR-Cas transcriptional control typically uses the catalytically inactive Cas9 protein (dCas9) with programmable guide RNAs that recognize DNA targets through Watson-Crick base pairing (Xu and Qi, 2019). Recently, we identified and optimized a variant of the transcriptional activator SoxS (R93A/S101A) that can be linked to a programmable CRISPR-Cas DNA binding domain to activate gene expression in *E. coli* (Dong et al., 2018; Fontana et al., 2020a). SoxS interacts with an interface on the α-subunit of RNA polymerase (RpoA) that is widely conserved throughout bacterial species, including in *P. putida*, suggesting that the CRISPRa system we developed in *E. coli* should also be effective in *P. putida* and other bacteria. However, in contrast to the relative permissiveness of CRISPRi (and CRISPRa in eukaryotes) (Gilbert et al., 2014; Konermann et al., 2015; Qi et al., 2013), CRISPRa in bacteria is known to be sensitive to several features of target promoters, including the precise distance from the transcription start site and the intervening sequence composition. (Fontana et al., 2020a; Ho et al., 2020; Liu et al., 2019). It is not known to what extent the rules characterized in one bacterial species are generalizable in others.

In this paper, we developed CRISPRa for programming heterologous gene expression in *P. putida* KT2440. We first constructed genetic components and

established experimental approaches to permit CRISPRa machinery developed in *E. coli* to be expressed and utilized in *P. putida*. By investigating promoter features that impact CRISPRa, such as guide RNA target sites and promoter strengths, we identified designs permitting 30- to 100-fold activation of heterologous reporter gene expression. We also demonstrated that CRISPRa can be coupled with CRISPRi for multi-gene programming and endogenous gene activation. Using an inducible system derived from *P. putida*, we have developed an inducible CRISPRa/CRISPRi platform with low leakage in the uninduced state. We showed that CRISPRa can drive the expression of heterologous genes to produce desirable metabolic products including biopterin derivatives and mevalonate. We further showed that the inducible CRISPRa system can generate 40-fold increases in mevalonate production, achieving titers comparable to those from a previously reported IPTG-inducible system. Taken together, this work provides a toolbox of components and validated workflows for implementing CRISPRa to program heterologous gene expression in *P. putida*. More broadly, these efforts establish a framework for the further development of CRISPRa tools for programming gene expression in industrially-promising bacteria.

2. Materials and methods

2.1 General Procedures

Plasmids pBBR1-MCS2(pBBR1-KmR), pBBR1-MCS5(pBBR1-GmR) (Kovach et al., 1995), pTNS1, pUC18T-miniTn7T-GmR (Choi and Schweizer, 2006), pRK2013, pFLP2, and *P. putida* KT2440 were a gift from the Harwood lab at the University of Washington. pRK2-AraE (Cook et al., 2018) was a gift from the Pfleger lab at the University of

Wisconsin-Madison (Addgene #110141). pMVA2RBS035 (Jervis et al., 2019) was a gift from the Scrutton lab at the University of Manchester (Addgene #121051). *S. pyogenes* dCas9 (*Sp*-dCas9) was expressed from the endogenous *Sp*.pCas9 promoter and the MCP-SoxS (R93A, S101A) (abbreviated MCP-SoxS) transcriptional activator fusion protein was expressed from the BBa_J23107 promoter (Fontana et al., 2020a) (http://parts.igem.org). The modified single guide RNAs (sgRNA) (Dong et al., 2018), scaffold RNAs b2.1xMS2 (scRNAs), were expressed from the BBa_J23119 promoter in the pBBR1-GmR plasmid, unless specified. 20 bp scRNA/sgRNA target sequences are provided in Table S4. mRFP1 and sfGFP reporters were expressed from the weak BBa_J23117 minimal promoter (http://parts.igem.org), unless specified, either by integrating into the genome or in the pBBR1-GmR plasmid together with the scRNA(s). All plasmids were constructed and propagated in *E. coli* NEB turbo cells (New England Biolabs). All *P. putida* strains were constructed from the wild type strain KT2440. DNA sequences are provided in the Supplementary Material. See Table 1 for a complete list of bacterial strains and plasmid constructs.

2.2 Plasmid Construction

All PCR fragments were amplified with Phusion DNA Polymerase (Thermo-Fisher Scientific) for Infusion Cloning (Takara Bio). Transformants were cultured or selected either on Lysogeny Broth (LB) or agar plates, with appropriate antibiotics, used in the following concentrations: 100 µg/mL Carbenicillin, 25 µg/mL Chloramphenicol, 30 µg/mL Kanamycin, 30 µg/mL Gentamicin. Successful constructs were confirmed by Sanger sequencing (GENEWIZ). Details for cloning strategies are described in the

Supplementary Methods and Tables S1-S3. sgRNA/scRNA target sequences are provided in Table S4.

2.3 *Pseudomonas putida* Strain Construction

Pseudomonas putida genome integrations were performed using the tri-parental conjugation for the mini-Tn7 method (Choi and Schweizer, 2006) or electroporation for the pGNW2 method (Wirth et al., 2019). Plasmid transformations into *P. putida* were performed either by electroporation (Choi and Schweizer, 2006) or heat-shock of $CaCl_2$ chemically competent cells (Zhao et al., 2013). Detailed methods for the preparation and transformation of chemically competent cells are described in the Supplementary Methods.

2.4 Fluorescence measurements of reporter gene expression

Fluorescence measurements of reporter gene expression were carried out either by flow cytometry or plate reader. Single colonies from LB plates were inoculated in 500 µL of EZ-RDM (Teknova) supplemented with the appropriate antibiotics and grown in 96-deep-well plates at 30 °C with shaking overnight 225 rpm. For small-molecule induction, overnight cultures were diluted 100-fold into a new culture with appropriate antibiotics and inducers, then shaken overnight at 30 °C, 225 rpm. For flow cytometry, overnight cultures were diluted 1:50 in Dulbecco's phosphate-buffered saline (PBS) and analyzed on a MACSQuant VYB flow cytometer with the MACSQuantify 2.8 software (Miltenyi Biotec) using the methods and instruments settings as described (Dong et al., 2018). For plate reader measurements, 150 µL of overnight culture were transferred into a flat, clear-

bottomed black 96-well plate. OD_{600} and fluorescence values were measured in a Biotek Synergy HTX plate reader and analyzed using the BioTek Gen5 2.07.17 software. For mRFP1 detection, the excitation wavelength was 540 nm and emission wavelength was 600 nm. For sfGFP detection, the excitation wavelength was 485 nm and the emission wavelength was 528 nm. Data were plotted using Prism (GraphPad).

2.5 Mevalonate production and quantitation by GC-MS

For mevalonate production experiments, the GC-MS method was adapted from prior methods (Pfleger et al., 2007). Single colonies from LB plates were inoculated in 500 µL of EZ-RDM (Teknova) supplemented with the appropriate antibiotics and grown in 96-deep-well plates at 30 °C with shaking overnight at 225 rpm. Overnight cultures were subcultured by 1:100 dilution into 3 mL of EZ-RDM media with 1% glucose as the carbon source, supplemented with the appropriate antibiotics, and shaken at 225 rpm for 72 hours at 30 °C. After 72 hours, 560 µL of cell suspension was acidified with 140 µL of 0.5 M HCl and vortexed. 700 µL ethyl acetate was added and samples were then vortexed again vigorously for 3 minutes and centrifuged at maximum speed in a benchtop centrifuge (15,000 rcf) for 10 min. The organic phase was then transferred into GC-MS vials for analysis. GC-MS analysis was performed using an Agilent 5973 instrument with a temperature program as follows. The inlet temperature was 250 °C (splitless mode). The column flow was kept at 1 mL/min in HP-5MS (Agilent). The temperature cycle started at 80 °C and was followed by a gradient of 20 °C/min to 260 °C, a second gradient of 40 °C/min to 300 °C, and a hold at 300 °C for 2 min. m/z = 71, the second most abundant peak corresponding to mevalonolactone, was used for quantitation (Pfleger et al., 2007).

A calibration curve was generated using freshly-prepared D,L-mevalonolactone (Sigma) dissolved in ethyl acetate. The calculated concentration was adjusted by the addition of HCl. Data were plotted using Prism (GraphPad).

2.6 Biopterin production and measurement

For the biopterin production experiments, single colonies from LB plates were inoculated in 500 µL of EZ-RDM supplemented with the appropriate antibiotics and grown in 96-deep-well plates at 30 °C with shaking overnight. Each sample was then sub-cultured at 100-fold dilution in 5 mL of EZ-RDM supplemented with the appropriate antibiotics and grown in 14 mL culture tubes at 30 °C and shaking for 24 hours. The overnight cultures were spun down and pteridine concentrations were determined by measuring the OD_{340} and comparing the results to a standard calibration curve prepared with purchased reagents (Cayman Chemical). The HPLC-MS measurements were performed as described (Ehrenworth et al., 2015). A detailed HPLC-MS protocol is provided in the Supplementary Methods. Data were plotted using Prism (GraphPad).

3. Results

3.1 Enabling CRISPRa in *P. putida*

3.1.1 Plasmid-based CRISPRa in *P. putida*

The first challenge to enable a CRISPRa system in *P. putida* is to express the components from *E. coli* in *P. putida*. The bacterial CRISPRa system developed in *E. coli* consists of three components, dCas9, MCP-SoxS, and scRNA (Dong et al., 2018), delivered in a p15A plasmid that is present at ~10 copies/cell (Shetty et al., 2008) (Figure

1A). The scRNA is a modified sgRNA with a 3' MS2 hairpin to recruit the MCP-SoxS activator. The reporter gene(s) were delivered in a pSC101** plasmid which is present at ~5 copies/cell (Lee et al., 2011). We used *E. coli* SoxS as the activator domain because it recognizes a motif on RpoA that is conserved between *E. coli* and *P. putida*(Dong et al., 2018), and there is no direct homolog of SoxS in *P. putida* (Park et al., 2006). To test this system in *P. putida*, the three CRISPRa components need to be expressed at levels sufficient to activate the target gene without dCas9 expression being so high that cellular functions are inhibited (Depardieu and Bikard, 2020; Zhang and Voigt, 2018). We first moved components from two *E. coli* plasmid constructs, a CRISPRa system plasmid and a reporter plasmid, directly into two *P. putida* expression plasmids, pBBR1 and pRK2 (each present at 25-30 copies/cell according to (Cook et al., 2018)) (Figure 1B). We observed reporter gene expression that depended on the presence of an on-target scRNA (Figure 1C). Reporter gene expression in the presence of an off-target scRNA was indistinguishable from a strain without scRNA/sgRNA present (Figure S1).

3.1.2 Growth-defect mitigation elevates CRISPRa efficiency

P. putida strains with the initial implementation of the CRISPRa system grew poorly on both agar and liquid media (Figure S2B). To mitigate the growth defect, we tested multiple different plasmid and genome-integrated delivery methods for the CRISPRa components. We first reduced the expression levels of dCas9 and MCP-SoxS by moving these genes from the pBBR1 plasmid to the pRK2 plasmid, which expresses transgenes at a lower level in *P. putida* (Damalas et al., 2020) (Figure S1). This change partially mitigated the growth defect and improved the CRISPRa reporter gene expression (Figure S2). We reduced the expression levels of dCas9 and MCP-SoxS further by integrating

the dCas9/MCP-SoxS cassette into the *P. putida* KT2440 genome (generating strain PPC01). We then delivered the scRNA and reporter gene cassettes on plasmids with different combinations of two origins of replication (pBBR1 and pRK2) and two antibiotic markers (GmR and KmR) to test whether variations in the plasmid backbones impart different metabolic burdens (Mi et al., 2016). We observed the highest level of activation (~5-fold) with the scRNA and reporter both expressed from a single pBBR1-GmR backbone, while the plasmid with either pRK2 origin or KmR marker yielded weaker activation (~2-fold) (Figure 1C and Figure S2A). The presence of the second plasmid reduced both fold-activation by CRISPRa and basal expression of mRFP significantly (Figure S3). In general, both CRISPRa fold-activation and the corresponding basal mRFP expression (off-target control) increased in strains that grew faster (Figure S2 & S3), suggesting that there are different metabolic burdens associated with different delivery methods and plasmid expression systems. Taken together, these results suggest that optimizing expression levels will be important for implementing CRISPRa in new bacterial species. To improve *P. putida* CRISPRa beyond the five-fold activation obtained in Figure 1C, we proceeded with the genomically integrated dCas9/MCP-SoxS strain (PPC01) for further optimization. While there is no specific target value for fold-activation, we aim for the largest dynamic range possible to provide the highest possible tunable range in future applications.

3.2 Characterization of promoter elements for optimal CRISPRa efficiency in *P. putida*

To improve the fold-activation of CRISPRa in *P. putida*, we investigated the criteria for effective CRISPRa that we previously observed in *E. coli* (Fontana et al., 2020a).

Specifically, factors known to affect CRISPRa efficiency in *E. coli* include i) the distance of target sequence to transcription start-site (TSS), ii) the sequence composition of the 20 bp scRNA targeting sequence, iii) the basal minimal promoter strength, and iv) the 5'-proximal sequence composition between target sequence and minimal promoter (Figure 2A).

3.2.1 Distance to transcription start-site (TSS)

In *E. coli*, the most effective CRISPRa target sites are in the region of -60 to -100 bp before the TSS, with sharp peaks of activity every 10 bases, separated by regions of inactivity (Fontana et al., 2020a). We constructed an integrated reporter that can be targeted at multiple sites (J1-sfGFP, previously characterized in *E. coli* (Fontana et al., 2020a) and delivered plasmids with scRNAs targeting different sites as shown in Figure 2B. With target sites spaced 10 bp apart, the optimal sites in *P. putida* were located in the -60 to -100 bp range before the TSS, similar to that in *E. coli*. When we tested sites at single base resolution between -81 to -93 bp, we observed peaks of activity ~10-11 bases apart, similar to what we observed in *E. coli* (Figure 2C). The efficiency of CRISPRa diminished after a 4-5 bp shift and was recovered at 10-12 bp. This finding suggests that CRISPRa has a periodic dependence on distance from the TSS, and similar effects have been observed in multiple bacterial species (Fontana et al., 2020a; Ho et al., 2020).

3.2.2 scRNA target sequences

Next, we examined the 20 bp target sequence that is recognized by the scRNA. The experiments described above were performed with the J1 promoter, which contains an array of 20 base target sites. We proceeded to test an alternative promoter, termed J3, that has a different set of 20 base target sites (see Methods and Supplemental

Information for complete sequence details). We tested multiple target sites in the J3 promoter and found that the J306 site, located 81 bases upstream of the TSS, yielded the highest fold-activation (Figure S4). Compared to the J1 promoter, where we observed 4-fold activation (J106 target site), the fold-activation with the J3 promoter increased to 34-fold (J306 target site) (Figure 2D). For both J1 and J3, the CRISPRa-induced expression levels were similar. The large difference in fold-activation results from an unexpected difference in basal expression levels. The basal expression of J3-mRFP is 11-fold lower than that of J1-mRFP, which leads to much higher fold-activation. This difference in basal expression was not observed in *E. coli*, where J1 and J3 reporters produced 27-fold and 36-fold activation, respectively (Fontana et al., 2020a).

To test whether the different basal expression levels were due to differences in the 20 base target sites or to other features of the promoters, we constructed hybrid promoters where the 20 base J106 target site in J1 was replaced by J306 (J1(306)) and vice versa (J3(106)). We observed low basal expression only with the hybrid J3(106) promoter (Figure 2D), suggesting that other sequence features of the J3 promoter besides the 20 base target site are responsible for the low basal expression of the J3 promoter. These sequence features could be upstream of the target sequence or between the target sequence and the minimal BBa_J23117 promoter. We therefore turned our focus to the J3 upstream sequence as a basis for further optimization of CRISPRa, as it yielded the best dynamic range from the promoter sequences tested.

3.2.3 Minimal promoter strength

The promoters that we tested in this work consist of a 35 base minimal promoter that binds the sigma subunit of RNA polymerase and an upstream 170 base sequence

region with scRNA target sites. The 35 bp minimal promoter sequence is also a key factor that governs the dynamic range of CRISPRa. In *E. coli*, we found that minimal promoter strength and the sigma factor regulating the promoter have large effects on CRISPRa (Fontana et al., 2020a). However, the alternative sigma factor regulons in *P. putida* are less characterized compared to those in *E. coli*. We therefore decided to focus on the sigma-70 regulon, the house-keeping sigma factor, that covers the majority of *E. coli* and *P. putida* endogenous promoters (Fujita et al., 1995). To test the effects of promoter strength, we introduced 11 minimal 35 base promoters from the Anderson promoter collection (BBa_J231XX, http://parts.igem.org) into the J3-mRFP reporter (Figure 3A). The variations in promoter strength arise from point mutations in the -10 and -35 sites that tune transcriptional activity; no significant changes in the transcription start sites (TSS) were detected when these promoters were experimentally characterized (Kosuri et al., 2013) (see Supplemental Information for annotated sequences).

CRISPRa-mediated expression from the Anderson promoter series followed trends similar to that previously observed in *E. coli* (Fontana et al., 2020a). When the promoter strengths are extremely weak (BBa_J23109 and BBa_J23113), the CRISPRa fold-activation dropped significantly to 3.1-fold and 1.4-fold compared to 27-fold with the moderately weak BBa_J23117 minimal promoter. As promoter strength increases from BBa_J23117 to the strong BBa_J23110 promoter, CRISPRa fold-activation decreases because basal expression increases ~10-fold, while the maximal CRISPRa output varies by <4-fold (Figure 3A). CRISPRa with the strongest promoter tested (BBa_J23111), could not be measured because no colonies were obtained when the CRISPR machinery was delivered to *P. putida* with this reporter, possibly due to the metabolic burden of

expressing high levels of mRFP and the CRISPR system at the same time. The minimal BBa_J23117 promoter yields the highest fold-activation in both *E. coli* and *P. putida* (36-fold and 27-fold, respectively) presumably because basal expression is weak enough that significant activation is possible, but not so weak that the promoter is difficult to activate. Thus, we used reporters with the BBa_J23117 minimal promoter for further characterization and application. We note that if a higher absolute expression level is preferred, the stronger BBa_J23106 promoter yielded the highest absolute expression level (2.4-fold higher than CRISPRa-mediated activation of BBa_J23117), although the fold-activation was smaller (Figure 3A).

3.2.4 5'-Proximal sequences

The last factor we tested is the intervening sequence between the 20 base target site and the 35 base minimal promoter, termed the 5'-proximal sequence. This sequence is 26 bp long when using an optimal target site located at -81 bp from the TSS. We constructed a pooled library of reporter gene plasmids with variable 26 base 5' proximal sequences using a randomized oligo pool (see Supplemental Methods). Each reporter retains the same 20 base J306 scRNA target site and the BBa_J23117 minimal promoter. We transformed this library into a *P. putida* reporter strain and functionally characterized a large number of single colonies without sequencing each colony. The random 5'-proximal sequences led to a broad range of mRFP levels from CRISPRa (Figure 3B), similar to what we observed in *E. coli* (Fontana et al., 2020a). Random 5' proximal sequences also affect basal expression levels in the absence of CRISPRa, although these effects are relatively small (Figure S5A). To determine if 5'-proximal sequence preferences are correlated between *E. coli* and *P. putida*, we also tested several known

sequences previously characterized in *E. coli*. We observed that high-efficiency 26 bp sequences from *E. coli* yield high CRISPRa efficiency in *P. putida* while a weak sequence from *E. coli* remains weak in *P. putida* (Figure 3C). Across the set of sequences we analyzed, one of these (5'-PS5) exhibited a higher fold activation (32-fold) compared to the J3-BBa_J23117 promoter (27-fold). The basal expression in 5'-PS5 is 15% lower than J3-BBa_J23117, and both sequences gave similar activated levels. We also tested whether the 26 bp 5' proximal sequence from the J1 promoter was responsible for the high basal activity of the J1 promoter (Figure 2D). When the 26 bp 5' proximal sequence from J1 was inserted into the J3 promoter, we observed relatively low basal expression (5'-PS2 in Figure 3C), similar to the J3 promoter. This result suggests that the 5' proximal sequence of J1 is not the cause of the high basal activity of the complete J1 promoter, and that sequence features upstream of the 5' proximal sequence and the 20 bp target site could be responsible.

The variation in CRISPRa outputs with different promoter features suggests that a set of distinct and orthogonal heterologous promoters could be developed for tunable control of gene expression. Promoters with orthogonal 20 base target sequences, together with different 5' proximal sequences, minimal promoters, and target site positions could be used to access a broad range of CRISPRa-mediated gene expression levels. Further, systematically varying the 5'-proximal sequence could allow us to identify promoters with lower basal expression and higher dynamic range of activation, similar to the case of the 5'-PS5 sequence mentioned above. We expect to be able to construct combinatorial libraries of multi-gene programs to explore how independently tuning gene expression levels in metabolic pathways affects product titers.

3.2.5 Correlation of CRISPRa efficiency between organisms

To compare CRISPRa in *P. putida* to that in *E. coli*, we constructed a correlation plot of mRFP expression from CRISPRa strains with different promoter sequence variations (Figure 3D). This plot indicates that the expression level induced by CRISPRa in *E. coli* correlates well with CRISPRa in *P. putida* (R^2 = 0.80). The fold-activation is also correlated (R^2 = 0.69 Figure S5B), although the fold-activation of *P. putida* CRISPRa tends to be lower than that of *E. coli*. The discrepancies across organisms might arise from variations in genetic context, transcription machinery, or cellular compositions between bacterial species. Despite these modest discrepancies, CRISPRa behaves largely similarly in *E. coli* and *P. putida*, suggesting that optimized CRISPRa circuits will be portable between species and that further modifications and improvements to CRISPRa systems should be readily transferable. While we do not expect these trends to be generalizable across all bacterial species, the metrics that we describe here can be systematically evaluated in alternative bacterial hosts to assess whether design principles and optimized CRISPRa circuits can be easily ported to new hosts.

3.3 Using *P. putida* CRISPRa for sophisticated transcriptional control strategies

With an optimized CRISPRa system in *P. putida*, we explored several strategies to enable more sophisticated control over gene expression programs. We constructed multi-gene CRISPRa/CRISPRi programs, demonstrated endogenous gene activation, and developed an inducible CRISPRa system for tunable, dynamically-regulated expression. These strategies will enable the construction of multi-gene programs to rewire metabolic networks for optimal biosynthesis in *P. putida*.

3.3.1 Multi-gene regulation by CRISPRa and CRISPRi

With optimized expression levels and a delivery strategy for the CRISPRa system in *P. putida* in place, we tested whether CRISPRa and CRISPRi can be used together to activate and repress multiple genes. This strategy has been previously successful in *E. coli* (Dong et al., 2018). We constructed a dual-reporter plasmid with weakly expressed mRFP (J3-BBa_J23117-mRFP) and highly expressed sfGFP (J3(106)-BBa_J23111-sfGFP). We inserted a dual scRNA/sgRNA cassette in this plasmid with a J306 scRNA for mRFP activation and an sgRNA that targets within the sfGFP open reading frame (ORF) for repression. We delivered this plasmid to a *P. putida* strain with integrated dCas9/MCP-SoxS and observed simultaneous activation of mRFP (6.6-fold) and repression of sfGFP (13-fold) (Figure 4). The magnitude of CRISPRa fold-activation in simultaneous CRISPRa/i was weaker than the 15-fold activation that was observed if just a single scRNA was delivered to activate the mRFP reporter, possibly due to competition between multiple scRNA/sgRNA cassettes for a limited pool of dCas9.

To determine if CRISPRa can be used to activate multiple genes simultaneously, we constructed a dual-reporter plasmid with weakly expressed mRFP (J3-BBa_J23117-mRFP) and weakly expressed sfGFP (J3(106)-BBa_J23117-sfGFP). We inserted a dual scRNA cassette into this plasmid with scRNAs that target mRFP and sfGFP for activation and delivered it to a *P. putida* strain with integrated dCas9/MCP-SoxS. We observed simultaneous activation of mRFP (19-fold) and sfGFP (69-fold) (Figure S6). As seen with simultaneous CRISPRa/CRISPRi, the CRISPRa effects with dual activation were weaker than those observed if each reporter was targeted individually (41-fold activation for mRFP and 105-fold activation for sfGFP), consistent with the idea that competition for dCas9 among multiple species of sgRNA/scRNA may be an issue for multi-gene

programs (Huang et al., 2020). We also observed simultaneous CRISPRa at multiple genes using the weak mRFP/strong sfGFP reporter described above; the strong sfGFP could be activated a further 2-3-fold when activated by an upstream scRNA (Figure 4).

We also demonstrated simultaneous CRISPRa/CRISPRi and dual CRISPRa on multi-gene reporters with integrated genomic reporters. The general trends were similar to what we observed with plasmid-based reporters (Figure S7-S8), but the magnitudes of the effects were smaller, likely due to the lower copy number of the reporter gene. The ability to activate genomically-integrated heterologous reporters suggests that CRISPRa may be effective at endogenous genomic targets in *P. putida*.

3.3.2 CRISPRa on *P. putida* endogenous promoters

To determine if CRISPRa can activate endogenous promoters, we identified a set of endogenous genes with appropriate upstream scRNA target sites. We analyzed thousands of reported TSSs for *P. putida* (D'Arrigo et al., 2016) and selected ten promoters with potentially activatable target sites located at the proper distance from the TSS. Specifically, we identified NGG protospacer adjacent motifs (PAMs), which are required for recognition of *Sp*-dCas9/guide-RNA complex (Qi et al., 2013), at distances corresponding to the J105-J112 target sites (Figure 2B) with ± 2 bp flexibility (Figure 2C). For each endogenous promoter, we built a reporter cassette with the promoter, flanking sequences, and an mRFP reporter gene following a strategy previously described for an *E. coli* endogenous promoter library (Zaslaver et al., 2006). We introduced on-target or off-target scRNAs into the reporter plasmid and delivered it to a *P. putida* strain with integrated dCas9/MCP-SoxS. We observed >1.5-fold activation at 4 of the 10 promoters tested, with the highest fold-activation (2.8-fold) from scRNA G2 targeting *katG*

(PP_3668) promoter (Figure 5A & S9). The magnitudes of fold-activation from endogenous promoters are significantly lower than those under control of synthetic heterologous promoters (up to 40-fold and 100-fold for mRFP and sfGFP, respectively) (Table S5). Although higher fold-activation values may be desirable for future applications, we note that relatively modest effects can still be physiologically significant. For example, external stresses can produce a wide range of expression changes in stress-responsive genes in *P. putida*. While some changes are quite large, others are in the 2-fold to 5-fold range (Bojanovič et al., 2017; Molina-Santiago et al., 2017). We suggest that tools to perturb endogenous gene expression in this range may still be effective for modulating bacterial physiology and redirecting metabolic flux. Further, we note that the ability to combine endogenous gene activation with heterologous gene activation and CRISPRi repression enables access to a vastly expanded space of gene expression programs compared to other synthetic gene regulatory methods.

This success rate and the magnitude of gene activation at endogenous targets in *P. putida* was similar to that observed previously in *E. coli* (Fontana et al., 2020a). To predictably activate any endogenous gene, we expect that it will be necessary to further elucidate the rules for effective CRISPRa. Accurate annotations of TSSs and PAM-flexible dCas9 variants to precisely target the optimal distance upstream of the endogenous gene may improve activation (Fontana et al., 2020a). Alternative bacterial activation domains are also available with different properties (Ho et al., 2020; Liu et al., 2019), and it may be possible to combine multiple activators as has been previously reported in eukaryotic systems (Chavez et al., 2015; Konermann et al., 2015).

3.3.3 Tunability of CRISPRa and CRISPRi with inducible promoter

To tune expression levels with CRISPRa and CRISPRi, we placed the CRISPR system components under the control of a small-molecule inducible promoter. We expressed dCas9 and/or MCP-SoxS using XylS-Pm, an inducible promoter system from the *P. putida* mt-2 toluene degradation pathway (Wirth et al., 2019). XylS-Pm provides a higher dynamic range compared to the widely-used LacI-Ptrc system (Figure S10A & S10B). We constructed strains with inducible dCas9, inducible MCP-SoxS, or double-inducible dCas9/MCP-SoxS (PPC08-PPC10). Using a weak J3-BBa_J23117-mRFP reporter, we induced with *m*-toluic acid (0-5 mM) and observed tunable gene activation as a function of inducer concentration in all three inducible strains (Figure S11). This approach will enable tunable and dynamically-regulatable expression control for further applications in metabolic engineering.

Using a strong reporter (J3-BBa_J23110-mRFP) that can be either activated or repressed, we showed that the extent of CRISPRa or CRISPRi could be tuned with different inducer levels. We delivered this reporter with either an activating scRNA or a repressing sgRNA to the inducible dCas9 strain (PPC08) and observed 3-fold activation with CRISPRa or 7-fold repression with CRISPRi at 1 mM *m*-toluic acid (Figure 5B). This result suggests another potential strategy for improving the dynamic range of activation from heterologous genes. By targeting CRISPRi and CRISPRa to the same locus, we may be able to obtain lower basal expression and higher induced expression. Such a strategy would require expression of only the sgRNA for repression in the off state and only the scRNA for activation in the on state, which could potentially be achieved with orthogonal induction systems or with multi-layer CRISPR circuits.

3.4 Metabolic Engineering with CRISPRa

3.4.1 Biopterin pathway activation

By characterizing the promoter features necessary for effective CRISPRa in *P. putida*, we were able to apply CRISPRa for metabolic pathway engineering. We used the J3-BBa_J23117 promoter described in the previous section to place genes of interest under the control of a CRISPRa system. In a strain with integrated dCas9/MCP-SoxS (PPC01), transcriptional units controlled by J3-BBa_J23117 can be activated by the cognate J306 scRNA (Figure 6A). Using this approach, we demonstrated that CRISPRa can be used to switch on two different heterologous biosynthesis pathways, one for tetrahydrobiopterin (BH4) production with multiple transcriptional units activated by the same scRNA and one for mevalonate production as a multi-gene transcriptional unit under a single promoter.

BH4 is an important cofactor in aromatic amino acid biosynthesis that can be produced from a three-enzyme pathway (Figure 6B). BH4 has been previously produced in yeast using the *E. coli* GTPCH enzyme and the *M. alpina* PTPS and SR enzymes (Ehrenworth et al., 2015; Trenchard et al., 2015). We used the *gtpch* gene from *E. coli* MG1655 and *ptps/sr* genes from *M. alpina* that were codon-optimized for expression in *E. coli*. Each gene was placed under control of the J3-BBa_J23117 promoter in a *P. putida* compatible plasmid (Figure 6C). Because BH4 can be readily oxidized by atmospheric oxygen into dihydrobiopterin (BH2) and then biopterin in yeast (Ehrenworth et al., 2015), we initially screened for pathway output by absorbance at 340 nm, which reports on BH2 and biopterin. We observed a significant increase in OD_{340} when the pathway was switched on with the cognate scRNA (Figure 12A). Subsequent analysis by HPLC-MS to

identify specific parental ions confirmed that BH2 is the major product (Figure 6D & FIgure S12B and Figure S13). BH2 was also detected in the off-target scRNA sample (Figure 6D), likely due to basal expression of the biopterin pathway enzymes. When the last gene in the pathway (*sr*) was omitted, no biopterin derivatives were detected by HPLC-MS, confirming that the full pathway is necessary for heterologous biopterin production (Figure S12A-B). Thus, biopterin pathway activation by CRISPRa was able to significantly increase heterologous production. We note that in some metabolic engineering applications, basal production may be problematic and pathway promoters may need to be modified to minimize leaky expression of the heterologous pathway genes. In future experiments, the CRISPRa system can be used to test whether product titers can be further optimized by independently activating biopterin pathway genes with orthogonal scRNAs and tuning their expression to different levels.

The major product of the biopterin pathway in *P. putida* is BH2, in contrast to *S. cerevisiae* where fully oxidized biopterin is the major product (Ehrenworth et al., 2015). The finding that BH2 is the major product suggests that the reducing potential of *P. putida* prevented BH2 from further oxidation. In *E. coli*, BH2 is the major product but the ratio of BH2:biopterin is significantly lower than in *P. putida* (Figure S12C). Even though the fully reduced BH4, which is the desired product, was not observed in our system, the low biopterin level in *P. putida* suggests that its reducing power is advantageous for biosynthesis of oxidation-sensitive compounds.

3.4.2 Mevalonate pathway activation

We next tested if CRISPRa could be used to produce mevalonic acid, a precursor to terpenoid natural products including fine chemicals, biofuels, and therapeutics

(Anthony et al., 2009; Jervis et al., 2019; Peralta-Yahya et al., 2011) Mevalonate has previously been produced in *P. putida* using two genes, *mvaE* and *mvaS*, expressed in a single operon under the control of LacI-Ptrc (Figure 7A) (Kim et al., 2019). We placed the *mvaES* operon under the control of J3-BBa_J23117 synthetic promoter (Figure 7B). The constitutively-active CRISPRa-regulated mevalonate production strain was cultured side-by-side with the LacI-Ptrc regulated *mvaES* strain as a control. We observed that the CRISPRa strain yielded 402 ± 21 mg/L mevalonate, which is similar to the highest mevalonate titer of 459 mg/L obtained with LacI-Ptrc after IPTG induction (Figure 7C). The CRISPRa-regulated *mvaES* operon enables tight control of mevalonate production, with basal mevalonate production from the off-target CRISPRa control strain indistinguishable from the empty plasmid control (Figure 7C). In contrast, the uninduced LacI-Ptrc strain produced mevalonate levels up to 214 ± 57 mg/L and yielded highly variable mevalonate levels in every IPTG concentration (ranging from 66 to 459 mg/L). We also observed highly variable IPTG-induced mRFP expression, suggesting that expression from the LacI-Ptrc promoter may be unstable in *P. putida* (Figure S10C). Taken together, our results demonstrate that we can effectively activate multi-gene biosynthesis pathways using a single operon (>40-fold increase in mevalonate biosynthesis, Figure 7) or with each enzyme produced from a separate transcriptional unit with its own CRISPRa-responsive promoter (5-fold increase in BH2 production, Figure 6).

To determine if an inducible CRISPRa system could effectively regulate mevalonate production, we tested a strain with toluic acid-inducible CRISPRa machinery (dCas9, MCP-SoxS, or both). In the absence of inducer we observed 84 ± 11 mg/L mevalonate from the inducible dCas9 strain. With inducer added to this strain (0.01 to 1.0

mM), we observed a similar mevalonate level to that with constitutively expressed dCas9 (345 to 397 mg/L and 402 ± 21 mg/L, respectively) (Figure 7). The inducible MCP-SoxS strain appeared to be leaky in the absence of inducer (112 ± 2 mg/L) and gave a lower mevalonate titer when induced (254 ± 9 mg/L). The double-inducible strain, with both dCas9 and MCP-SoxS controlled by XylS-Pm, had no significant leaky production in the absence of inducer but yielded the lowest mevalonate titer (199 ± 20 mg/L). The off-target scRNA yielded a level of mevalonate indistinguishable from the empty plasmid controls (less than 10 mg/L in Figure S14). The inducible CRISPRa system provides an additional layer of control that can be switched on at different growth phases and could be coupled with an inducible CRISPRi system for multi-gene programs with both activation and repression. Compared to the LacI-Ptrc regulated *mvaES* strain, which showed significant leaky production, the inducible dCas9 CRISPRa-regulated *mvaES* strain had minimal leakage and could provide advantages in situations where leaky metabolic gene expression could be toxic or burdensome to the cell.

4. Conclusions

In this work, we have ported a CRISPRa system from *E. coli* to *P. putida*. We optimized the expression methods of dCas9, MCP-SoxS, and scRNA in *P. putida* and demonstrated that the criteria for effective CRISPRa target sites in *P. putida* are similar to that in *E. coli*. We anticipate that a similar process of optimizing expression systems will enable effective CRISPRa-regulated gene expression in a wide range of bacterial species to enable complex CRISPR-based transcriptional programming in other industrially-relevant microbes.

As reported previously in *E. coli* and in many eukaryotic systems, CRISPRa and CRISPRi can be used to target multiple genes simultaneously for activation or repression. Further, the CRISPRa system can be induced with small molecules, which will enable dynamic control of heterologous pathway activation. In *P. putida*, we applied CRISPRa to metabolic pathway engineering for tetrahydrobiopterin and mevalonate biosynthesis, providing a proof-of-concept that CRISPRa-mediated gene regulation can be used to activate heterologous biosynthetic pathways.

In future work, we expect that an inducible CRISPR-Cas transcriptional control system will enable the rapid exploration of large combinatorial spaces of gene expression levels. A key advantage of CRISPR-Cas-mediated control is that, in principle, each gene of interest can be targeted by an orthogonal guide RNA and its expression level can be independently tuned. We can target endogenous genes for both activation and repression to redirect metabolic flux towards the desired pathway precursors, and we can tunably activate heterologous pathways to optimal expression levels to maximize the production of desired biosynthetic products. By learning the design principles for how to rewire metabolic networks, we expect to enable more efficient biosynthetic production pathways for valuable chemical products.

Author contributions

C.K., C.D., J.F., P.P.-Y., J.M.C., and J.G.Z. designed experiments and analyzed data. C.K., C.D., J.F., and W.S. performed experiments. C.K., C.D., J.F., J.M.C., and J.G.Z. wrote the manuscript with input from all of the authors.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at [*link here*].

Table 1: Bacterial strains and plasmids used in this study

Strains/Plasmids	Features	Sources
Strains		
P. putida KT2440	Wildtype strain	Harwood lab
PPC01	KT2440 with integrated *Sp*.pCas9-dCas9 and BBa_J23107_MCP-SoxS made from pPPC001	This study
PPC02	KT2440 with integrated J1-BBa_J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, made from pPPC002	This study
PPC03.N	KT2440 with integrated J1(+N)-BBa_J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, made from pPPC003.N	This study
PPC04	KT2440 with integrated BBa_J23111-mRFP, BBa_J1-J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, made from pPPC004	This study
PPC05	PPC01 with integrated J3-BBa_J23117-mRFP and BBa_J23111-sfGFP, made from pPPC024 and pPPC025	This study
PPC06	PPC01 with integrated J3-BBa_J23117-mRFP and J3(106)-BBa_J23117-sfGFP, made from pPPC024 and pPPC026	This study
PPC07	PPC01 with integrated J3-BBa_J23117-mRFP and J3(106)-BBa_J23111-sfGFP, made from pPPC024 and pPPC027	This study
PPC08	KT2440 with integrated XylS-Pm-dCas9, BBa_J23107-MCP-SoxS made from pPPC005	This study
PPC09	KT2440 with integrated *Sp*.pCas9-dCas9, XylS-Pm-MCP-SoxS made from pPPC006	This study
PPC10	KT2440 with integrated XylS-Pm-dCas9, XylS-Pm-MCP-SoxS made from pPPC007	This study
Plasmids		
pUC18T-miniTn7T-Gm	Plasmid backbone for integration into P. putida genome, GmR and AmpR	(Choi and Schweizer, 2006)
pTNS1	Tn7 transposase (tnsABCD) expressing plasmid, R6K origin, AmpR	(Choi and Schweizer, 2006)
pRK2013	Helper plasmid for triparental mating, KmR	(Choi and Schweizer, 2006)
pFLP2	*S. cerevisiae* Flippase expression plasmid for marker deletion, AmpR	(Choi and Schweizer, 2006)
pS448-CsR	CRISPR/Cas9 counterselection in Gram-negative bacteria with XylS/Pm promoter, SmR	(Wirth et al., 2019)
pBBR1-MCS5 (pBBR1-GmR)	Broad-host-range plasmid backbone with multiple cloning site, GmR	(Kovach et al., 1995)
pBBR1-MCS2 (pBBR1-KmR)	Broad-host-range plasmid backbone with multiple cloning site, KmR	(Kovach et al., 1995)

pRK2-AraE	Broad-host-range plasmid backbone with AraE expressing cassette, GmR	(Cook et al., 2018)
pRK2-GmR	Broad-host-range plasmid backbone with multiple cloning site, GmR	This study
pRK2-KmR	Broad-host-range plasmid backbone with multiple cloning site, KmR	This study
pGNW2	Integrative vector carrying P14g-msfGFP, KmR	(Wirth et al., 2019)
pS448-CsR	CRISPR/Cas9 counterselection in Gram-negative bacteria with XylS/Pm promoter, SmR	(Wirth et al., 2019)
pSEVA1213S	pRK2, PEM7-I-SceI; AmpR	(Wirth et al., 2019)
pGNW2-pp1	pGNW2 derivative with integration site at prophage1, KmR	This study
pGNW2-pp2	pGNW2 derivative with integration site at prophage2, KmR	This study
pCK241	pBBR1 bearing LacI-Ptrc-mRFP, GmR	This study
pCK243	pBBR1 bearing XylS-Pm-mRFP, GmR	This study
pCK255	pBBR1 bearing I-SceI and sacB genes, GmR	This study
pMVA2RBS035	p15A, LacI-Ptrc *mvaE*, *mvaS*, *mvaK1*, *mvaK2*, and *mvaD* from *E. faecalis*, and *idi* gene from *E. coli*, KmR	(Jervis et al., 2019)
pCD442	p15A, *Sp*.pCas9-dCas9, BBa_J23107-MCP-SoxS, CmR	(Fontana et al., 2020a)
pPPC001	pUC18T-miniTn7T, *Sp*.pCas9-dCas9, BBa_J23107-MCP-SoxS, AmpR/GmR	This study
pPPC002	pUC18T-miniTn7T, J1-J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, AmpR/GmR	This study
pPPC003.N	pUC18T-miniTn7T, J1(+N)-J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, AmpR/GmR	This study
pPPC004	pUC18T-miniTn7T, J23111-mRFP, J1-J23117-sfGFP, *Sp*.pCas9-dCas9, and BBa_J23107_MCP-SoxS, AmpR/GmR	This study
pPPC005	pUC18T-miniTn7T, XylS-Pm-dCas9, BBa_J23107-MCP-SoxS, AmpR/GmR	This study
pPPC006	pUC18T-miniTn7T, *Sp*.pCas9-dCas9, XylS-Pm-MCP-SoxS, AmpR/GmR	This study
pPPC007	pUC18T-miniTn7T, XylS-Pm-dCas9, XylS-Pm-MCP-SoxS, AmpR/GmR	This study
pPPC008	pBBR1, sgRNA or scRNA, GmR	This study
pPPC009	pBBR1, sgRNA or scRNA, KmR	This study
pPPC010	pBBR1, *Sp*.pCas9-dCas9, Bba_J23107-MCP-SoxS, scRNA, KmR	This study
pPPC011	pRK2, *Sp*.pCas9-dCas9, Bba_J23107-MCP-SoxS, KmR	This study
pPPC012	pBBR1, J1-J23117-mRFP, GmR	This study
pPPC013	pBBR1, J1-J23117-mRFP, KmR	This study
pPPC014	pRK2, J1-J23117-mRFP, GmR	This study
pPPC015	pRK2, J1-J23117-mRFP, KmR	This study

pPPC016	pBBR1, J1-J23117-mRFP, scRNA, GmR	This study
pPPC016(306)	pBBR1, J1-J23117-mRFP where J106 was replaced with J306, scRNA, GmR	This study
pPPC017	pBBR1, J1-J23117-mRFP, scRNA, KmR	This study
pPPC018	pRK2, J1-J23117-mRFP, scRNA, GmR	This study
pPPC019	pRK2, J1-J23117-mRFP, scRNA, KmR	This study
pPPC020	pBBR1, J3-J23117-mRFP, scRNA, GmR	This study
pPPC020(106)	pBBR1, J3-J23117-mRFP where J106 was replaced with J306, scRNA, GmR	This study
pPPC021.J231XX	pBBR1, J3-J231XX-mRFP, scRNA, GmR	This study
pPPC022.5PS	pBBR1, J3-Random-5PS-J23117-mRFP, scRNA-J306, GmR	This study
pPPC023.5PSN	pBBR1, J3-Ec-5PS-J23117-mRFP, scRNA, GmR	This study
pPPC024	pBBR1, J3(106)-J23111-sfGFP, J3-J23117-mRFP, scRNA, GmR	This study
pPPC025	pBBR1, J3(106)-J23117-sfGFP, J3-J23117-mRFP, scRNA, GmR	This study
pPPC026.XN	pBBR1, PP_NNNN-mRFP, scRNA, GmR where PP_NNNN is an endogenous promoter	This study
pPPC027	pBBR1, J3-J23117-GTPCH, J3-J23117-PTPS, J3-J23117-SR, scRNA, GmR	This study
pPPC028	pBBR1, J3-J23117-GTPCH, J3-J23117-PTPS, scRNA, GmR	This study
pPPC029	pBBR1, LacI-Ptrc-mvaES, GmR	This study
pPPC030	pBBR1, J3-J23117-*mvaES*, scRNA, GmR	This study
pPPC031	pGNW2 derivative with integration site at prophage1 for integration of J3-J23117-mRFP cassette, KmR	This study
pPPC032	pGNW2 derivative with integration site at prophage2 for integration of J23111-sfGFP, KmR	This study
pPPC033	pGNW2 derivative with integration site at prophage2 for integration of J3(106)-J23117-sfGFP, KmR	This study
pPPC034	pGNW2 derivative with integration site at prophage2 for integration of J3(106)-J23111-sfGFP, KmR	This study

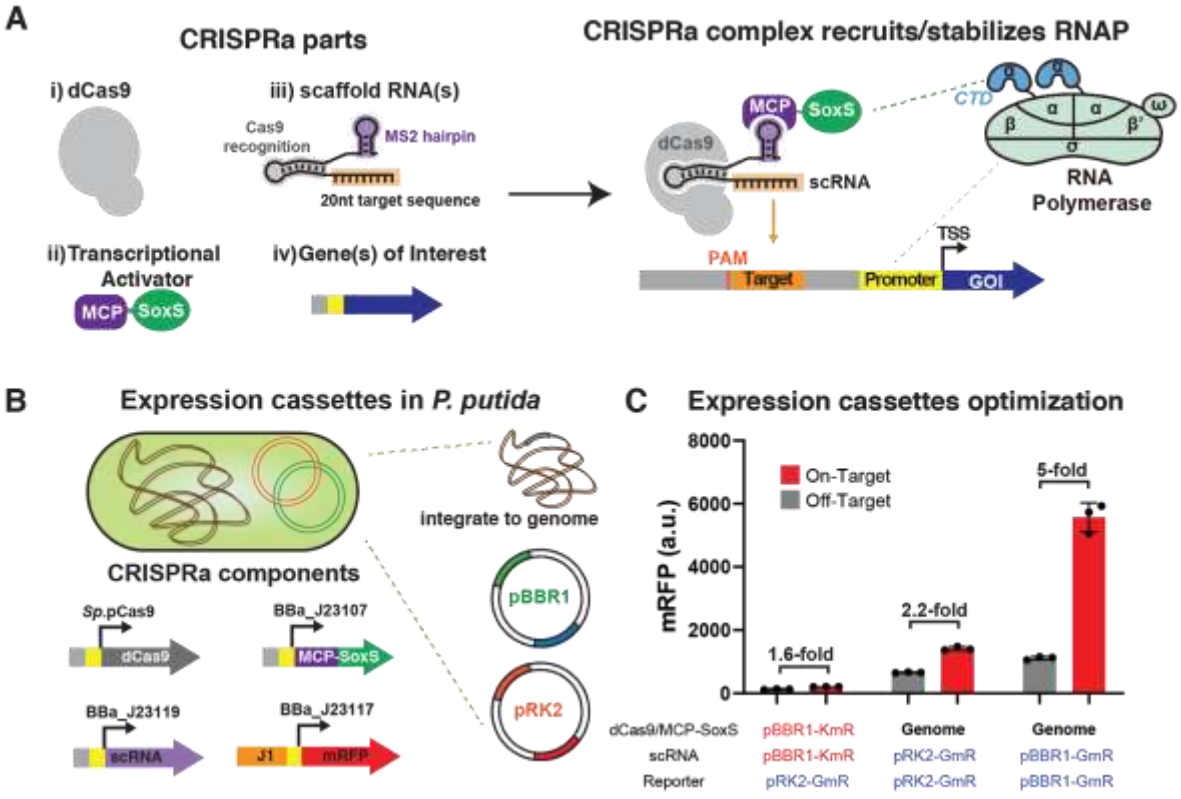
A
CRISPRa parts
i) dCas9
iii) scaffold RNA(s)
Cas9 recognition
MS2 hairpin
20nt target sequence
ii) Transcriptional Activator
MCP
SoxS
iv) Gene(s) of Interest
CRISPRa complex recruits/stabilizes RNAP
dCas9
scRNA
CTD
RNA Polymerase
PAM
Target
Promoter
TSS
GOI
B
Expression cassettes in P. putida
integrate to genome
CRISPRa components
Sp.pCas9
dCas9
BBa_J23107
MCP-SoxS
BBa_J23119
scRNA
BBa_J23117
J1
mRFP
pBBR1
pRK2
C
Expression cassettes optimization
mRFP (a.u.)
8000
6000
4000
2000
0
On-Target
Off-Target
1.6-fold
2.2-fold
5-fold
dCas9/MCP-SoxS
scRNA
Reporter
pBBR1-KmR
pBBR1-KmR
pRK2-GmR
Genome
pRK2-GmR
pRK2-GmR
Genome
pBBR1-GmR
pBBR1-GmR

Figure 1. Configuring CRISPRa in *P. putida*. (A) CRISPRa components (i-iii) are necessary to activate the gene of interest (iv). The CRISPRa ternary complex recruits and stabilizes RNA polymerase at the promoter region. (B) Available gene expression tools in *P. putida* include pBBR1 plasmid, pRK2 plasmid, and genome integration. We used two antibiotic selection markers, Gentamicin (GmR) and/or Kanamycin (KmR). (C) Testing CRISPRa in different expression systems. The CRISPRa fold-activation is highest when dCas9/MCP-SoxS were integrated into the genome and the scRNA/reporter genes were expressed on pBBR1-GmR plasmid. The J109 scRNA was used for activation and hAAVS1 was used as an off-target scRNA. Values in panel C represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

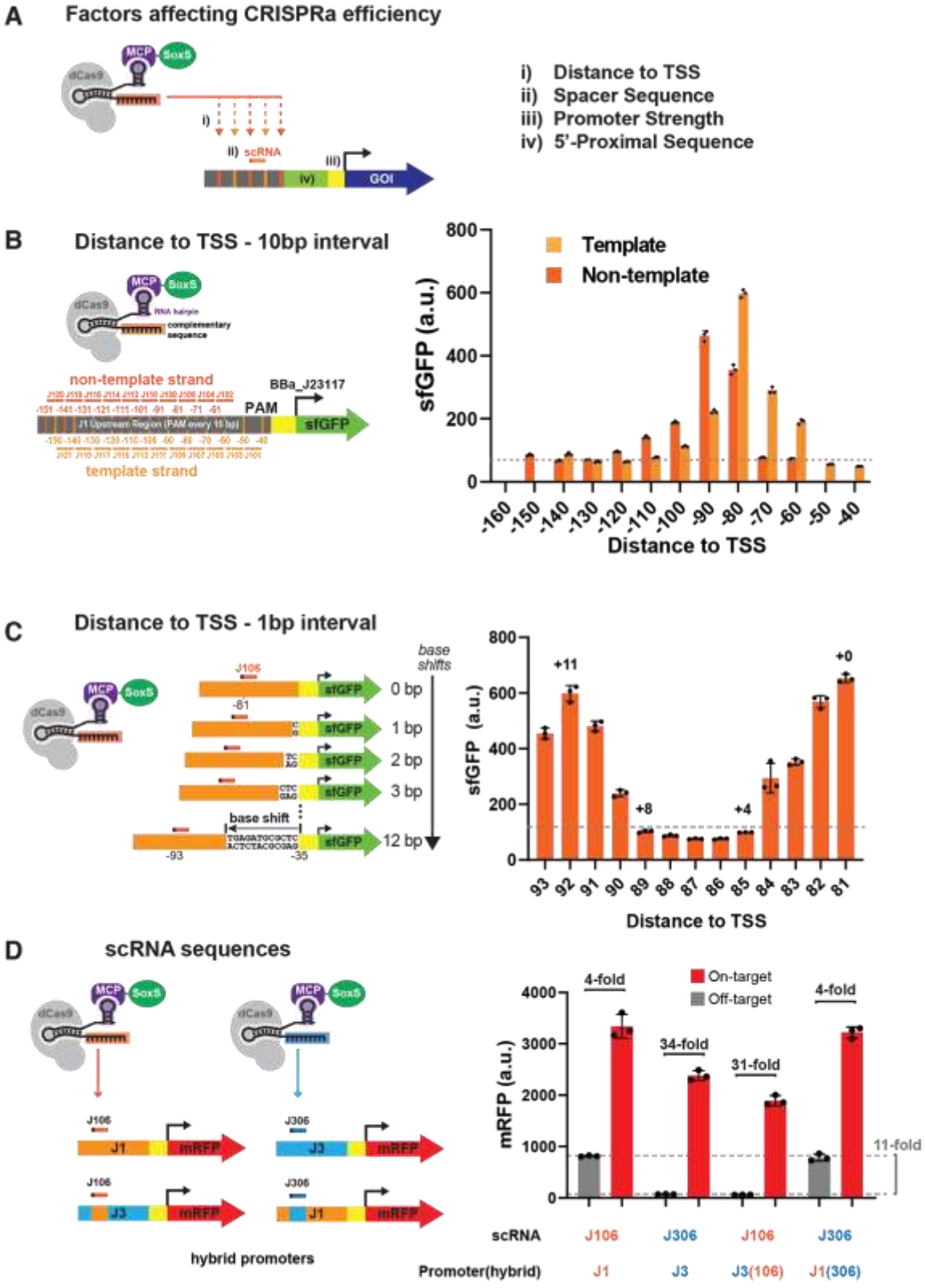
A
Factors affecting CRISPRa efficiency
MCP
SoxS
dCas9
i)
ii) scRNA
iii)
iv)
GOI
i) Distance to TSS
ii) Spacer Sequence
iii) Promoter Strength
iv) 5'-Proximal Sequence
B
Distance to TSS - 10bp interval
RNA hairpin
complementary sequence
non-template strand
BBa_J23117
PAM
sfGFP
J1 Upstream Region (PAM every 10 bp)
template strand
Template
Non-template
sfGFP (a.u.)
800
600
400
200
0
-160 -150 -140 -130 -120 -110 -100 -90 -80 -70 -60 -50 -40
Distance to TSS
C
Distance to TSS - 1bp interval
base shifts
J106
-81
0 bp
1 bp
2 bp
3 bp
base shift
12 bp
-93
-35
+11
+0
+8
+4
93 92 91 90 89 88 87 86 85 84 83 82 81
Distance to TSS
D
scRNA sequences
J106
J1
mRFP
J306
J3
hybrid promoters
On-target
Off-target
4-fold
34-fold
31-fold
4-fold
11-fold
mRFP (a.u.)
4000
3000
2000
1000
0
scRNA J106 J306 J106 J306
Promoter(hybrid) J1 J3 J3(106) J1(306)

Figure 2. Sensitivity of CRISPRa to distance from the TSS and promoter sequence composition in *P. putida*. (A) Factors known to affect CRISPRa efficiency in *E. coli* include i) distance to TSS, ii) scRNA target sequence, iii) minimal promoter strength, and 5'-proximal sequence between target sequence and minimal promoter. (B) Effect of distance to TSS on CRISPRa efficiency at 10 bp resolution. The J1 synthetic sequence upstream of the minimal promoter includes target sites every 10 bp in both the template strand (light orange), and the non-template strand (orange). scRNAs J101-J121 were expressed in the pBBR1-GmR backbone. The observed peaks of activation are slightly offset on the template and non-template strands because the distance is defined from the TSS to the PAM sites, which is proximal to the TSS on template strand targets and distal to the TSS on non-template strands. The most effective sites at -91 on the template strand (J108) and -80 on the non-template strand (J109) target overlapping 20-base sites. (C) Effect of distance to TSS on CRISPRa efficiency at single bp resolution. N bases were added upstream of the minimal promoter (N = 1 - 12), and the J106 scRNA was used to target sites at -81 to -93 upstream of the TSS. (D) The J3 upstream sequence has lower basal expression (11-fold) and higher fold-activation by CRISPRa than the J1 sequence. When the 20 bp target sequence J106 was inserted into the J3 promoter, the basal expression remains low. When the 20 bp target sequence J306 was inserted into the J1 promoter, basal expression remains high. See Methods and Supplemental Information for detailed descriptions of the J1 and J3 sequences. Values in panel B, C, and D represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

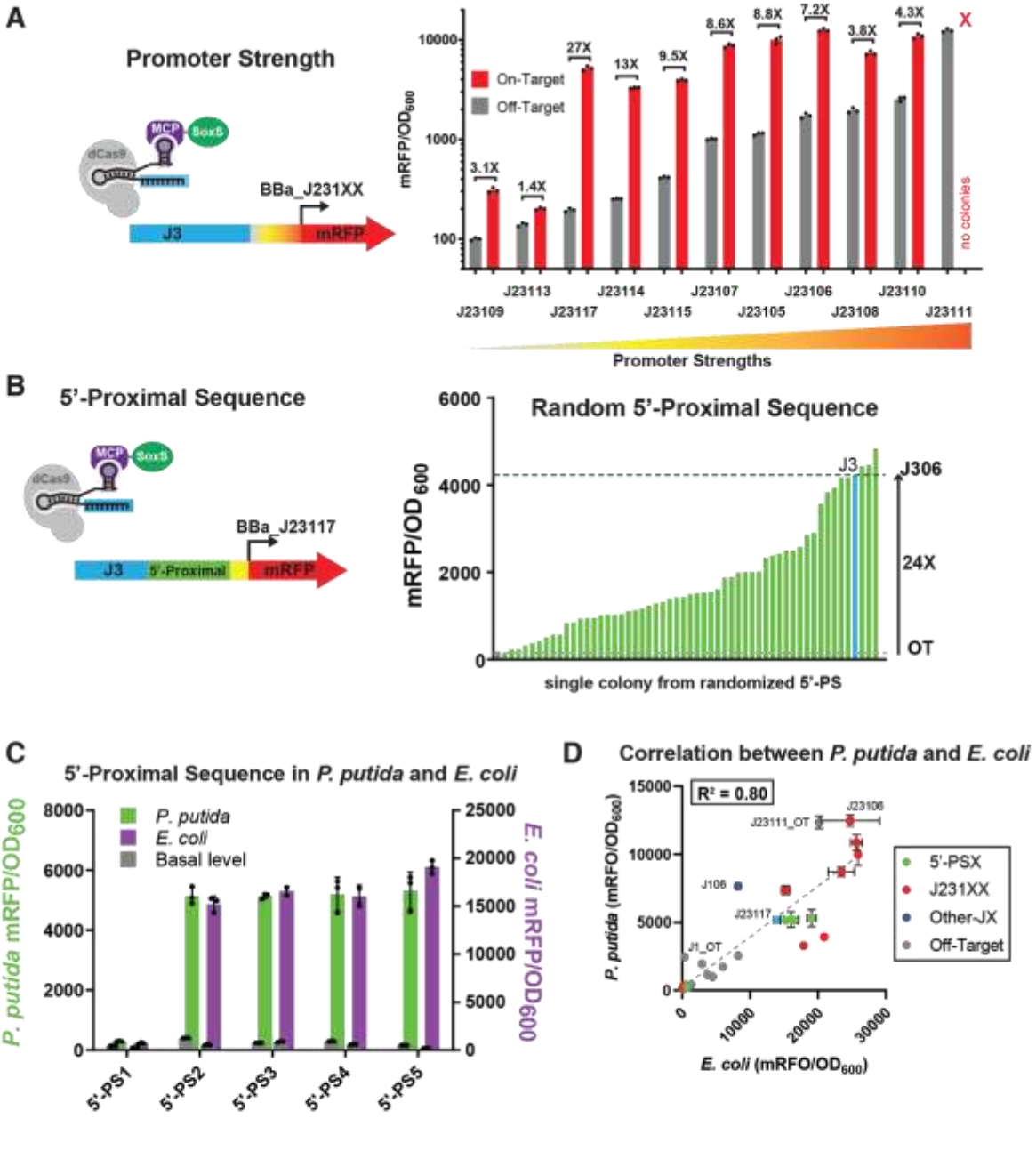
A
Promoter Strength
dCas9
MCP
8oxS
BBa_J231XX
J3
mRFP
mRFP/OD600
On-Target
Off-Target
3.1X
1.4X
27X
13X
9.5X
8.6X
8.8X
7.2X
3.8X
4.3X
X
no colonies
J23109
J23113
J23117
J23114
J23115
J23107
J23105
J23106
J23108
J23110
J23111
Promoter Strengths
B
5'-Proximal Sequence
BBa_J23117
5'-Proximal
Random 5'-Proximal Sequence
J306
24X
OT
single colony from randomized 5'-PS
C
5'-Proximal Sequence in P. putida and E. coli
P. putida
E. coli
Basal level
P. putida mRFP/OD600
E. coli mRFP/OD600
5'-PS1
5'-PS2
5'-PS3
5'-PS4
5'-PS5
D
Correlation between P. putida and E. coli
R² = 0.80
5'-PSX
J231XX
Other-JX
Off-Target
P. putida (mRFO/OD600)
E. coli (mRFO/OD600)

Figure 3. Sensitivity of CRISPRa to promoter strength and 5' upstream sequence in *P. putida.* (A) CRISPRa is sensitive to basal promoter strength. Variants of pPPC021.J231XX were constructed by changing the BBa_J23117 promoter into ten other minimal promoters. The promoters weaker than BBa_J23117 exhibited low CRISPRa efficiency while the fold-activation was maximized at BBa_J23117 and decreased as the promoter strength increased beyond that point. (B) CRISPRa is sensitive to the sequence composition of the 26 bp 5'-proximal sequence between the scRNA target site and the minimal BBa_J23117 promoter. (C) Comparison of CRISPRa-induced expression with different 5'-proximal sequences characterized in *E. coli* and *P. putida*. Sequences are available in the Supplemental Information. (D) Correlation between CRISPRa-induced mRFP expression levels from different promoter contexts in *E. coli* and *P. putida* (R^2 = 0.80). Values in panel A, C, D represent the mean ± standard deviation calculated from *n = 3* independent biological replicates. Bars in panel B represent the value of one (*n = 1*) sample.

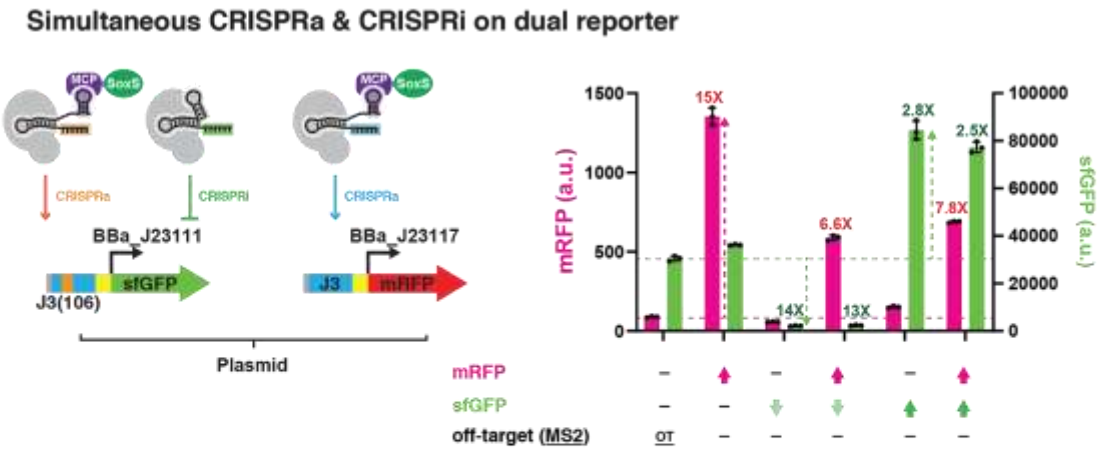
Simultaneous CRISPRa & CRISPRi on dual reporter
MCP
SoxS
CRISPRa
CRISPRi
CRISPRa
BBa_J23111
BBa_J23117
J3(106)
sfGFP
J3
mRFP
Plasmid
mRFP (a.u.)
sfGFP (a.u.)
1500
1000
500
0
100000
80000
60000
40000
20000
0
15X
14X
6.6X
13X
2.8X
7.8X
2.5X
mRFP
sfGFP
off-target (MS2)
OT

Figure 4. Multi-gene CRISPRa/CRISPRi in the plasmid-borne dual reporters. A multi-gene CRISPRa/CRISPRi reporter with weakly expressed mRFP (J3-BBa_J23117) and highly expressed sfGFP (J3(106)-BBa_J23111) shows simultaneous activation and repression when an activator scRNA for mRFP and a repressor sgRNA for sfGFP are delivered. The strong sfGFP reporter can also be further activated ~2-3-fold if targeted by an upstream activating scRNA. This strain exhibits noticeably slower growth in both agar and liquid media (data not shown). Values represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

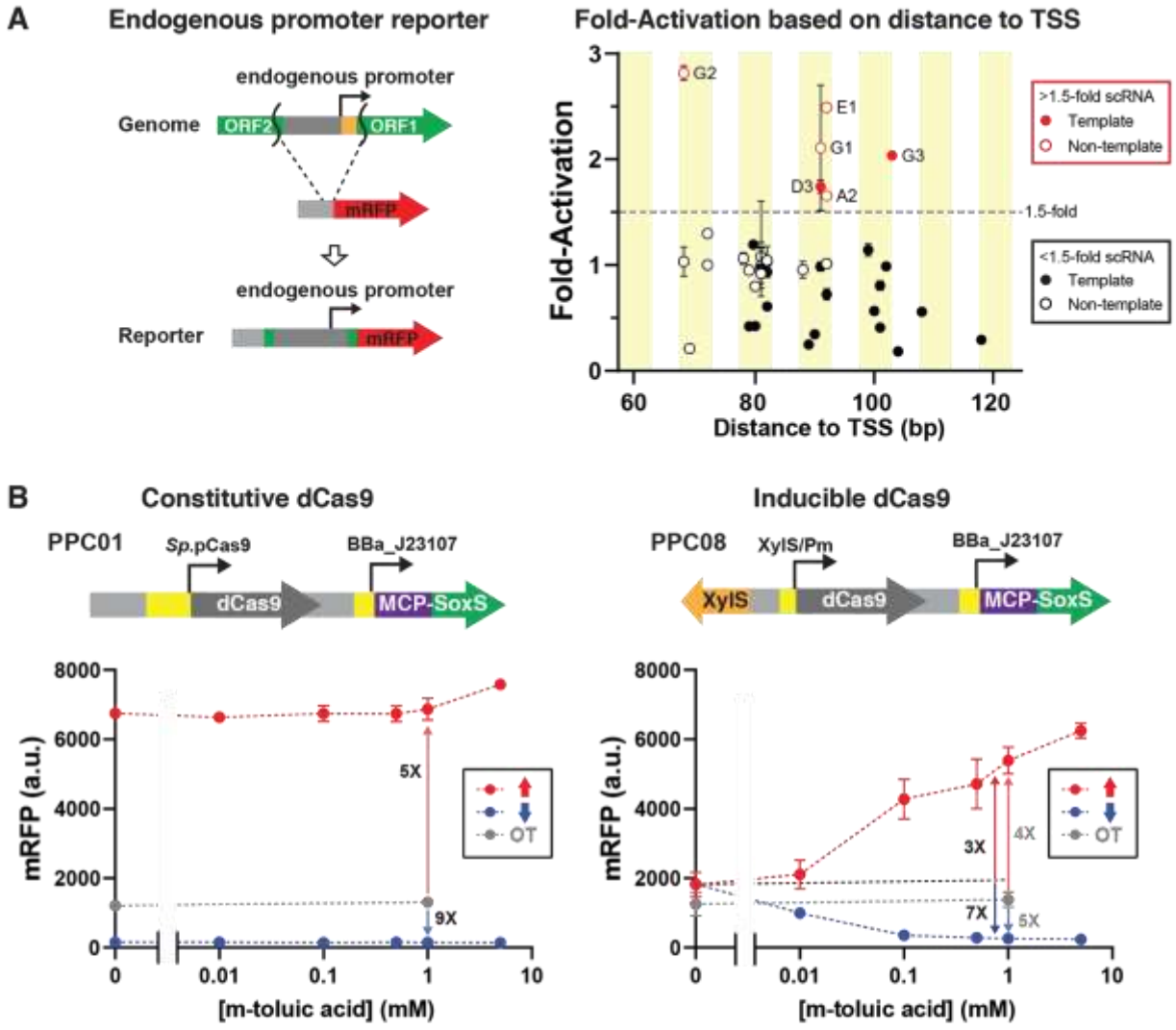
A
Endogenous promoter reporter
endogenous promoter
Genome
ORF2
ORF1
mRFP
endogenous promoter
Reporter
mRFP
Fold-Activation based on distance to TSS
Fold-Activation
Distance to TSS (bp)
G2
E1
G1
G3
D3
A2
1.5-fold
>1.5-fold scRNA
Template
Non-template
<1.5-fold scRNA
Template
Non-template
B
Constitutive dCas9
PPC01
Sp.pCas9
BBa_J23107
dCas9
MCP-SoxS
mRFP (a.u.)
[m-toluic acid] (mM)
5X
9X
OT
Inducible dCas9
PPC08
XylS/Pm
BBa_J23107
XylS
dCas9
MCP-SoxS
mRFP (a.u.)
[m-toluic acid] (mM)
3X
4X
7X
5X
OT

Figure 5. CRISPRa with endogenous promoters and inducible CRISPRa/CRISPRi in *P. putida*. (A) The putative promoter sequences between two open reading frames (ORFs) with 60 bp flanking sequences were incorporated into the mRFP reporter. scRNAs were introduced for all potentially activatable target sites corresponding to the effective distances in Figure 2B & C. Precise distances from the target site to the TSS are listed in Table S4. hAAVS1 was used as an off-target scRNA for all ten promoters. (B) Tunable activation of mRFP expression with CRISPRa and tunable repression of mRFP expression with CRISPRi were achieved with different inducer concentrations (0-5 mM *m*-toluic acid) in the inducible-dCas9 strain (right). The inducible-dCas9 strain yielded 3-fold activation with CRISPRa or 7-fold repression with CRISPRi at 1 mM m-toluic acid compared to the no-inducer control. Fold-changes compared to the off-target control were 4-fold and 5-fold, respectively. The constitutively expressed dCas9 strain (left) showed little to no response to inducer concentration. Values in panel A and B represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

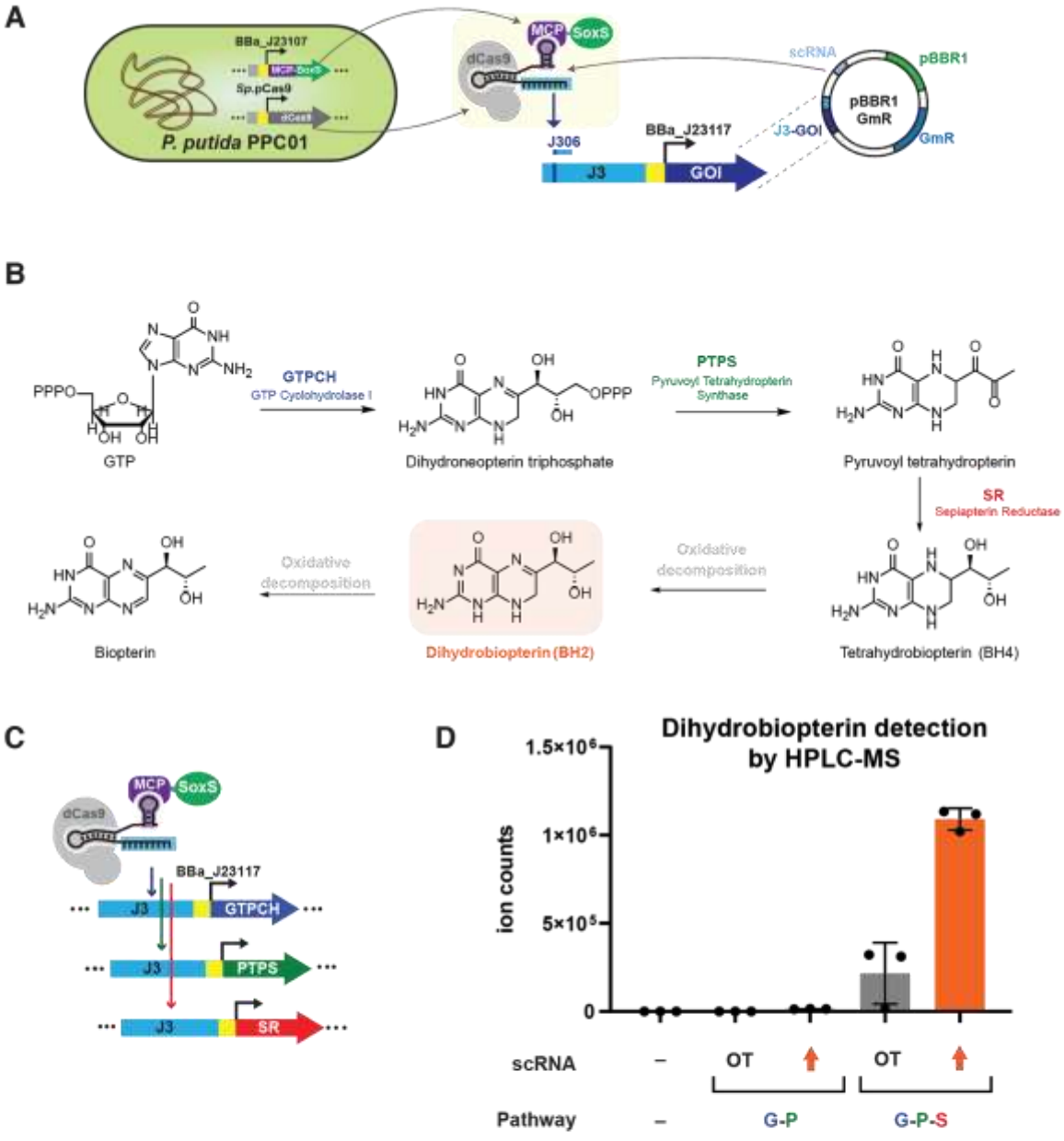
A
BBa_J23107
MCP-SoxS
Sp.pCas9
dCas9
P. putida PPC01
MCP
SoxS
dCas9
scRNA
pBBR1
pBBR1
GmR
GmR
J306
BBa_J23117
J3-GOI
J3
GOI
B
GTP
GTPCH
GTP Cyclohydrolase I
Dihydroneopterin triphosphate
PTPS
Pyruvoyl Tetrahydropterin Synthase
Pyruvoyl tetrahydropterin
SR
Sepiapterin Reductase
Tetrahydrobiopterin (BH4)
Oxidative decomposition
Dihydrobiopterin (BH2)
Oxidative decomposition
Biopterin
C
MCP
SoxS
dCas9
BBa_J23117
J3
GTPCH
J3
PTPS
J3
SR
D
Dihydrobiopterin detection by HPLC-MS
ion counts
1.5×10⁶
1×10⁶
5×10⁵
0
scRNA
–
OT
OT
Pathway
–
G-P
G-P-S

Figure 6. Multi-target CRISPR activation on a biopterin pathway. (A) Graphical depiction of CRISPRa implementation to any gene of interest by integrated dCas9/MCP-SoxS strains (PPC001) where scRNA(s) and heterologous genes were delivered on pBBR1-GmR plasmid. (B) Biosynthetic and spontaneous oxidation scheme from GTP into tetrahydrobiopterin (BH4) and its oxidized variants. The three-enzyme pathway consisted of E. coli gtpch, M. alpina ptps, and M. alpina sr. Tetrahydrobiopterin is reactive towards ambient oxygen and is readily oxidized into dihydrobiopterin (BH2) and biopterin, respectively. (C) Graphical depiction of CRISPRa activating three genes with a single scRNA. (D) Dihydrobiopterin (BH2) levels observed by HPLC-MS of PPC01 strains bearing pPPC024 (3-gene pathway) or pPPC025 (absence of sr gene). HPLC-MS data of three biopterin species are shown in Figure S13. Values in panel D represent the mean ± standard deviation calculated from $n = 3$ technical replicates.

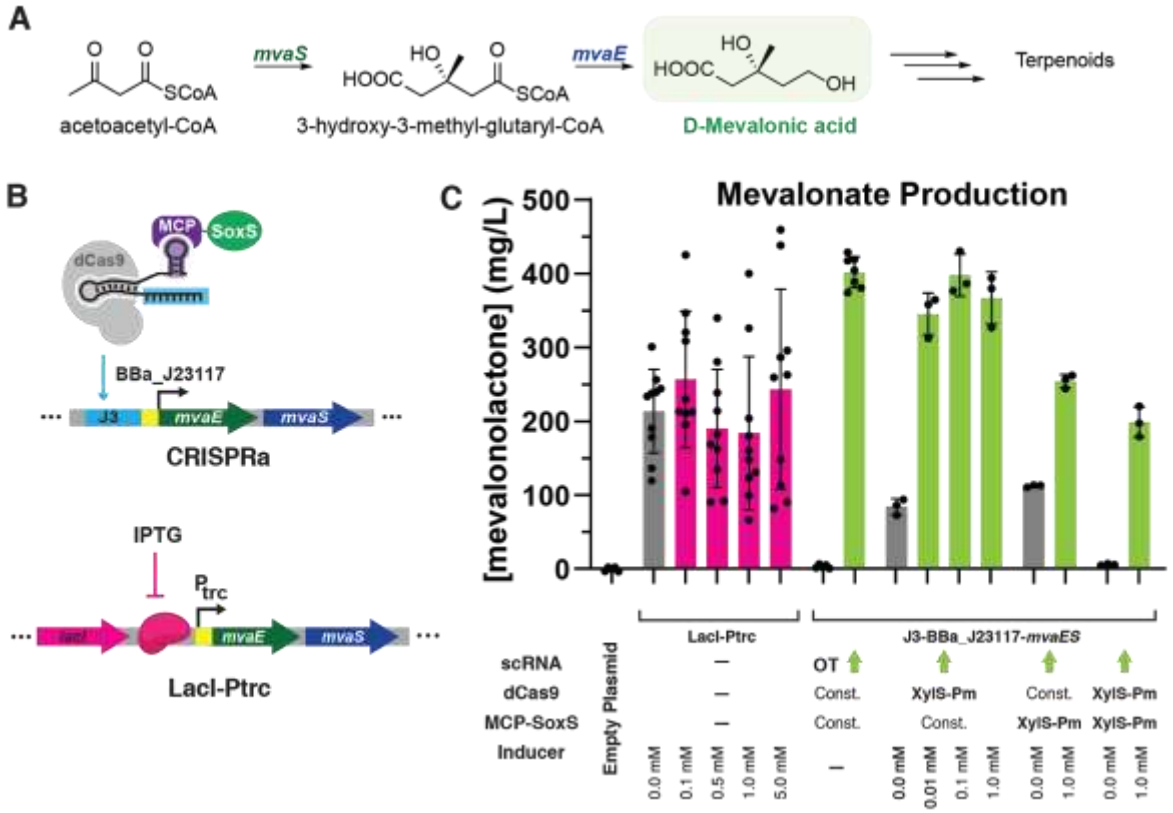
A
mvaS
mvaE
Terpenoids
acetoacetyl-CoA
3-hydroxy-3-methyl-glutaryl-CoA
D-Mevalonic acid
B
MCP
SoxS
dCas9
BBa_J23117
J3
mvaE
mvaS
CRISPRa
IPTG
Ptrc
lacI
LacI-Ptrc
C
Mevalonate Production
[mevalonolactone] (mg/L)
LacI-Ptrc
J3-BBa_J23117-mvaES
scRNA
dCas9
MCP-SoxS
Inducer
Empty Plasmid
OT
Const.
XylS-Pm

Figure 7. CRISPR activation on Mevalonate Production Operon. (A) Biosynthetic pathway for D-mevalonic acid production from acetoacetyl-CoA with heterologous *mvaS* and *mvaE* genes from *Enterococcus faecalis*. (B) Graphical depiction comparing CRISPR activation complex (pPPC029) with the LacI-Ptrc inducible system (pPPC030) for gene activation. (C) Titer of mevalonate calculated based on GC-MS detection of cyclized mevalonolactone (m/z = 71). The J306 scRNA was used as an on-target CRISPRa scRNA while hAAVS1 was used as an off-target scRNA. Up to 5.0 mM IPTG was used for induction of LacI-Ptrc and up to 1 mM *m*-toluic acid was used for induction of XylS-Pm. The off-target scRNA produced a mevalonate titer indistinguishable from the no plasmid control (less than 10 mg/L, see Figure S14). Values in panel C represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates, $n = 5$ for the no plasmid control and off-target scRNA, $n = 7$ for the constitutively expressed dCas9/MCP-SoxS strain, and $n = 10$ for the LacI-Ptrc strain.

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Supplementary Information

Portable bacterial CRISPR transcriptional activation enables metabolic engineering in *Pseudomonas putida*

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This file includes:

- **Supplementary Figures**
- **Supplementary Tables**
- **Supplementary Methods**
- **DNA Sequences**

Supplementary Figures

Figure S1: Basal mRFP expression on pBBR1 and pRK2 plasmids
Figure S2: Expression cassettes affect CRISPRa efficiency and growth of *P. putida*
Figure S3: Additional plasmid decreases CRISPRa efficiency
Figure S4: Distance effect of CRISPRa in J3
Figure S5: Correlation Plot of Fold-Activation between *P. putida* and *E. coli*
Figure S6: Dual CRISPR activation on the plasmid-borne dual reporter
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Supplementary Tables

Table S1: Description of bacterial strains and plasmids used in each figure
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Table S4: Target sequences of scRNA and sgRNA
Table S5: Summary of CRISPRa-mediated fold-changes in gene expression and metabolite production

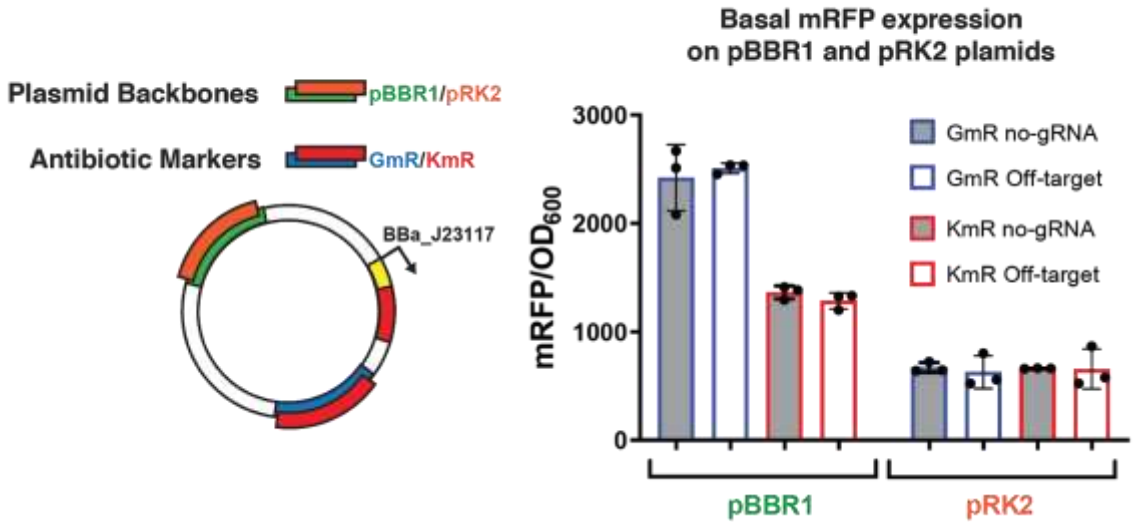
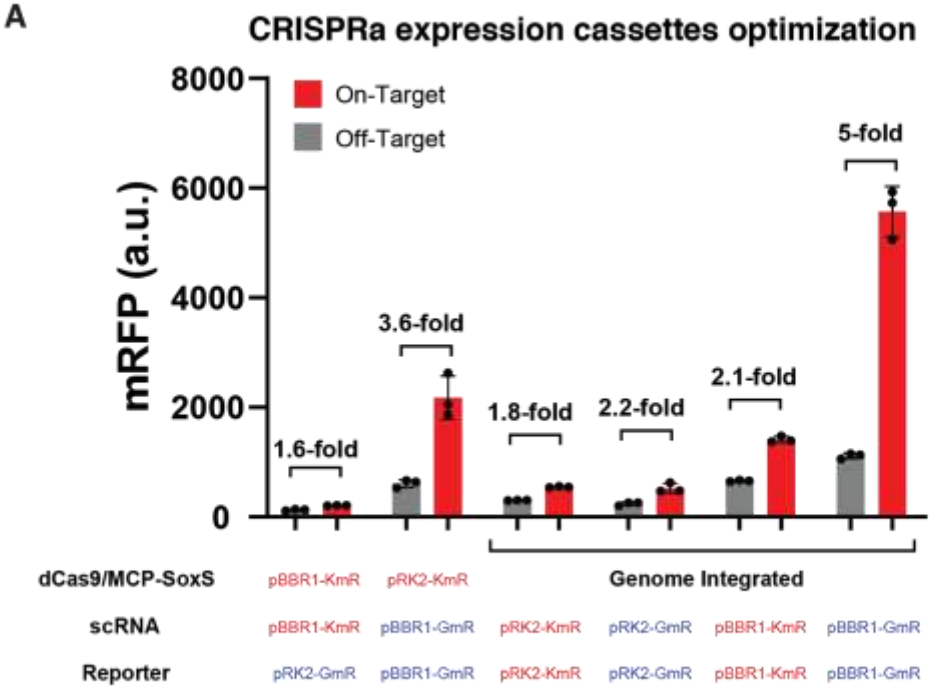


Figure S1: Basal mRFP expression on pBBR1 and pRK2 plasmids. Basal expression of mRFP reporter gene from J1-BBa_J23117-mRFP in different plasmid backbones (pBBR1 or pRK2 origins), with either GmR (blue) or KmR (red) antibiotics. The plasmids expressing an off-target scRNA were tested side-by-side to the no-gRNA control and exhibited indistinguishable expression levels. See Table S2 for plasmid constructs used here. Values represent the mean ± standard deviation calculated from *n* = *3* independent biological replicates. independent samples.

A


CRISPRa expression cassettes optimization
mRFP (a.u.)
8000
6000
4000
2000
0
On-Target
Off-Target
1.6-fold
3.6-fold
1.8-fold
2.2-fold
2.1-fold
5-fold
dCas9/MCP-SoxS
pBBR1-KmR
pRK2-KmR
Genome Integrated
scRNA
pBBR1-KmR
pBBR1-GmR
pRK2-KmR
pRK2-GmR
pBBR1-KmR
pBBR1-GmR
Reporter
pRK2-GmR
pBBR1-GmR
pRK2-KmR
pRK2-GmR
pBBR1-KmR
pBBR1-GmR

B

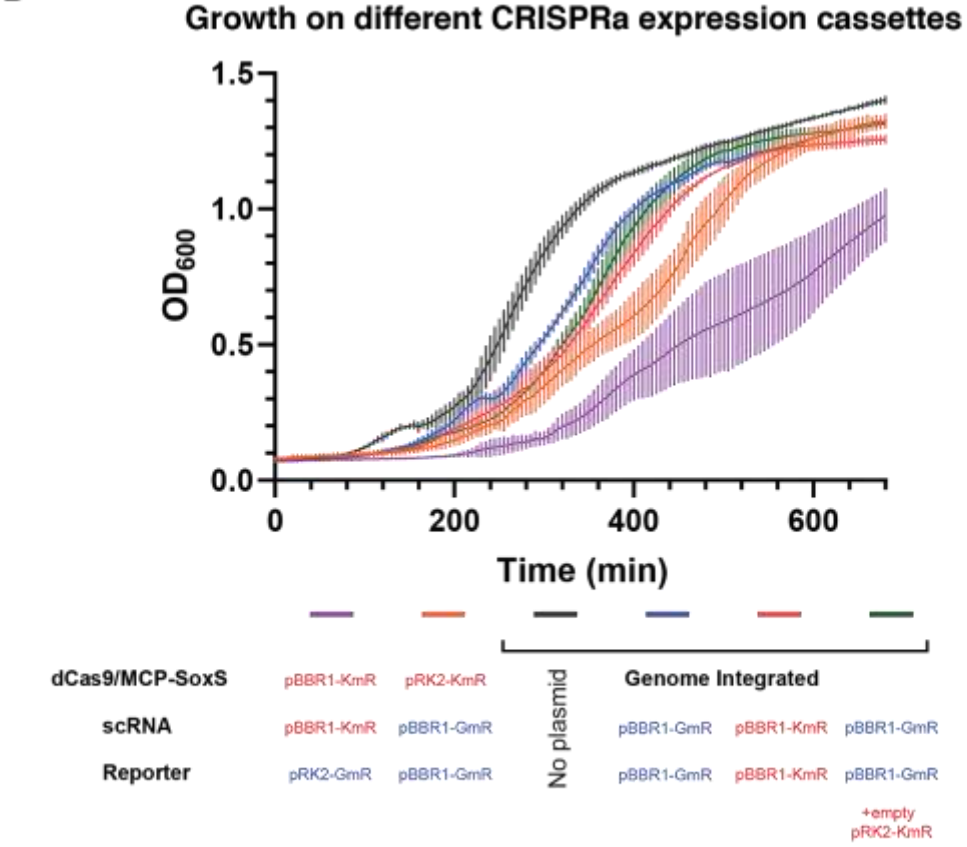

Growth on different CRISPRa expression cassettes
OD600
1.5
1.0
0.5
0.0
0
200
400
600
Time (min)
dCas9/MCP-SoxS
pBBR1-KmR
pRK2-KmR
Genome Integrated
scRNA
pBBR1-KmR
pBBR1-GmR
No plasmid
pBBR1-GmR
pBBR1-KmR
pBBR1-GmR
Reporter
pRK2-GmR
pBBR1-GmR
pBBR1-GmR
pBBR1-KmR
pBBR1-GmR
+empty
pRK2-KmR

Figure S2: Expression cassettes affect CRISPRa efficiency and growth of *P. putida*. (A) CRISPRa from different expression methods for dCas9/MCP-SoxS, scRNA, and reporter. *P. putida* compatible plasmids are pBBR1 and pRK2 in which GmR (blue text) and KmR (red text) can be used as antibiotic selection markers. Both basal expressions and fold-activation varied with different expression systems. The genomically-integrated dCas9/MCP-SoxS cassettes give the highest fold-activation in pBBR1-GmR (5-fold compared to that of an off-target scRNA control). (B) Growth curve (OD_{600} vs. time) of *P. putida* strains in liquid culture with dCas9/MCP-SoxS cassette either on plasmid or integrated into the genome. Every strain expresses an off-target scRNA. Expressing dCas9/MCP-SoxS on plasmid systems (purple or orange lines) significantly reduces the growth rate compared to that of 2-plasmid strains with integrated dCas9/MCP-SoxS (green line). Qualitatively similar growth defects were observed when colonies were grown on agar plates. Values in panel A and B represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

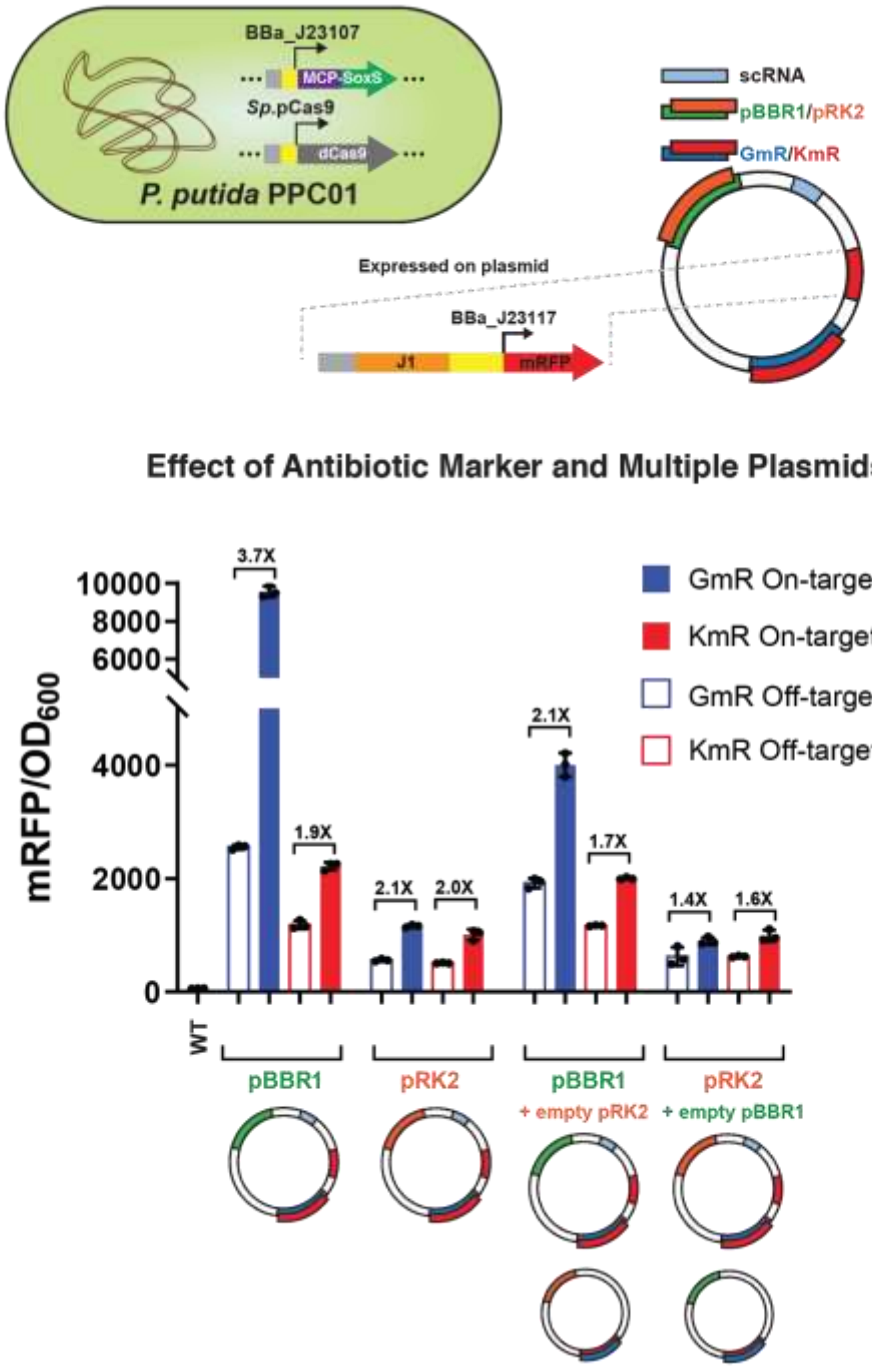


Figure S3: Additional plasmid decreases CRISPRa efficiency. *P. putida* (PPC01) strains bearing J1-mRFP and scRNA plasmids with different origin of replications and antibiotic markers (Table S1 and S2) were further transformed with a second empty plasmid of different origin of replication and antibiotic marker. Expressing a second empty plasmid led to significant drops in both basal expression levels and fold-activation. Values represent the mean ± standard deviation calculated from *n = 3* independent biological replicates.

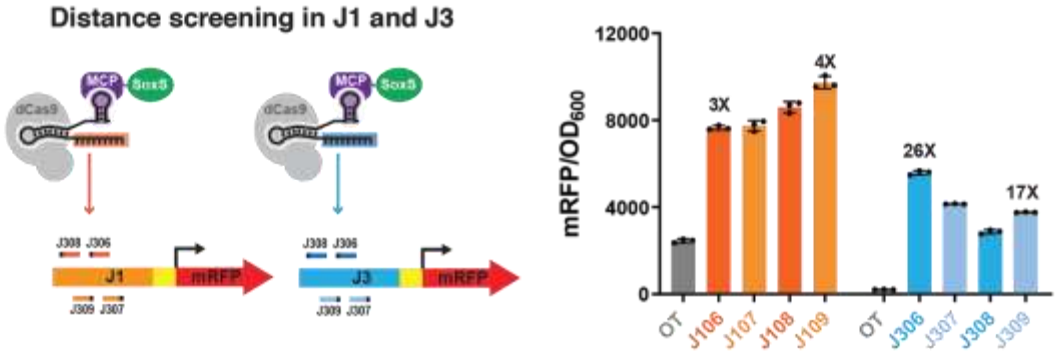


Figure S4: Distance effect of CRISPRa in the J3 promoter. Comparison of optimal target sites of the J1 promoter (J106-J109) and the J3 promoter (J306-J309). The highest fold-activation was obtained with the J306 scRNA. Values represent the mean ± standard deviation calculated from *n = 3* independent biological replicates.

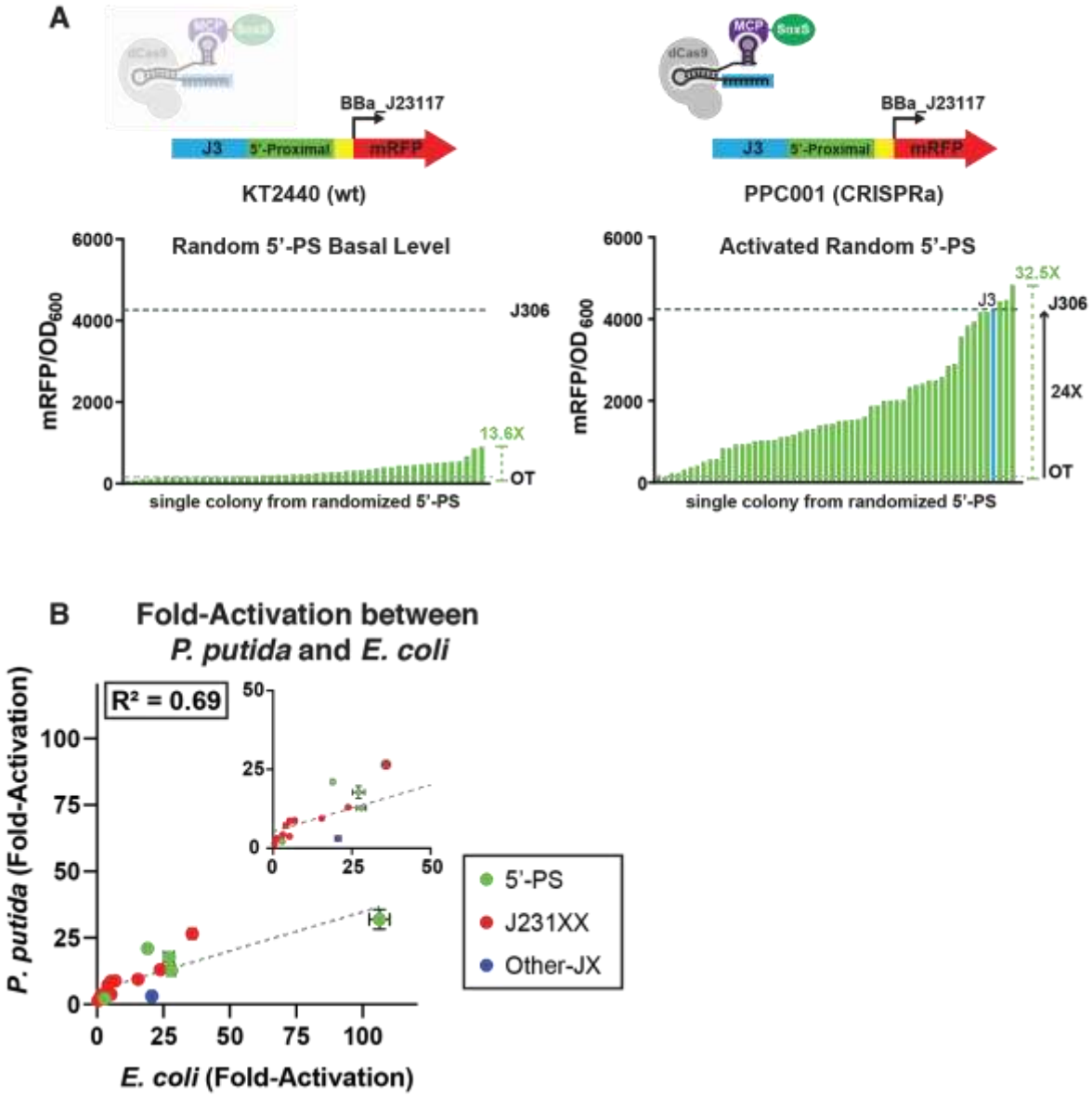


Figure S5: Correlation plot of fold-activation between *P. putida* and *E. coli.* (A) Randomized 5'-proximal sequences (5'-PS) were transformed into either a no-CRISPR (KT2440) strain or a CRISPRa (PPC01) strain. Differences in basal expression with variable 5'-PS are detectable but are relatively small compared to the effects on CRISPRa fold-activation. (B) Correlation plot of fold-activation between CRISPRa strains with cognate scRNAs and off-target scRNAs ($R^2 = 0.69$). *E. coli* data are from (Fontana et al., 2020). Bars in panel A represent the value of *n = 1* independent biological replicates. Values in panel B represent the mean ± standard deviation calculated from *n = 3* independent biological replicates.

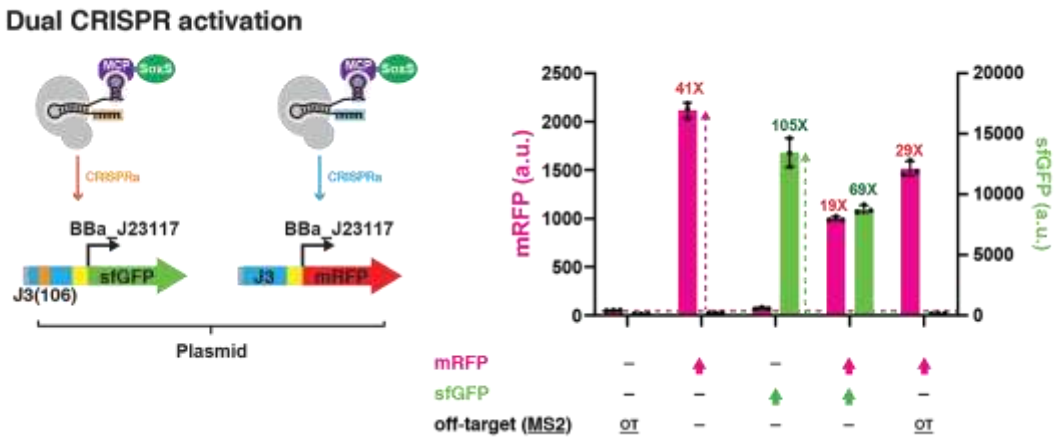


Figure S6: Dual CRISPR activation on the plasmid-borne dual reporter. A multi-gene CRISPRa reporter with weakly expressed mRFP (J3-BBa_J23117) and weakly expressed sfGFP (J3(106)-BBa_J23117) can be simultaneously activated by targeting the reporters with two cognate scRNAs. The observed fold-activations with two scRNAs expressed are weaker than with only one scRNA expressed, possibly due to competition for a limited pool of dCas9. A similar effect is observed with one on-target and one off-target scRNA. Values represent the mean ± standard deviation calculated from *n = 3* independent biological replicates.

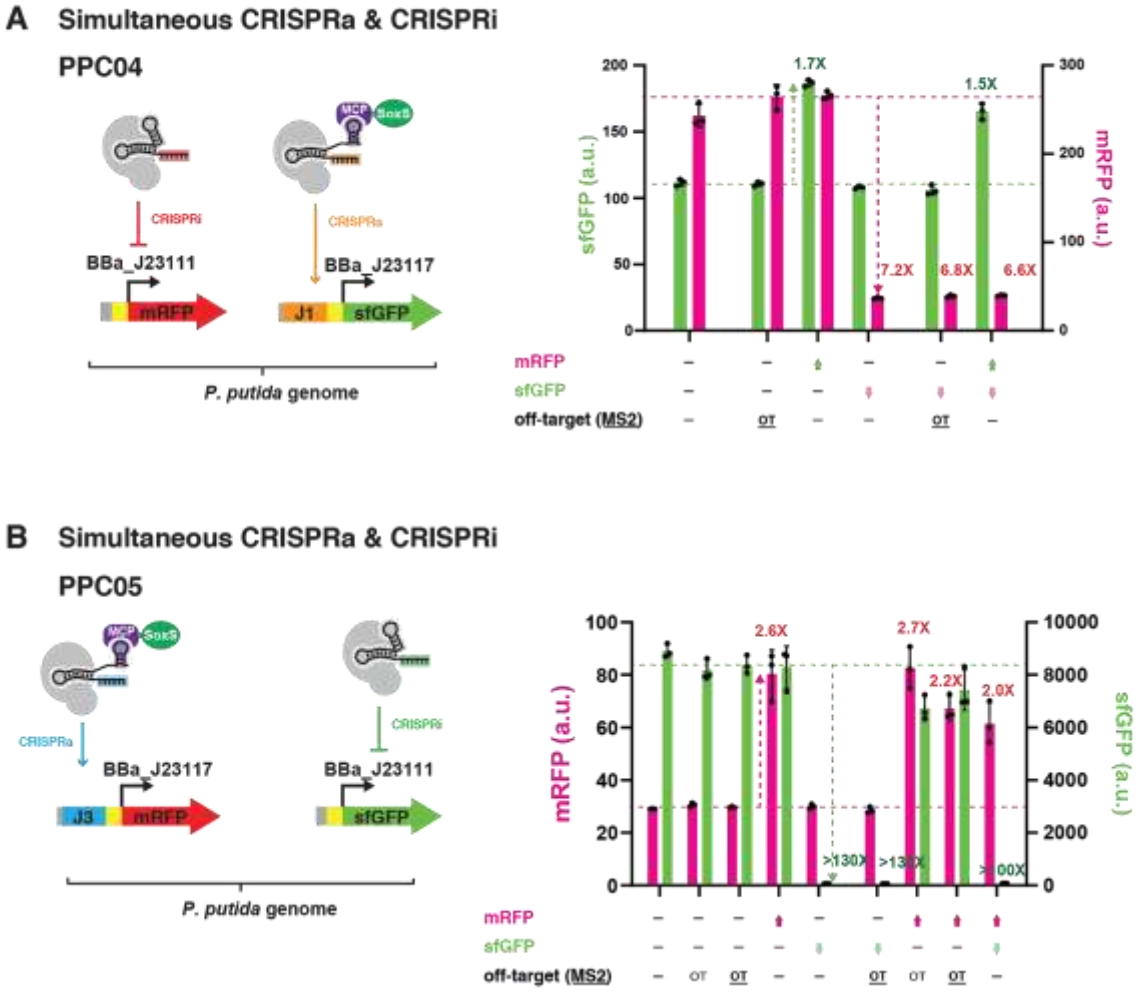


Figure S7: Simultaneous CRISPRa/CRISPRi in strains with integrated dual reporters. (A) Highly expressed mRFP (BBa_J23111) and weakly expressed sfGFP (J1-BBa_J23117) were integrated together with a dCas9/MCP-SoxS construct to produce strain PPC04. Simultaneous CRISRPa on sfGFP and CRISPRi on mRFP are detectable, but the CRISPRa fold-activation is modest compared to that observed with the plasmid reporter (main text Figure 4). (B) Weakly expressed mRFP (J3-BBa_J23117) and highly expressed sfGFP (BBa_J23111) were integrated into the PPC01 strain at the pp1 and pp2 sites, respectively, to produce strain PPC05. The fold-change from CRISPRa and CRISPRi marginally improved compared to the PPC04 strain (panel A). The magnitude of CRISPRa fold-activation in simultaneous CRISPRa/CRISPRi was weaker than that observed if just a single scRNA was delivered to activate the mRFP reporter, possibly due to competition between multiple scRNA/gRNA cassettes for a limited pool of dCas9. A similar effect was observed with one activating scRNA and one off-target scRNA. Values in panel A and B represent the mean ± standard deviation calculated from *n* = *3* independent biological replicates.

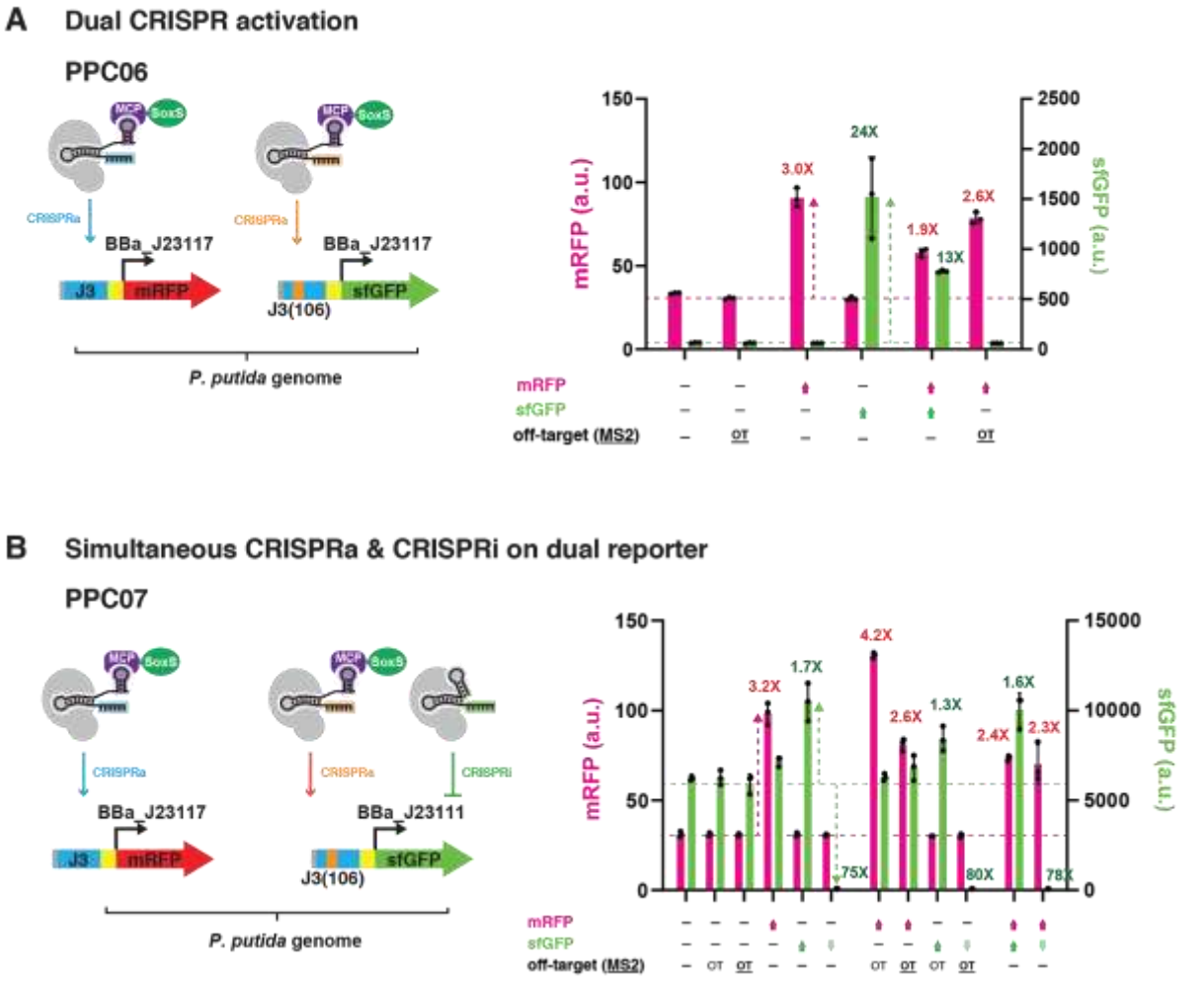


Figure S8: Multi-gene CRISPRa/CRISPRi regulation in the integrated dual reporters. (A) An integrated dual reporter with weakly expressed mRFP (J3-BBa_J23117) and weakly expressed sfGFP (J3(106)-BBa_J23117) can be activated with two scRNAs, one targeting each reporter. The presence of the second sgRNA/scRNA reduced CRISPRa efficiency compared to single gene activation. (B) An integrated dual reporter with weakly expressed mRFP (J3-BBa_J23117) and a highly expressed sfGFP (J3(106)-BBa_J23111) can be activated or repressed at the sfGFP reporter. Simultaneous activation of mRFP and repression of sfGFP occurs with a J306 scRNA for mRFP activation and an sgRNA that targets within the sfGFP ORF for repression. Activation of both mRFP and sfGFP occurs a J306 scRNA for mRFP and a J106 scRNA for sfGFP. An unexpected improvement in CRISPRa mRFP expression from the second off-target sgRNA was observed, while sfGFP CRISPRa suffered from the presence of the second guide-RNA, similar to previous conditions. Values in panel A and B represent the mean ± standard deviation calculated from *n* = *3* independent biological replicates.

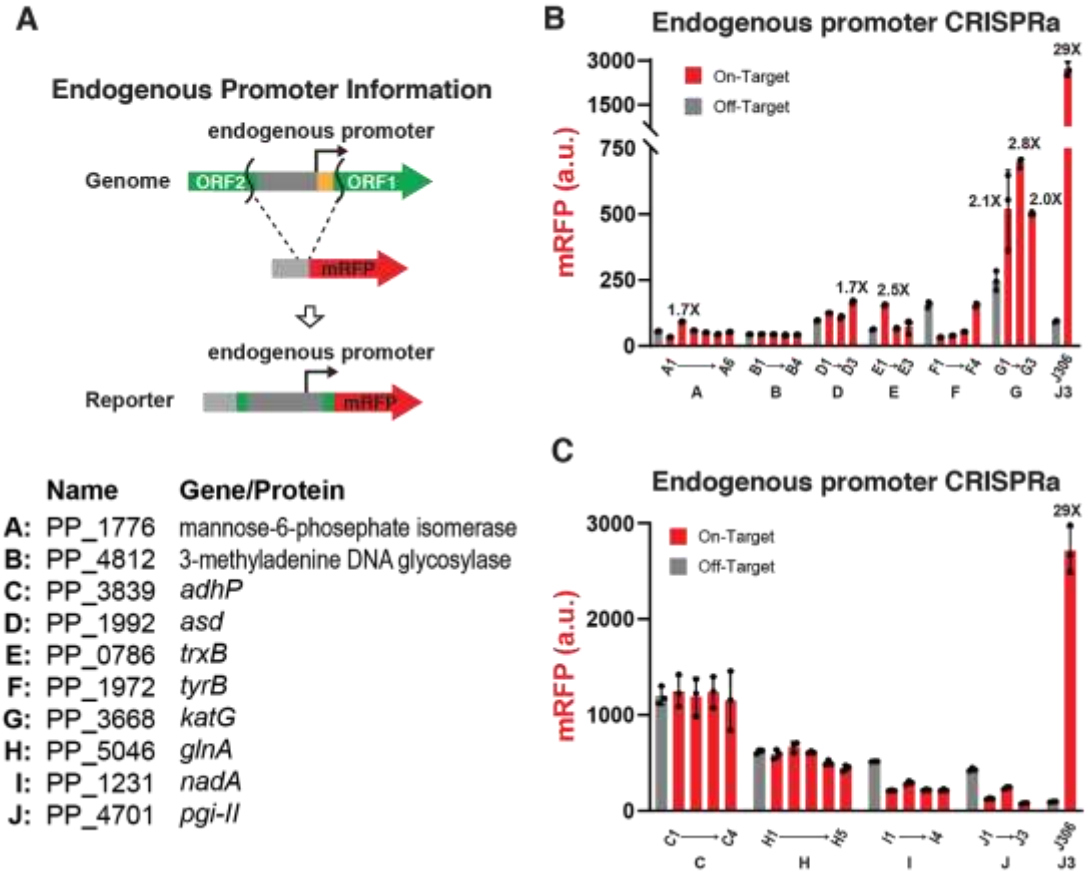
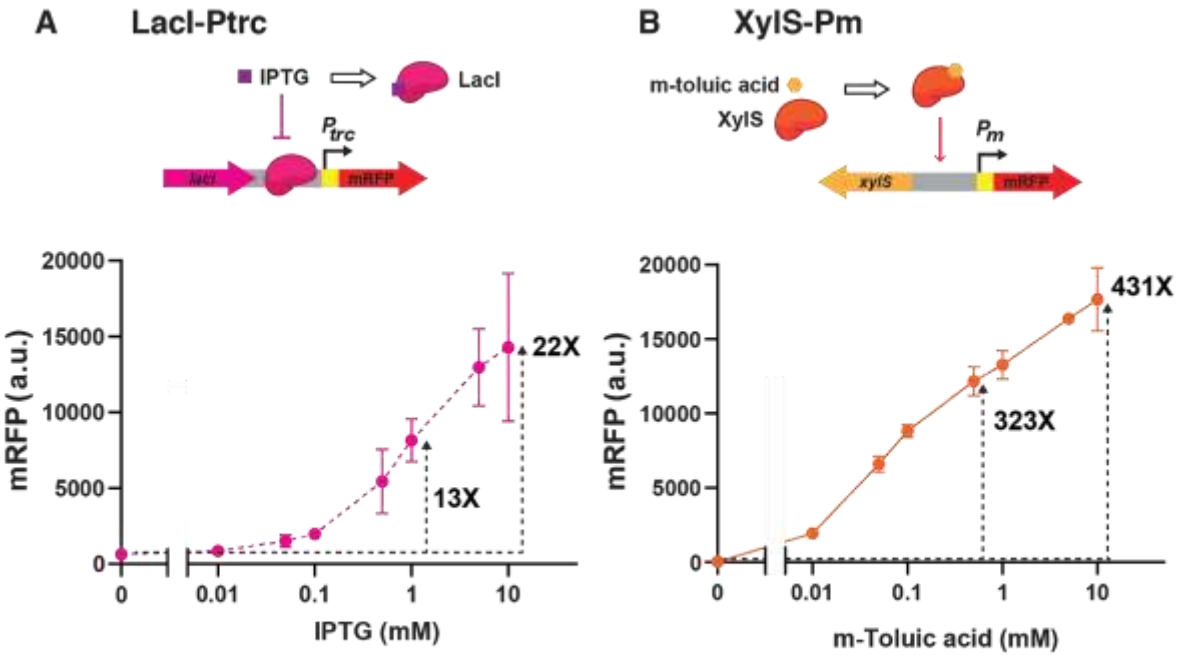


Figure S9: CRISPR activation of *P. putida* endogenous promoters (A) Ten *P. putida* endogenous promoters were selected based on available scRNA target sites at the appropriate phase and distance from reported transcription start sites (TSSs; see main text) and were coupled with mRFP reporter. Each gene was given an abbreviated code (A-J). (B) Activation profiles of CRISPRa on the endogenous promoters with relatively low basal expression (promoters A-G except promoter C) were plotted with the corresponding scRNAs (A1-A6, for example). Fold-changes were provided for instances where >1.5-fold activation was observed compared to an off-target scRNA (hAAVS1). (C) Activation profiles of CRISPRa on the endogenous promoters with relatively high basal expression promoter (promoters C and H-J) revealed no significant CRISPRa activity with the scRNAs that were tested. The J3-BBa_J23117 promoter with J306 scRNA was included as a positive CRISPRa control. Values in panel B and C represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.


A
LacI-Ptrc
IPTG
LacI
P_{trc}
lacI
mRFP
B
XylS-Pm
m-toluic acid
XylS
P_m
xylS
mRFP
mRFP (a.u.)
20000
15000
10000
5000
0
22X
13X
0
0.01
0.1
1
10
IPTG (mM)
431X
323X
m-Toluic acid (mM)

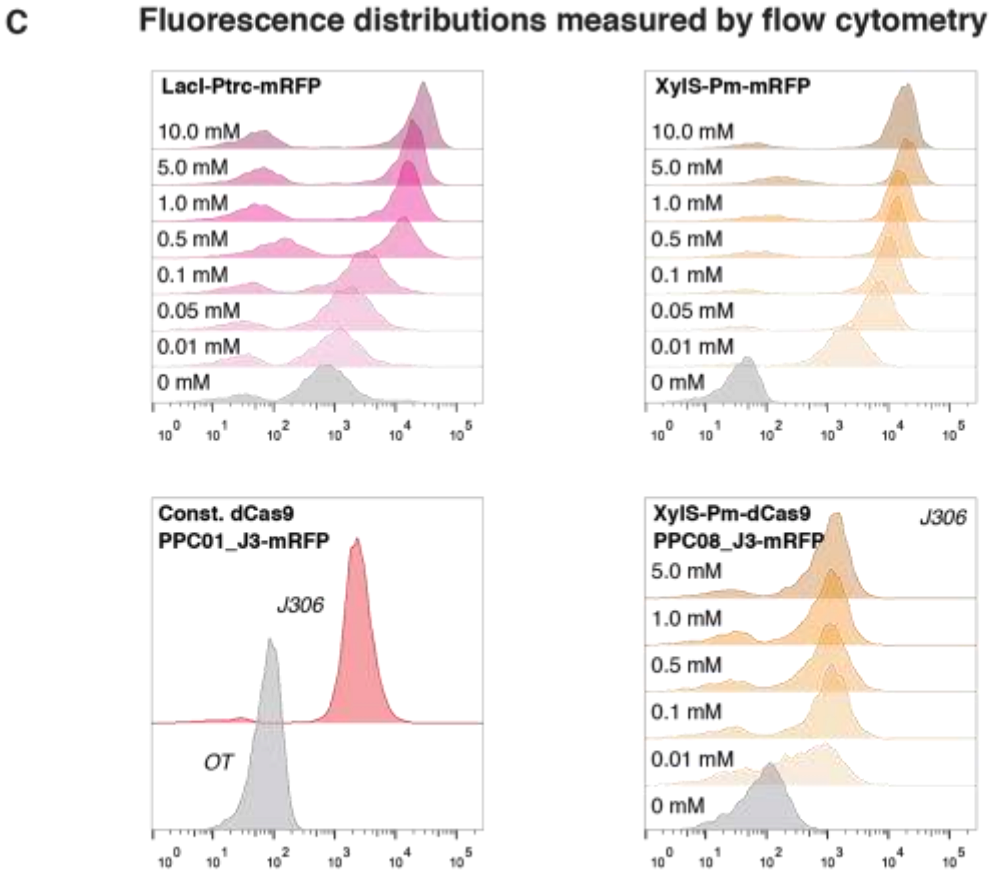

C
Fluorescence distributions measured by flow cytometry
LacI-Ptrc-mRFP
10.0 mM
5.0 mM
1.0 mM
0.5 mM
0.1 mM
0.05 mM
0.01 mM
0 mM
XylS-Pm-mRFP
Const. dCas9
PPC01_J3-mRFP
J306
OT
XylS-Pm-dCas9
PPC08_J3-mRFP

Figure S10: Inducible promoter in *P. putida* (A) Inducible LacI-Ptrc-mediated activation of an mRFP reporter gene in *P. putida* KT2440 with 0-1 mM IPTG. We observed 13-fold activation at 1 mM IPTG compared to the no inducer condition. (B) Inducible XylS-Pm-mediated activation of an mRFP reporter gene in P. putida KT2440 with 0-5 mM *m*-toluic acid. We observed >300-fold activation at 1 mM *m*-toluic acid compared to the no inducer condition. The XylS-Pm promoter provides a better dynamic range compared to LacI-Ptrc promoter mainly due to its relatively low basal expression. (C) mRFP reporter gene fluorescence distributions measured by flow cytometry. At every inducer concentration, the LacI-Ptrc promoter demonstrated higher variability within the population than XylS-Pm. We observed bimodal population distributions with LacI-Ptrc, suggesting that this promoter is unstable in *P. putida*. No bimodal distributions were detected with constitutive CRISPRa, and only a small bimodal population was observed with XylS-Pm inducible mRFP or dCas9. The strains shown for constitutive CRISPRa are on-target (J306) and off-target (OT) in the PPC01 background. For inducible CRISPRa, the strain background is PPC08. Values in panel A represent the mean ± standard deviation calculated from $n = 6$ independent biological replicates.. Values in panel B represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates. Values in panel C represent the data (n =1) from different inducer concentrations or scRNAs.

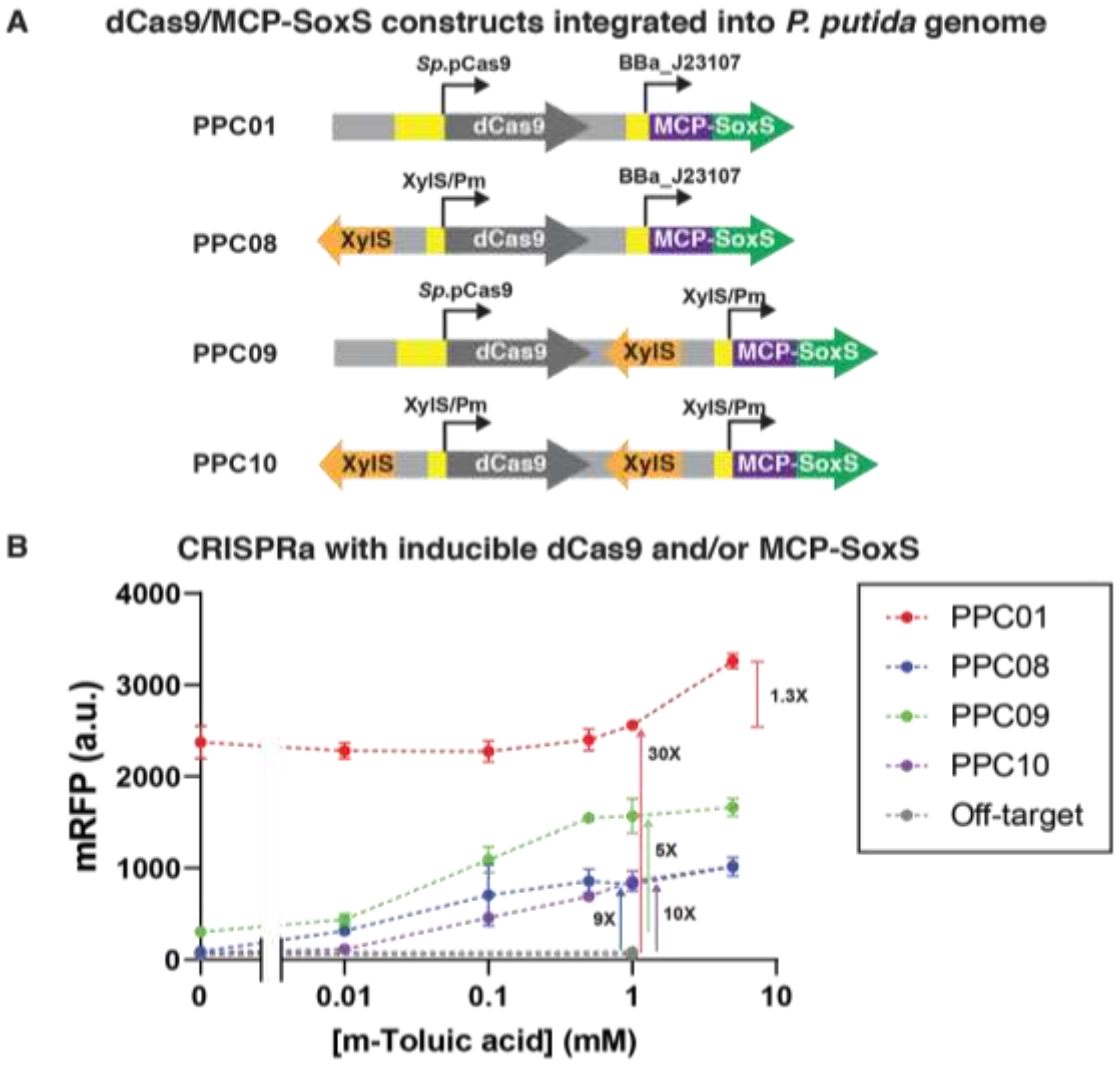


Figure S11: Inducible CRISPRa by XylS-Pm on CRISPRa machinery. (A) Inducible dCas9/MCP-SoxS constructs were integrated into the *P. putida* genome with the mini-Tn7 method. XylS-Pm promoters were introduced in place of the *Sp*.pCas9 promoter regulating dCas9 and/or the J23107 promoter for MCP-SoxS. (B) Strains were transformed with a vector (pPPC020) carrying either an off-target scRNA or a J306 scRNA. The fold-activation in the presence of *m*-toluic acid as an inducer are 9-fold, 5-fold, and 10-fold from PPC08, PPC09, and PPC10, respectively. Strains with inducible dCas9 only, or both dCas9 and MCP-SoxS inducible, showed minimal leaky activation in the absence of inducer. If only MCP-SoxS is under control of the inducible promoter, leaky activation is detectable. In a strain with constitutively expressed CRISPRa machinery that should not be responsive to inducer, high inducer concentrations (5 mM) also led to a modest increase in mRFP expression (1.3-fold). Values in panel B represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates.

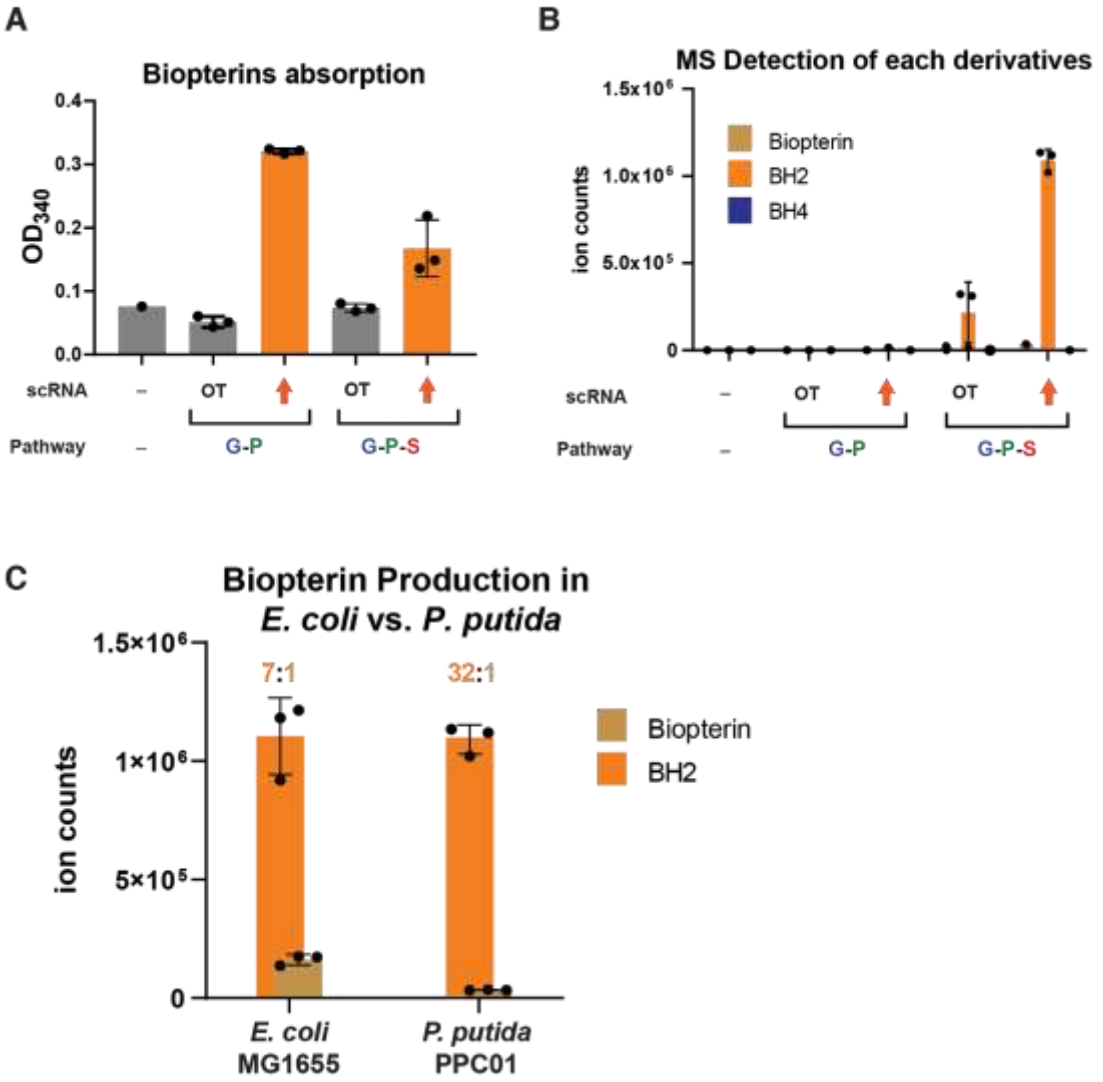


Figure S12: Biopterins Production in *P. putida* with CRISPRa tool. (A) OD_{340} absorption of *P. putida* supernatant, which corresponds to absorption of dihydrobiopterin and biopterin. (B) The HPLC-MS signals of three biopterin derivatives with pathway genes regulated by different CRISPRa programs. (C) Comparison of biopterin and dihydrobiopterin (BH2) production from a CRISPR-activated tetrahydrobiopterin pathway in *E. coli* MG1655 (transformed with pCK015 and pCK005.AAV/pCD581 bearing hsAAV1/J306 scRNA) and *P. putida* PPC01 (transformed with pPPC027 bearing either hsAAV1 or J306 scRNA). The ratio of signal between BH2 and biopterin produced from *P. putida* (32:1) is higher than that of *E. coli* (7:1). Values in panel A represent the mean ± standard deviation calculated from $n = 3$ independent biological replicates. The no pathway control in panel A represents one ($n = 1$) sample. Values in panel B, and C represent the mean ± standard deviation calculated from $n = 3$ technical replicates.

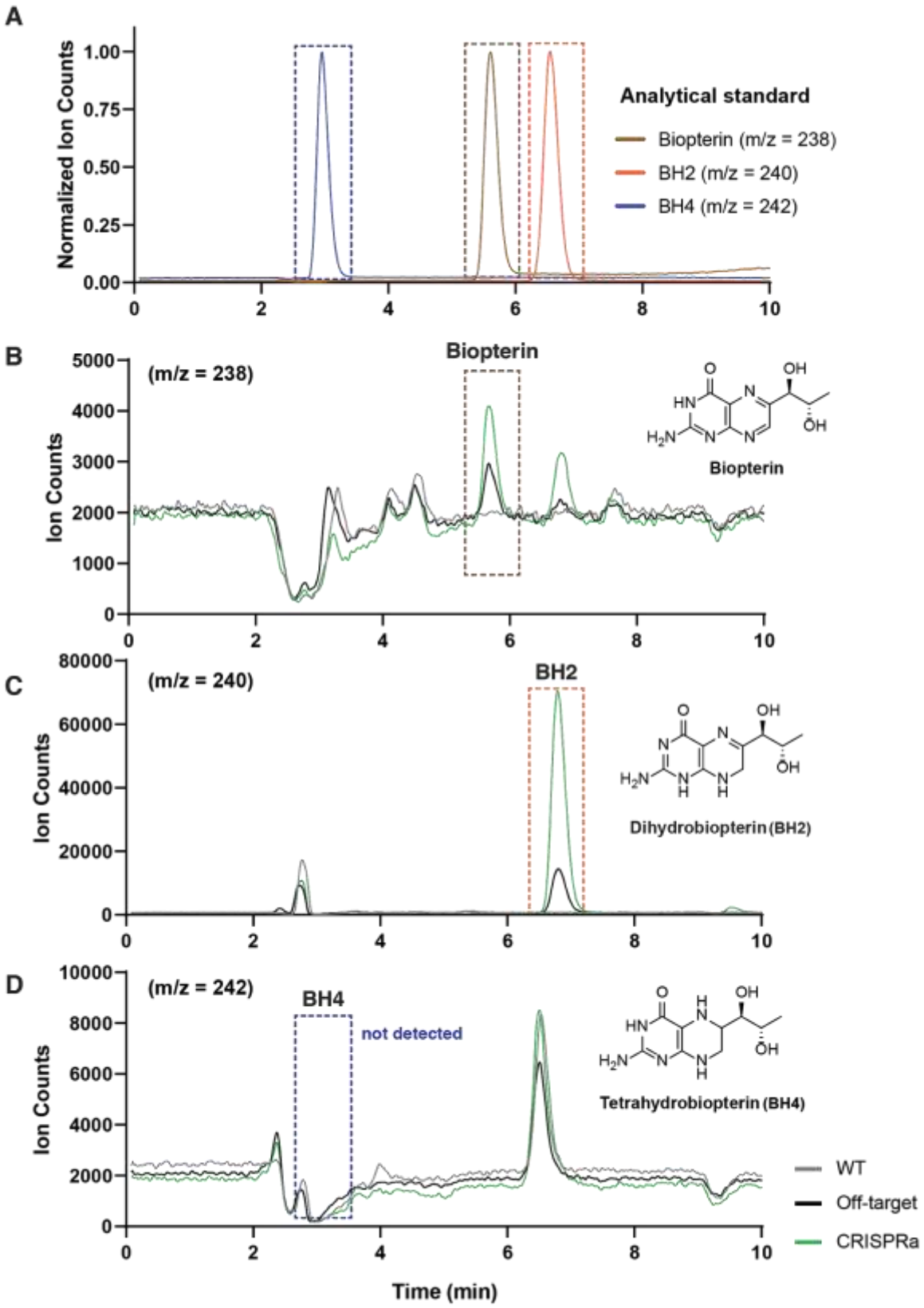

A
Normalized Ion Counts
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
Analytical standard
Biopterin (m/z = 238)
BH2 (m/z = 240)
BH4 (m/z = 242)
B
(m/z = 238)
Biopterin
Ion Counts
5000
4000
3000
2000
1000
0
Biopterin
C
(m/z = 240)
BH2
Ion Counts
80000
60000
40000
20000
0
Dihydrobiopterin (BH2)
D
(m/z = 242)
BH4
not detected
Ion Counts
10000
8000
6000
4000
2000
0
Tetrahydrobiopterin (BH4)
WT
Off-target
CRISPRa
Time (min)

Figure S13: HPLC-MS Spectra of Biopterin Products in *P. putida*. (A) Overlaid chromatograms of commercial standards for biopterin, BH2, and BH4 normalized to maximum signal of each corresponding ion count. (B-D) Biopterin pathway outputs from a *P. putida* strain with CRISPRa-mediated activation of the metabolic pathway. Panels correspond to m/z ion count signals for Biopterin (B), BH2 (C), and BH4 (D). The parental strain KT2440 (grey) was used as a negative control (no heterologous pathway). PPC01 carrying pPPC027 with a J306 scRNA (green) showed significant improvement in BH2 product compared to that of an off-target scRNA (black). BH4 (r.t. ~ 3 min) was not observed in any tested condition.

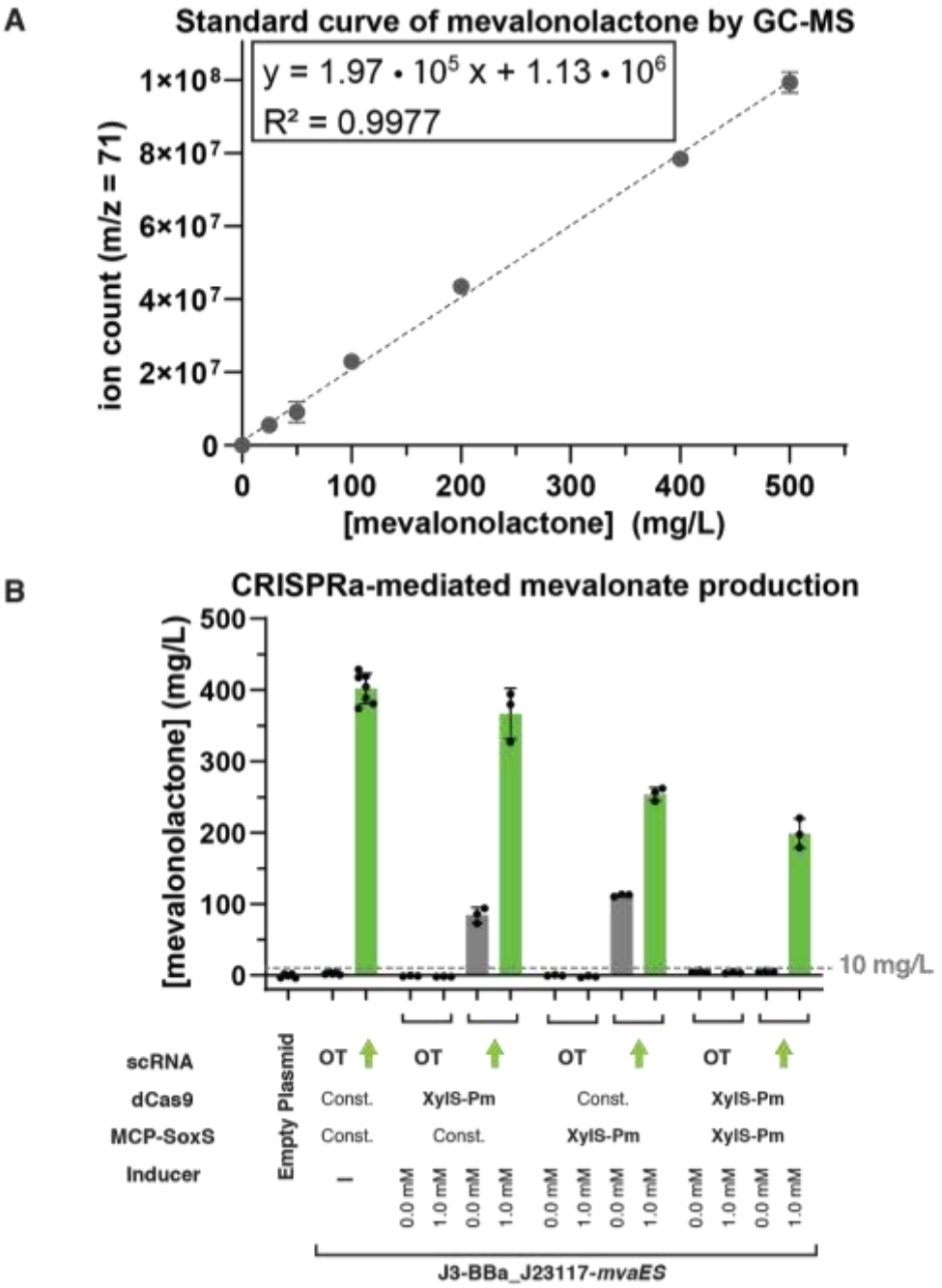


Figure S14: GC-MS Detection of Mevalonic Acid. (A) A representative standard curve of mevalonolactone in ethyl acetate at different concentrations (0, 25, 50, 100, 200, 400, and 500 mg/mL) measured by GC-MS at m/z = 71. (B) CRISPRa-mediated mevalonate production with additional off-target controls (see also main text Figure 7). Strains with an off-target scRNA yielded mevalonate levels that were indistinguishable to that of an empty plasmid control (less than 10 mg/L). Values in panel A represent the mean ± standard deviation calculated from *n = 3* technical replicates. Values in panel B represent the mean ± standard deviation calculated from *n = 3* independent biological replicates, *n = 5* for the no plasmid control and off-target scRNA of constitutively expressed dCas9/MCP-SoxS strain, and *n = 7* for the J306 scRNA.

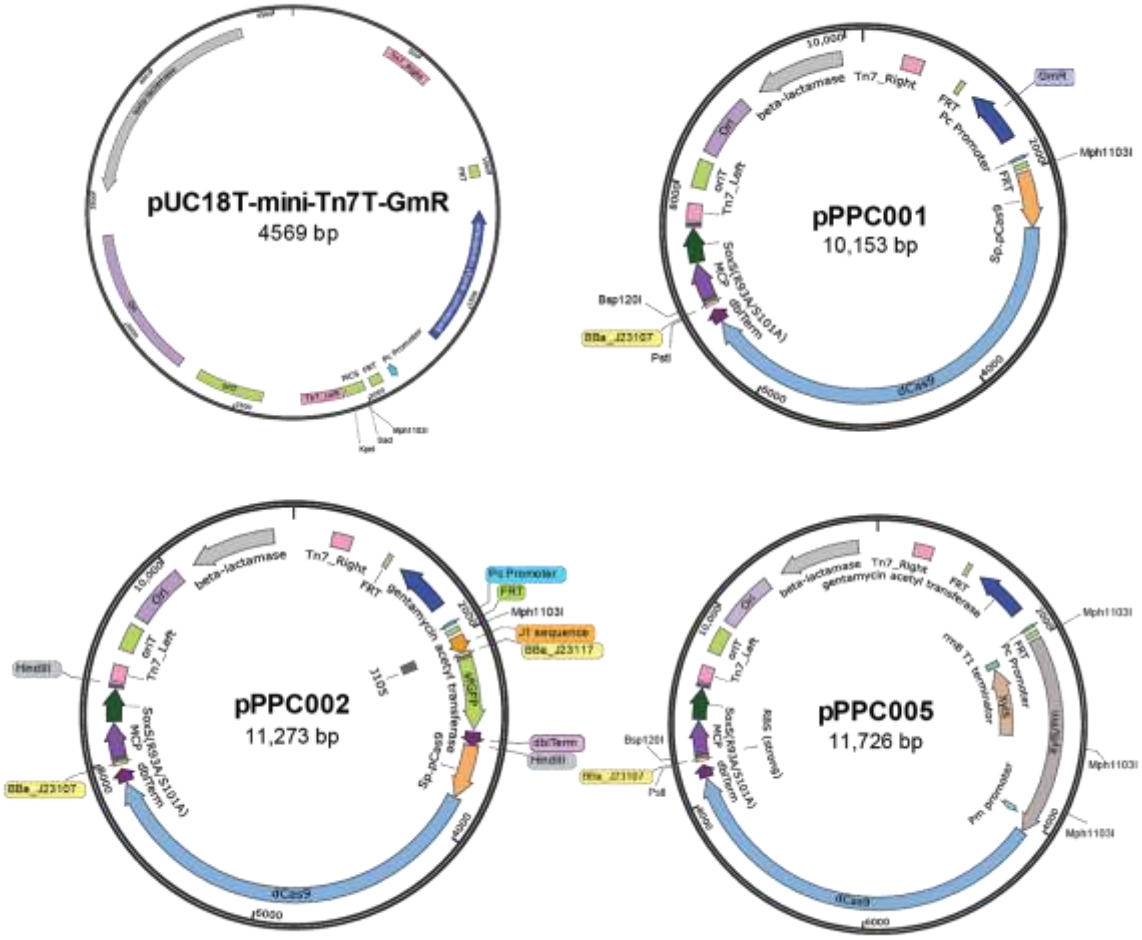


Figure S15: Mini-Tn7T Plasmid Maps. Selected examples of integration plasmid maps with labeled important parts and restriction sites of pPPC001, pPPC002, and pPPC005.

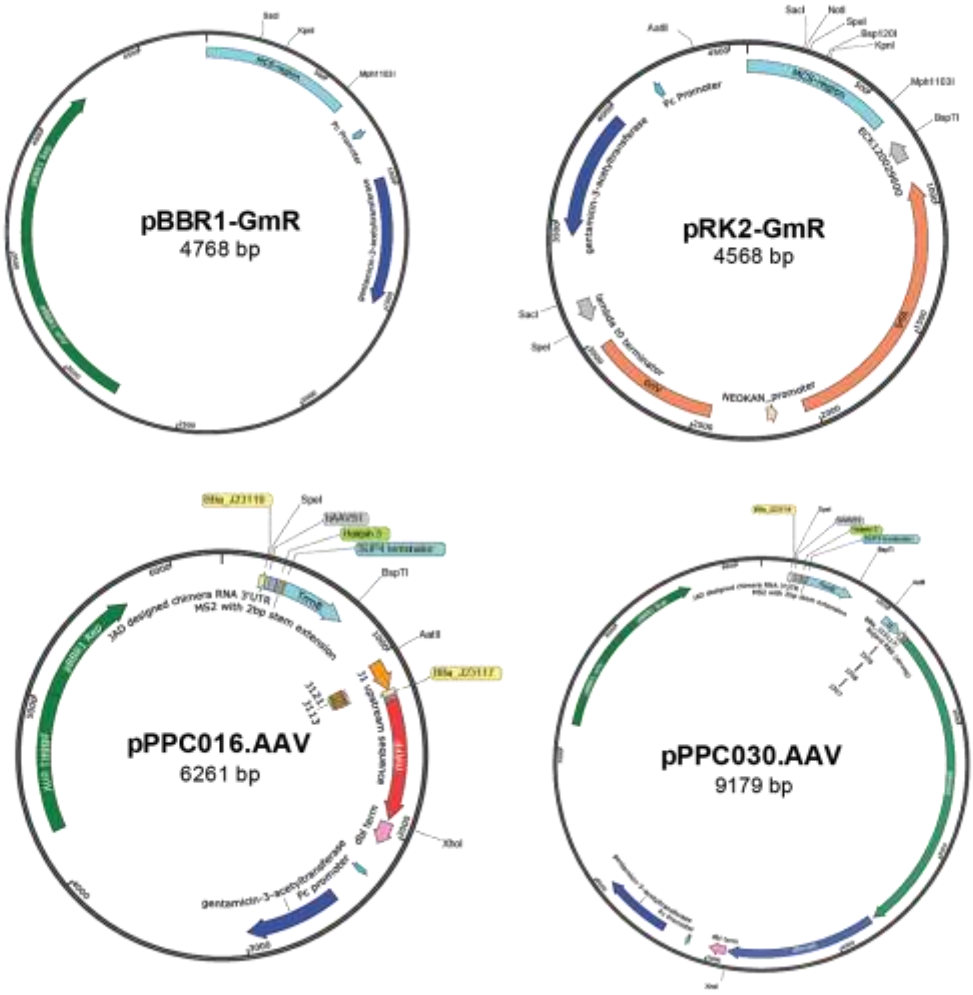


Figure S16: Replicable Plasmid Maps. Selected examples of replicable plasmid maps with labeled important parts and restriction sites of pBBR1-GmR, pRK2-GmR, pPPC016, and pPPC030.

Table S1: Description of bacterial strains and plasmids used in each figure

Figures	Strains	Plasmid	scRNA target (or sgRNA)
1C	KT2440, PPC01	pPPC010 + pPPC014, pPPC014, pPPC016	hAAVS1, J109
2B	PPC02	pPPC008	hAAVS1, J101-121
2C	PPC02, PPC03.1-12	pPPC008	hAAVS1, J106
2D	PPC01	pPPC016, pPPC020, pPPC020.106, pPPC016.306,	hAAVS1, J106, J306
3A	PPC01	pPPC020, pPPC021.J231XX (10 promoters)	hAAVS1, J306
3B	PPC01	pPPC022.5PS	J306
3C	PPC01, MG1655	pPPC023.5PSN (PS1 to PS5), co-transform with pCD442 in *E. coli*	hAAVS1, J306
3D	PPC01, MG1655	pPPC016, pPPC020, pPPC021.J231XX, pPPC023.5PSN *E. coli* plasmids according to (Fontana et al., 2020)	hAAVS1, J106, J306
4	PPC01	pPPC024	hAAVS1, J306, jfGFP1, J306-jfGFP1, J106, J306-J106
5A	PPC01	pPPC026.XN	hAAVS1, AN-JN as listed in Table S4
5B	PPC01, PPC08	pPPC021.J23110	hAAVS1, J306, RR2
6D	PPC01	pPPC027, pPPC028	hAAVS1, J306
7C	KT2440, PPC01, PPC08, PPC09, PPC10	pBBR1-GmR, pPPC029, pPPC030	hAAVS1, J306
S1	KT2440	pPPC012, pPPC013, pPPC014, pPPC015, pPPC016, pPPC017, pPPC018, pPPC019	hAAVS1
S2A	KT2440, PPC01	pPPC010 + pPPC014, pPPC011 + pPPC016, pPPC019, pPPC018, pPPC017, pPPC016	hAAVS1, J109
S2B	KT2440, PPC01	pPPC010 + pPPC014, pPPC011 + pPPC016, pPPC016, pPPC017, pPPC016 + pRK2-KmR	hAAVS1
S3	PPC01	pPPC016, pPPC017, pPPC018, pPPC019, pBBR1-GmR, pBBR1-KmR, pRK2-GmR, pRK2-KmR	hAAVS1, J109
S4	PPC01	pPPC016, pPPC020	hAAVS1, J106, J107, J108, J109, J306, J307, J308, J309
S5A	KT2440, PPC01	pPPC022.5PS	J306
S5B	PPC01, MG1655	pPPC016, pPPC020, pPPC021.J231XX, pPPC023.5PSN *E. coli* plasmids according to (Fontana et al.,	hAAVS1, J106, J306

		2020)	
S6	PPC01	pPPC025	hAAVS1, J306, J106, J306-J106, J306-hAAVS1
S7A	PPC04	pBBR1-KmR, pPPC009	hAAVS1, J106, RR2, hAAVS1-RR2, J106-RR2
S7B	PPC05	pBBR1-GmR, pPPC008	hAAVS1(sgRNA), hAAVS1, J306, jfGFP1, hAAVS1-jfGFP1, J306-hAAVS1(sgRNA), J306-hAAVS1, J306-jfGFP1
S8A	PPC06	pBBR1-GmR, pPPC008	hAAVS1, J306, J106, J306-J106, J306-hAAVS1
S8B	PPC07	pBBR1-GmR, pPPC008	hAAVS1(sgRNA), hAAVS1, J306, J106, jfGFP1, J306-hAAVS1(sgRNA), J306-hAAVS1, hAAVS1(sgRNA)-J106, hAAVS1-jfGFP1, J306-J106, J306-jfGFP1
S9	PPC01	pPPC026.XN	hAAVS1, AN-JN as listed in Table S4
S10A	KT2440	pCK241	
S10B	KT2440	pCK243	
S10C	KT2440, PPC01, PPC08	pCK241, pCK243, pPPC020	hAAVS1, J306
S11	PPC01, PPC08, PPC09, PPC10	pPPC020	hAAVS1, J306
S12A, S12B	PPC01	pPPC027, pPPC028	hAAVS1, J306
S12C	PPC01, MG1655	pPPC027, pCD442, pCD581, pCK015	hAAVS1, J306
S13	KT2440, PPC01	pPPC027	hAAVS1, J306
S14	KT2440, PPC01, PPC08, PPC09, PPC10	pBBR1-GmR, pPPC030	hAAVS1, J306

Table S2: Description of biological parts in each plasmid

Integration plasmid	**Backbone**	**Mph1103I**	**SacI/KpnI**	**HindIII**
pPPC001	pUC18T-miniTn7T-Gm		*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS	
pPPC002	pUC18T-miniTn7T-Gm		J1-BBa_J23117-sfGFP	*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS
pPPC003.N	pUC18T-miniTn7T-Gm		J1(+N)-BBa_J23117-sfGFP	*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS
pPPC004	pUC18T-miniTn7T-Gm	BBa_J23111-mRFP	J1-BBa_J23117-sfGFP	*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS
pPPC005	pUC18T-miniTn7T-Gm		XylS-Pm-dCas9/BBa_J23107-MCP-SoxS	
pPPC006	pUC18T-miniTn7T-Gm		*Sp*.pCas9-dCas9/XylS-Pm-MCP-SoxS	
pPPC007	pUC18T-miniTn7T-Gm		XylS-Pm-dCas9/XylS-Pm-MCP-SoxS	
Integration plasmid	**Backbone**	**Integration site**	**Gene Island**	
pGNW2-pp1	pGNW2	pp1		
pPPC031	pGNW2	pp1	J3-BBa_J23117-mRFP	
pGNW2-pp2	pGNW2	pp2		
pPPC032	pGNW2	pp2	BBa_J23111-sfGFP	
pPPC033	pGNW2	pp2	J3(106)-BBa_J23117-sfGFP	
pPPC034	pGNW2	pp2	J3(106)-BBa_J23111-sfGFP	
Replicable plasmid	**Backbone**	**SacI/KpnI (NotI/Bsp120I)**	**Mph1103I (AatII/XhoI or KpnI/XhoI)**	**scRNA or sgRNA**
pPPC008	pBBR1-GmR	scRNA, sgRNA, scRNA/sgRNAs		hAAVS1, J101-121, J306, jfGFP1, hAAVS1(sgRNA), hAAVS1-jfGFP1, J306-hAAVS1(sgRNA), J306-hAAVS1, J306-jfGFP1,

				J306-J106, hAAVS1(sgRNA)-J106
pPPC009	pBBR1-KmR	scRNA, sgRNA, scRNA/sgRNAs		hAAVS1, J106, RR2, hAAVS1-RR2, J106-RR2
pPPC010	pBBR1-KmR	scRNA	*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS	hAAVS1, J109,
pPPC011	pRK2-KmR		*Sp*.pCas9-dCas9/BBa_J23107-MCP-SoxS	
pPPC012	pBBR1-GmR		J1-BBa_J23117-mRFP	
pPPC013	pBBR1-KmR		J1-BBa_J23117-mRFP	
pPPC014	pRK2-GmR		J1-BBa_J23117-mRFP	
pPPC015	pRK2-KmR		J1-BBa_J23117-mRFP	
pPPC016	pBBR1-GmR	scRNA	J1-BBa_J23117-mRFP	hAAVS1, J106,J107, J108, J109
pPPC016.306	pBBR1-GmR	scRNA	J1-BBa_J23117-mRFP (swap J106 to J306)	hAAVS1, J306
pPPC017	pBBR1-KmR	scRNA	J1-BBa_J23117-mRFP	hAAVS1, J109
pPPC018	pRK2-GmR	scRNA	J1-BBa_J23117-mRFP	hAAVS1, J109
pPPC019	pRK2-KmR	scRNA	J1-BBa_J23117-mRFP	hAAVS1, J109
pPPC020	pBBR1-GmR	scRNA	J3-BBa_J23117-mRFP	hAAVS1, J306, J307, J308, J309
pPPC020.106	pBBR1-GmR	scRNA	J1-BBa_J23117-mRFP (swap J306 to J106)	hAAVS1, J106
pPPC021.J231XX	pBBR1-GmR	scRNA	J3-BBa_J231XX-mRFP where BBa_J231XX refers to either of J23109, J23113, J23114, J23115, J23107, J23105, J23106, J23108, J23111	hAAVS1, J306
pPPC021.J23110	pBBR1-GmR	scRNA	J3-BBa_J23110-mRFP	hAAVS1, J306, RR2
pPPC022.5PS	pBBR1-GmR	scRNA	J3(random-5PS)-BBa_J23117-mRFP	J306
pPPC023.5PSN	pBBR1-GmR	scRNA	J3(5PSN)-BBa_J23117-mRFP	hAAVS1, J306
pPPC024	pBBR1-GmR	scRNA	J3(106)-BBa_J23111-sfGFP_J3-BBa_J23117-mRFP	hAAVS1, J306, jfGFP1, J306-jfGFP1, J106, J306-J106

pPPC025	pBBR1-GmR	scRNA	J3(106)-BBa_J23117-sfGFP_J3-BBa_J23117-mRFP	hAAVS1, J306, J106, J306-J106, J306-hAAVS1
pPPC026.XN	pBBR1-GmR	scRNA	PP_NNNN-mRFP where PP_NNNN refers to 10 endogenous promoters as listed in DNA Sequences	hAAVVS1, AN-JN as listed in Table S4
pPPC027	pBBR1-GmR	scRNA	J3-BBa_J23117-GTPCH, J3-BBa_J23117-PTPS, J3-BBa_J23117-SR	hAAVS1, J306
pPPC028	pBBR1-GmR	scRNA	J3-BBa_J23117-GTPCH, J3-BBa_J23117-PTPS	hAAVS1, J306
pPPC029	pBBR1-GmR		LacI-Ptrc-mvaE-mvaS	
pPPC030	pBBR1-GmR	scRNA	J3-J23117-mvaE-mvaS	hAAVS1, J306
pCK241	pBBR1-GmR		LacI-Ptrc-mRFP	
pCK243	pBBR1-GmR		XylS-Pm-mRFP	
pCK255	pBBR1-GmR		I-SceI_sacB	

Table S3: Cloning Primers

Name	Sequence	Descriptive name
oCDP057	ATTCGATCATGCATGTTACGAAATCATCCTGTGGAGCTT	pPPC001_*Sp*.pCas9_F
oCDP058	CAAGGCCTTCGCGAGGCGAAAAAACCCCGCCG	pPPC001_BBa_B1002_R
oCDP003	attcgatcatgcatgCTGCAGGCCTACGGTATCCACCGG	pPPC002_J1
oCDP002	caaggccttcgcgagAAGCTTtataaacgcagaaaggcccac	pPPC002_dblTerm_R
oCDP021	tgcgtttataAAGCTTTTACGAAATCATCCTGTGGAGC	pPPC002_*Sp*.pCas9_F
oCDP022	ccttcgcgagAAGCTTgcgaaaaaaccccgccg	pPPC002_BBa_B1102_R
oCDP061	AAGCTAATTCGATCatgcatttgacggctagctcagtcc	pPPC003_BBa_J23111_F
oCDP062	CGTAGGCCTGCAGcatataaacgcagaaaggcccacc	pPPC003_dblTerm_F
oCK257	gaagctaattcgatcatgcatgatttgtcctactcaggagagcg	pPPC005_XylS_F
oCK258	attgagtatttcttatccatatgtttttcctcc	pPPC005_Pm_R
oCK259	cggtttgcgtattgggcgcaatttgtcctactcaggagagcg	pPPC006_XylS_F
oCK260	ACGTCTTCGCTACTCGCCATatgtttttcctcctaaccgcg	pPPC006_Pm_R
oCK101	ccagctggcaattccgacgtcgtcgaatttgctttcgaatttctgc	pRK2-GmR_MCS_F
oCK102	aagaccggcggtcttaagtttttttggctgaagaattcgcaaatattatacgcaaggcgac	pRK2-GmR_MCS_R
oCK085	aacaggagtccaagcgcatggaagccatcacaaacg	pRK2-KmR_KmR_F
oCK086_short	gacgtcggaattgccagctggg	pRK2-KmR_KmR_R
oCDP023	tatagggcgaattggagctcTTGACAGCTAGCTCAGTCC	pPPC008_scRNA_F
oCDP008	gggaacaaaagctggTCTAGCTTAAGAGTTCACCGACAAACAACAGATA	pPPC008_scRNA_R
oCDP056	TCGGTGAACTCTTAAcGGATCCTTGACAGCTAGCTCAGTCCTAGG	2nd-gRNA_F
oCDP051	gctggTCTAGCTTAAGAGTTCACCGACAAACAACAGAT	2nd-gRNA_R
oCK079	GCTCAGTCCTAGGTATAATACTAGT	change-gRNA_F
oCK287	TAGGTATAATACTAGTNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGT	new-gRNA_F
oCK077	ttgcgtattgggcgcaTTACGAAATCATCCTGTGGAGCTT	pPPC010_*Sp*.pCas9_F
oCK078	attacaacagtttttagcgaaaaaaccccgccg	pPPC010_*Sp*.pCas9_R
oCK097	ttgcgtattgggcgcatggcaattccgacgtc	pPPC012_J1_F
oCK098	attacaacagtttttaTATAAACGCAGAAAGGCCC	pPPC012_dblTerm_R
oCK237	GCGTTCTGGACACAATTGGGTTCCACCGGATACCTCCGGACttgacagctagctcagtcc	pPPC016_J106-to-J306_F

oCK279	TTGTGTCCAGAACGCTCCGTAGGACACCGCAGGATACCTGAGGTCGCCCG	pPPC016_J106-to-J306_R
oCK130	ccaccgcggtggcggccgcTTGACAGCTAGCTCAGTCCTAG	pPPC018_gRNA_F
oCK146	agctgggtaccgggcccAGTTCACCGACAAACAACAGATA	pPPC018_gRNA_R
oJF365	GCGGTTACCAAAGGCGTCCTCGTCGTCTTGAAGTTGCG	pPPC020_J306-to-J106_F
oJF366	GCCTTTGGTAACCGCAGGAGAAGTGAGGAGACGAGC	pPPC020_J306-to-J106_R
oCK177	ttgggcgcatggcaattccg	pPPC021_J3_F
oCK219	GCCTGGagatccttactcga	pPPC021_dblTerm_R
oJF447	GCGTTCTGGACACAANNNNNNNNNNNNNNNNNNNNNNNNNNNNttgacagctagctcagtcc	pPPC022_BBa_J23117_F
oJF448	TTGTGTCCAGAACGCTCCGTAGGAGAAG	pPPC022_J3_R
oCK084	cggtgcttaaaaactcgagtaaggatctCCAGG	pPPC022_dblTerm_F
oBT110	gctagcactatacctaggactgagctagccgtcaa	pPPC024_BBa_J23111_R
oBT072	CAGTCCTAGGTATAGTGCTAGCGAATTCATTAAAGAGGAG	pPPC024_BBa_J23111-RBS_F
oCK383	ATGATCGCAAATGCTgAGTACTtataaacgcagaaaggcccacccg	pPPC024_dblT_R
oCDP059	TTAAAGAGGAGAAAGGTACCATGGCGAGTAGCGAAGACG	pPPC026_mRFP_F
oCK251	tgcgcccaatacgcaaaccg	pPPC026_MCS_R
oCK253	cggtttgcgtattgggcgcagttctcagggctcgccgaga	pPPC026_PP_1776-A_F
oCK354	GGTACCTTTCTCCTCTTTAATGAATTCtgatactggccacagacggct	pPPC026_PP_1776-A_R
oCK169	gcgcatggcaattccgatatcAGCATTT	pPPC027_J3_F
oCK170	GGagatccttactcgagtttTTATTCGTCGTAGAAATCAATGTGG	pPPC027_SR_R
oCK073	ccagctggcaattccgacgtc	pPPC029_LacI_F
oCK072_Short	agatccttactcgagtttttaattacgatagctacgcacgg	pPPC029_mvaS_R
oCDP046	TTAAAGAGGAGAAAGGTACCatgaaaaccgtggtgattattgatg	pPPC030_mvaE_F
oCK325	tgcctgcaggtcgactctagatgaccgacctgatcgaagtgaagac	pGNW2-pp1_HR1_F
oCK326	atggcggATGCATgggctcggttctctactggcg	pGNW2-pp1_HR1_R
oCK327	cgagcccATGCATccgccattacgatctgacttgcc	pGNW2-pp1_HR2_F
oCK328	ataacagggtaatctgaatttcacttcactcgggaaaaatcagggg	pGNW2-pp1_HR2_R
oCK344	ccagtagagaaccgagcccAgcatggcaattccgacgtc	pPPC031_J3_F
oCK345	agtcagatcgtaatggcggATATAAACGCAGAAAGGCCC	pPPC031_dblT_R
oCK369	tgcctgcaggtcgactctagaccaggatgaataccttaaggacgcc	pGNW2-pp2_HR1_F
oCK370	acaccttATGCATgcgcgtgatgcgctgcacac	pGNW2-pp2_HR1_R
oCK371	cacgcgcATGCATaaggtgtattcccccggcattca	pGNW2-pp2_HR2_F

oCK372	ataacagggtaatctgaatttccgctcggcgcgtgatccgc	pGNW2-pp2_HR2_R
oCK373	agcgcatcacgcgcATGCATttgacggctagctcagtcctaggt	pPPC032_BBa_J23111_F
oCK374	cgggggaatacaccttAGTACTtataaacgcagaaaggcccacccg	pPPC032_dblT_R
oCK384	cagcgcatcacgcgcATGCATAGCATTTGCGATCATTCACGCAGC	pPPC033_J3_F
oCDP004	GGTACCTTTCTCCTCTTTAATGAATTC	RBS_R
oCD292	GAATTCATTAAAGAGGAGAAAGGTACC	RBS_F
oCD281	ATGGCGAGTAGCGAAGACGT	mRFP_F
oCK320	TTCGCTACTCGCCATGGTACCTTTCTCCTCTTTAATGAATTCtgaaattgtt atccgctc	Ptrc_RBS_R
oCK259	cggtttgcgtattgggcgcaatttgtcctactcaggagagcg	XylS-Pm_F
oCK277	GGTACCTTTCTCCTCTTTAATGAATTCttgcataaagcctaaggggtaggcc ttactaga	Pm_RBS_R

Table S4: Target sequences of scRNA and sgRNA

Name	Sequence	Target Promoter/Gene	Target Strand	Distance to TSS
sgRNA	NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT GGCACCGAGTCGGTGCTTTTTTT			
scRNA_1x MS2.b2	NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT GGCACATGAGGATCACCCATGTGCTTTTTTT			
hAAVS1	GGGGCCACTAGGGACAGGAT	Off-target	N.A.	N.A.
RR2	TGGAACCGTACTGGAACTGC	mRFP (CRISPRi)	Template	185 from ATG
jfGFP1	CATCTAATTCAACAAGAATT	sfGFP (CRISPRi)	Template	38 from ATG
J101	TGGGTTCCACCGGATACCTC	J1	Non-template	40
J102	AGGTATCCGGTGGAACCCAA	J1	Template	61
J103	AGGCGTCCTTTGGGTTCCAC	J1	Non-template	50
J104	TGGAACCCAAAGGACGCCTT	J1	Template	71
J105	CGGTTACCAAAGGCGTCCTT	J1	Non-template	60
J106	AGGACGCCTTTGGTAACCGC	J1, J3(106)	Template	81
J107	CGGTGTCCTGCGGTTACCAA	J1	Non-template	70
J108	TGGTAACCGCAGGACACCGC	J1	Template	91
J109	AGGTATCCTGCGGTGTCCTG	J1	Non-template	80
J110	AGGACACCGCAGGATACCTG	J1	Template	101
J111	GGGCGACCTCAGGTATCCTG	J1	Non-template	90
J112	AGGATACCTGAGGTCGCCCG	J1	Template	111
J113	GGGCCACCACGGGCGACCTC	J1	Non-template	100
J114	AGGTCGCCCGTGGTGGCCCA	J1	Template	121
J115	TGGTGACCATGGGCCACCAC	J1	Non-template	110
J116	TGGTGGCCCATGGTCACCAT	J1	Template	131
J117	GGGTGACCTATGGTGACCAT	J1	Non-template	120
J118	TGGTCACCATAGGTCACCCT	J1	Template	141
J119	TGGTTGCCAAGGGTGACCTA	J1	Non-template	130
J120	AGGTCACCCTTGGCAACCAA	J1	Template	151
J121	AGGACACCTTTGGTTGCCAA	J1	Non-template	140

J306	TTGTGTCCAGAACGCTCCGT	J3, J1(306)	Template	81
J307	ACTTCTCCTACGGAGCGTTC	J3	Non-template	70
J308	AACGCTCCGTAGGAGAAGTG	J3	Template	91
J309	TCGTCTCCTCACTTCTCCTA	J3	Non-template	80
A1	TTCATGTAGCTTGTCCCCCG	PP_1776-A	Template	82
A2	CCGACTGAAGATGCGCTCTC	PP_1776-A	Non-template	92
A3	ATGCGCTCTCTGGCGCTCCT	PP_1776-A	Non-template	82
A4	TGCGCTCTCTGGCGCTCCTC	PP_1776-A	Non-template	81
A5	GCGCTCTCTGGCGCTCCTCG	PP_1776-A	Non-template	80
A6	CGCTCTCTGGCGCTCCTCGG	PP_1776-A	Non-template	79
B1	ACTGGGATTTGTGTAGGAGC	PP_4812-B	Non-template	92
B2	GGGTTTACCCGCGAAAGGGC	PP_4812-B	Non-template	72
B3	GGCAGTGCCGGCCCTTTCGC	PP_4812-B	Template	82
B4	TGGCAGTGCCGGCCCTTTCG	PP_4812-B	Template	81
C1	AATGCGTGGTCGCTTAATCC	PP_3839-C	Non-template	82
C2	ATGCGTGGTCGCTTAATCCT	PP_3839-C	Non-template	81
C3	TAATCCTGGGTTAACCGGAC	PP_3839-C	Non-template	68
C4	TGCGCCGGTCCGGTTAACCC	PP_3839-C	Template	81
D1	GGCCCCTGCGCTGCGCTCCG	PP_1992-D	Non-template	72
D2	CAGCGCAGGGGCCGGATGAT	PP_1992-D	Template	99
D3	CCGGAGCGCAGCGCAGGGGC	PP_1992-D	Template	91
E1	TATCGATGAAATCGCAGCAT	PP_0786-E	Non-template	92
E2	CAGCATAGGCGATGCCTATG	PP_0786-E	Non-template	78
E3	CCTTAGACAATCCACCTCAT	PP_0786-E	Template	81
F1	AAAGCTGCGCCAGAGTGTCG	PP_1972-F	Non-template	69
F2	ACACTCTGGCGCAGCTTTTG	PP_1972-F	Template	89
F3	GACACTCTGGCGCAGCTTTT	PP_1972-F	Template	90
F4	CGACACTCTGGCGCAGCTTT	PP_1972-F	Template	91
G1	GGCGTCCTGGGCAAAGGGTA	PP_3668-G	Non-template	91
G2	CTGTGTATTGAAGCATGGCG	PP_3668-G	Non-template	68
G3	CACAGCCATACCCTTTGCCC	PP_3668-G	Template	103
H1	TATCCCACCCTCGCCATTTT	PP_5046-H	Non-template	88

H2	CCCTCGCCATTTTCGGGCAC	PP_5046-H	Non-template	81
H3	TGCCCGAAAATGGCGAGGGT	PP_5046-H	Template	102
H4	GTGCCCGAAAATGGCGAGGG	PP_5046-H	Template	101
H5	TGCATGCCAGTGCCCGAAAA	PP_5046-H	Template	92
I1	GGTTTTTGTAGTGCTTGTGC	PP_1231-I	Template	101
I2	GGGTTTTTGTAGTGCTTGTG	PP_1231-I	Template	100
I3	CAATCCAGCGATTACTAAAG	PP_1231-I	Template	80
I4	ACAATCCAGCGATTACTAAA	PP_1231-I	Template	79
J1	TGGGTATGGCAGGGGGATTT	PP_4701-J	Template	118
J2	GTGCTGGGAATGGGTATGGC	PP_4701-J	Template	108
J3	CCACGTGCTGGGAATGGGTA	PP_4701-J	Template	104

Table S5: Summary of CRISPRa-mediated fold-changes in gene expression and metabolite production

Description	Reporter gene/ Metabolic genes	scRNA	Fold-change	Figure
Activation of plasmid-bourne mRFP with J1 promoter using a 2-plasmid system	J1-mRFP (2-plasmid)	J109	1.6-fold	Figure 1C
Activation of plasmid-bourne mRFP with J1 promoter using a strain with integrated dCas9/MCP-SoxS (PPC01)	J1-mRFP (pBBR1-GmR)	J109	5-fold	Figure 1C
Activation of plasmid-bourne mRFP with J3 promoter	J3-mRFP (pBBR1-GmR)	J306	34-fold	Figure 2D
Dual activation of mRFP and sfGFP reporters (plasmid-bourne)	J3-mRFP (pBBR1-GmR)	J306	41-fold	Figure S6
	J3(106)-sfGFP (pBBR1-GmR)	J106	105-fold	Figure S6
Dual activation of mRFP and sfGFP reporters (genomically-integrated)	J3-mRFP (integrated)	J306	3-fold	Figure S8A
	J3(106)-sfGFP (integrated)	J106	24-fold	Figure S8A
Activation of plasmid-bourne endogenous promoter reporter constructs (mRFP)	PP_1776-A-mRFP (pBBR1-GmR)	A2	1.7-fold	Figure 5A
	PP_1992-D-mRFP (pBBR1-GmR)	D3	1.7-fold	Figure 5A
	PP_0786-E-mRFP (pBBR1-GmR)	E1	2.5-fold	Figure 5A
	PP_3668-G-mRFP (pBBR1-GmR)	G2	2.8-fold	Figure 5A
Metabolite production upon activation of biopterin pathway genes	J3-GTPCH, J3-PTPS, J3-SR (pBBR1-GmR)	J306	5-fold	Figure 6D
Metabolite production upon activation of mevalonate pathway genes	J3-mvaES (pBBR1-GmR)	J306	>40-fold	Figure 7B

Fold-change values were calculated relative to a strain with an off-target scRNA. The J1 and J3 promoters shown in this table contain the BBa_J23117 minimal promoter.

Supplementary Methods

Media and Chemicals

E. coli and *P. putida* culture and engineering were generally performed in LB media. *Pseudomonas* isolation agar (Difco) was used in the tri-parental mating for mini-Tn7 cloning (Choi and Schweizer, 2006). Fluorescent protein reporter gene activation and metabolic engineering experiments were performed in EZ rich-defined media (Teknova) with 0.2% glucose as the carbon source, unless specified. Appropriate concentration of antibiotics were included for plasmid maintenance: 100 μg/mL for carbenicillin, 25 μg/mL for chloramphenicol, 30 μg/mL for gentamicin, 30 μg/mL for kanamycin. For two-plasmid transformations in this work, the antibiotic concentration was reduced by half to 15 μg/mL each of gentamicin and kanamycin. IPTG was prepared in water as a 1 M stock solution prior to use. *m*-Toluic acid was prepared as a 0.5 M stock solution in 50% DMSO/water. Biopterin, BH2, and BH4 (Cayman Chemical) were stored in DMSO and diluted into water prior to use. D,L-mevalonolactone (Sigma) was freshly prepared in ethanol as a 20 mg/mL solution before dilution in ethyl acetate.

Plasmids construction strategy

Plasmids used in this study can be separated into genome integration plasmids and replicable plasmids. Integration plasmids were made based on pUC18T-mini-Tn7T-Gm. Replicable plasmids were constructed from pBBR1_MCS2 (named pBBR1-KmR in this study), pBBR1_MCS5 (named pBBR1-GmR in this study), and pRK2-AraE (Table 1). The AraE cassette from pRK2-AraE (bearing GmR marker) was replaced with multiple-cloning-site regions of pBBR1 to generate pRK2-GmR. pRK2-KmR was made by replacing the GmR marker and AraE cassette with KmR marker and its multiple-cloning-site from pBBR1-KmR. The CRISPRa components and genes of interest were incorporated into each backbone at the multiple-cloning-site region. The detailed methodology for construction of each backbone is provided below. Table S1 shows the list of strains and plasmids used in each figure. Plasmids descriptions are listed in Table S2.

MCP-SoxS(R93A/S101A) was used in this study and will be abbreviated as MCP-SoxS. Both dCas9 and MCP-SoxS were obtained from the pCD442 plasmid (Fontana et al., 2020). The 1xMS2 scRNA.b2 was used in this study with variable 20 bp target sequences (Dong et al., 2018). The fulll sequence of sgRNA and scRNA are provided in the DNA sequences section. Any plasmid with sg/scRNA have different 20bp target sequences according to Table S2. sg/scRNA sequences were provided in Table S4.

Integration plasmids

pPPC001 and pPPC005-007

For pPPC001, the dCas9/MCP-SoxS cassette was amplified from pCD442 and inserted into pUC18T-miniTn7T-Gm with KpnI/SacI. For pPPC005, the dCas9/MCP-SoxS coding sequence was amplified from pCD442 and inserted together with XylS-Pm as a promoter of dCas9, amplified from pS448-CsR (Wirth et al., 2019). Further modification of the MCP-SoxS promoter was achieved by digestion of pPPC001/pPPC05 with PstI/Bsp120I and XylS-Pm was inserted into the corresponding site to give pPPC006/007, respectively. See Figure S9 for representative plasmid maps.

pPPC002-004

For integration plasmids with the reporter gene included, J1-BBa_J23117-sfGFP was amplified from pJF076Sa (Fontana et al., 2020) and inserted into the KpnI/SacI site of pUT18T-miniTn7T-Gm. Then, dCas9/MCP-SoxS was added to the HindIII site to give pPPC002. For pPPC003.N, the J1(+N)-BBa_J23117-sfGFP fragments were amplified from pJF155.1-12 (Fontana et al., 2020) instead of pJF076Sa. In the case of pPPC004 with additional BBa_J23111-mRFP reporter, the corresponding reporter fragment was amplified from the pJF143.J3.J23111 (Fontana et al., 2020), with BBa_J23111 promoter instead of a BBa_J23117, and inserted into pPPC002 at the Mph1103I cut site.

Replicable plasmids

pRK2-GmR was made by digesting pRK2-AraE (containing GmR marker) with AatII/BspTI and the multiple-cloning-site (MCS) from pBBR1 was inserted into the pRK2 backbone. pRK2-KmR was made by digestion of pRK2-AraE with SacI/BspTI and insert KmR and MCS fragments from pBBR1-KmR. Then, the further modification of these plasmids followed the general manipulation at the MCS. See Figure S10 for representative plasmid maps.

scRNA (or sgRNA) was inserted into the replicable plasmid at the SacI/KpnI site of the MCS. Then, the reporter fragment was inserted at the Mph1103I region. The dCas9/MCP-SoxS cassette was inserted into Mph1103I. For pRK2-GmR and pRK2-KmR, the scRNA fragment was amplified from pPPC013 and inserted into pRK2 backbones at NotI/Bsp120I site due to conflicting SacI/KpnI cut sites in the pRK2 backbone.

To change the scRNA target sequence, the existing scRNA cassette was excised with SpeI/BspTI and the new scRNA fragment was inserted. To express multiple scRNAs from the same plasmid, additional scRNA (or sgRNA) cassettes can be inserted at the BspTI site. To generate a new scRNA fragment, any existing scRNA construct can be amplified with a forward primer binding at the promoter region, oCK079_GCTCAGTCCTAGGTATAATACTAGT. To introduce a new 20 base target sequence, a forward primer with the same overhang can be used, oCK287_TAGGTATAATACTAGTNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGT, where the variable 20nt in oCK287 can be replaced with the desired target sequence.

To insert J1-mRFP reporter cassettes, the PCR fragment was amplified from pJF076Sa (Fontana et al., 2020) as a template and cloned into the Mph1103I site. The J3-mRFP variants were constructed in the same manner with pJF143.J3 (Fontana et al., 2020) as a PCR template. To insert other genes of interest under control of J1 or J3 promoters, several approaches are available. AatII was introduced upstream of J1 and J3 sequences. KpnI was added at the end of strong RBS and XhoI was added between the stop codon and terminator. The desired cassette can be cloned into the Mph1103I site directly or inserted at the aforementioned sites. In our hands, biopterin pathway genes were inserted with AatII/XhoI using pCK015 (J3-GTPCH-J3-PTPS-J3-SR) and pCK014 (J3-GTPCH-J3-PTPS) as templates for pPPC027-028. LacI-Ptrc was added into AatII/XhoI and mvaES was added into KpnI/XhoI using pMVA2RBS035 as a template for PCR to give pPPC029-030 respectively.

pCK014 and pCK015 were analogs of pPPC028 and pPPC027, respectively, in pSC101** origin for *E. coli* experiment which can be double transformed with pCK005.AAV and pCD581 (Fontana et al., 2020). The *gtpch* gene was amplified from the *E. coli* MG1655 genome. *ptps* and *sr* from *M. alpina* were synthesized from GeneArt (Thermo-Fisher) with codon-optimization for

expression in *E. coli* using Gene Designer (Atum). Each J3-CDS was individually added into the reporter cassette. Then, an additional J3-CDS construct was inserted into the existing one at the EcoRV site (altered from AatII due to the presence of cut-site in *sr* gene) to get pCK014 and pCK015.

Changing scRNA target sequence of J1 or J3

To alter the scRNA 20 base target sequence, we used a single-fragment PCR to change the existing 20 bp target of J106 in pPPC016 to the desired J306 using oCK237/oCK279 (Table S3). Then, the fragment was treated with DpnI, gel purified, and circularized with Infusion. The same method as used for converting J306 to J106 with oJF365/oJF366.

Construction of 5'-proximal sequence library (pPPC022)

To generate a library of different 26bp sequences upstream of a minimal promoter, a fragment with randomized 26bp region (5'-PS-BBa_J23117-mRFP) was constructed with the oJF447 and oCK219 primers (Table S3). pPPC020 bearing J306 scRNA was linearized by PCR with oJF448/oCK084 and treated with DpnI to remove the parent vector. Then the linearized pPPC016 backbone fragment and a randomized 26bp library fragment were assembled with Infusion.

Construction of 5'-proximal sequence variants characterized in *E. coli* (pPPC023)

pPPC023 was constructed similar to pPPC022 as described above. Five oJF447 variants with known 26bp sequences (provided in the DNA Sequences section) were used to generate 5'-PSN-BBa_J23117-mRFP fragments (PS1 to PS5) for insertion into the linearized backbone.

Construction of dual reporter plasmids (pPPC024-025, pPPC031-034)

For the plasmid-based dual reporter for multi-gene CRISPRa with two strongly expressed fluorescent reporters, a J3(106)-BBa_J23117-sfGFP cassette was inserted at the AatII site of J3-BBa_J23117-mRFP (pPPC020) to generate pPPC024. The plasmid-based dual reporter for CRISPRi/a with weakly expressed mRFP and strongly expressed sfGFP was constructed by delivering J3(106)-BBa_J23111-sfGFP to pPPC020 to generate pPPC025. Multiple sgRNA/scRNA cassettes were delivered as described above in the Replicable plasmids section.

The genomically-integrated dual reporter strains were constructed by sequentially integrating separate mRFP and sfGFP reporters at different genomic sites. Plasmids, pGNW2-pp1 and pGNW2-pp2 were constructed from pGNW2 (Wirth et al., 2019) by addition of prophage1 (pp1) or prophage2 (pp2) regions into the XbaI/EcoRI site. Flanking homology sites (HR1 and HR2) were separated by an Mph1103I site for insertion of the desired heterologous gene. J3-BBa_J23117-mRFP was inserted into pGNW2-pp1 at the Mph1103I site to construct pPPC031. sfGFP constructs with different promoters were cloned into pGNW2-pp2 at the Mph1103I site to generate pPPC032-034.

Construction of endogenous promoter reporter (pPPC026)

The J3-BBa-J23117 reporter (pPPC020) was modified into an endogenous promoter reporter by replacing the J3-BBa_J23117 promoter with an intergenic region from each gene of interest. The intergenic region contained 60 bases from the ORF of interest on the 3' end. On the

5’ end, the intergenic region extended 60 bp into the next upstream ORF, following a previously reported strategy (Zaslaver et al., 2006). The mRFP cassette, along with its original strong RBS, was included downstream of the 60 bp fragment of the ORF of interest. Complete sequences are provided below.

$CaCl_2$ chemically competent cell preparation

The chemically competent cell preparation was adapted from a prior method (Zhao et al., 2013). From an overnight culture seeded from a single colony of a *P. putida* strain in LB, the cell suspension was 100-fold diluted into 50 mL LB without antibiotic in a 250 mL Erlenmeyer flask. The culture was incubated at 30 °C to OD_{600} = 0.8 - 1.0, transferred to 2 x 50 mL conical tubes, and placed on ice for 5 minutes. The cell suspension was centrifuged at 4 °C for 10 min at 5000 rpm. After discarding the supernatant, the cells were washed with an ice-cold solution of 50 mM $MgCl_2$ + 10 mM $CaCl_2$ twice. The final pellets were resuspended in 15% glycerol + 100 mM $CaCl_2$ solution to give chemically competent cells. The competent cells can be stored at -80 °C for a month with negligible loss of activity.

For transformation, 50 ng of a *P. putida* compatible plasmid was added to 100 µL of $CaCl_2$ chemically competent cells in a 1.5 mL microcentrifuge tube. Cells were mixed gently and incubated on ice for 30 minutes. The incubated competent cells were subjected to heat-shock at 42 °C for 3 minutes and cooled on ice for another 5 minutes. Then, 900 µL of LB was added to the competent cells and cultures were shaken at 30 °C for 1.5 hours. The outgrowth competent cells were spun down at 10000 rcf, room temperature for 1 minute. After discarding ~900 µL of supernatant, cells were resuspended in residual media for plating on a pre-warmed agar plate with appropriate antibiotic selection.

Biopterin Quantification by HPLC-MS

The LC-MS quantification was adapted from the prior method (Ehrenworth et al., 2015). LC−MS analysis was completed using an Agilent 1100/1260 series system equipped with a 1260 ALS autosampler and a 6120 Single Quadrupole LC−MS with a Poroshell 120 SB-Aq 3.0 mm × 100 mm × 2.7 µm column and an electrospray ion source. LC conditions: solvent A—150 mM acetic acid with 0.1% formic acid; solvent B—methanol with 0.1% formic acid. Gradient: 4 min ramp from 95%:5%:0.2 (A:B:flow rate in mL/min) to 70%:30%:0.2, 6 min ramp to 40%:60%:0.2, 2 min ramp to 2%:98%:0.2, 2 min ramp to 2%:98%:0.5, 4 min at 2%:98%:0.5, 1 min ramp to 95%:5%:0.5, 7 min at 95%:5%:0.5, and 1.5 min post time. MS acquisition (positive ion mode) included 25% scan from m/z 100−600, 25% scan from m/z 230−260, 25% scan from m/z 145−165, and 25% selective ion monitoring (SIM) for BH4 (m/z 242.1), dihydrobiopterin (m/z 240.1), and biopterin (m/z 238.1). Retention times were determined using commercially available standards (BH4, BH2, and biopterin from Cayman Chemical).

Determination of *P. putida* growth rate

Single colonies from LB plates were inoculated in 500 µL of EZ-RDM (Teknova) supplemented with the appropriate antibiotics and grown in 96-deep-well plates at 30 °C with shaking overnight. From the overnight cultures, OD_{600} of each replicate was measured in a 1-cm cuvette, then diluted to OD_{600} = 0.1 (30-50 fold dilution) and 200 µL of each diluted culture were

grown in flat bottom microplate at 30 °C in a Biotek Synergy HTX plate reader for 16 hours with continuous slow orbital shaking.

DNA sequences

The DNA sequence of biological parts used in this study were provided below in the 5'-to-3' format of the non-template strand. Each part was color-coded based on its function: red-promoter, blue-CDS, brown-upstream sequence, dark green-5'-UTR, purple-terminator, and others as specified. The **transcription start site** (TSS), **start codon** and **stop codon** are **bolded**.

>*Sp*.pCas9-dCas9-dblTerm

TTACGAAATCATCCTGTGGAGCTTAGTAGGTTTAGCAAGATGGCAGCGCCTAAATGTAGAATGATAAAAGGATTAAG
AGATTAATTTCCCTAAAAATGATAAAACAAGCGTTTTGAAAGCGCTTGTTTTTTTGGTTTGCAGTCAGAGTAGAATA
GAAGTATCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATG
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CAGCCATAAAACAATACTTAATACTATAGAATGATAACAAAATAAACTACTTTTTAAAAGAATTTTGTGTTATAATC
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>BBa_J23107-MCP-GGGGS_linker-SoxS (R93A, S101A) (5'-UTR)

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>XylS-Pm-CDS (5'-UTR)

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>J1-BBa_J23117-sfGFP (5'-UTR)

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>J3-BBa_J23111-mRFP (5'-UTR)

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GAAGACTACACCATCGTTGAACAGTACGAACGTGCTGAAGGTCGTCACTCCACCGGTGCT**TAA**

>J3-BBa_J23117-EcGTPCH (5'-UTR)

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C**ATG**CATCACCATCACCATCACCCATCACTCAGTAAAGAAGCGGCCCTGGTTCATGAAGCGTTAGTTGCGCGAGGAC
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GAAATCATGCAGCTGCTGAATCTCGACCTGGCTGATGACAGTTTGATGGAAACGCCGCATCGCATCGCTAAAATGTA
TGTCGATGAAATTTTCTCCGGTCTGGATTACGCCAATTTCCCGAAAATCACCCTCATTGAAAACAAAATGAAGGTCG
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CTTGGTGGATTGTTCAAATCCAGTCAGAATACGCGCCACGAGTTTCTGCGCGCTGTGCGTCATCACAAC**TAA**

>J3-BBa_J23117-MaPTPS (5'-UTR)

AGCATTTGCGATCATTCACGCAGCGCTTATTCAGTTGCTCACTGCGATGTCATAATCATCGCTACGAGCTGTGAAAG
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CTGCGCACAGATTGAACTCCGTCCACCTCTCGCCTGCTGAGAACGTCAAGCTCTTCGGTAAGTGCAACCACACTTCC
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ACTTCGAGTCCAGACCCTCCACTACTGAGAACCTCGCTGTCTTCTTGTGGGAGAATATCAAGAGCCACTTGCCACCT
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>J3-BBa_J23117-MaSR (5'-UTR)

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TCCTTGGAGCAAGTCTTAACGGAGCTGCACGAGGCTGCATCCCACGCTGGTGTCGTCTTCAAGGGTGTCGTTGTGTC
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TCTCTTCCCTATTAGCTGTTCAGGCTTTCCCAAACTGGGGTTTGTACGCTGCTGGTAAGGCAGCTAGAGATAGACTA
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AATGCAGGCTGACGTCCGCAGAACTTTGGGTGATAAGGAACAACTGAAGATCTACGACGACATGCATAAGTCTGGTT
CCTTGGTGAAGATGGAGGACTCCTCTAGAAAGTTGATTCATTTGTTAAAGGCTGACACCTTCACCTCCGGTGGCCAC
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>LacI-Ptrc-*mvaE-mvaS* (5'-UTR)

CCAGCTGGCAATTCCGACGTCGACACCATCGAATGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGA
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>J3-BBa_J23117-*mvaE-mvaS* (5'-UTR)

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TTACCGGTGAACTGGTTGCAGGTTATCAGAATCATCTGCAGAAAGAAACCCATCTGGCCCTGCTGGATAATCGTACC
GAACTGAGCATTGCAGAATATGAAGCAATGTTTGCAGAAACCCTGGATACCGATATTGATCAGACCCTGGAAGACGA
ATTAAAATATAGCATTAGCGCCATTAATAACACCGTGCGTAGCTATCGTAAT**TAA**

>dblTerm
TAAAAACTCGAGTAAGGATCTCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATC
TGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATA

Endogenous promoters

Underlined sequences are 60bp from two ORF adjacent to the promoter. **Bolded** nucleotides are **TSS** according to (D'Arrigo et al., 2016) or **start codon** of the gene or mRFP.

>PP_1776-A-mRFP
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ACGTTCACGCGCGCAGTTGTCTCAAACCTGCCATTTGAGTTTCGCCGCCCGACGGTGCAGTTGCTAAAACGGCGGTT
GAACAGCCGACTGAAGATGCGCTCTCTGGCGCTCCTCGGGGGACAAGCTACATGAAAAAAACTCTGGTATTCTACGA
GCCTCAGCTCGGGTCTGGAGCCTGGATGGCCAGGAATTGCC**T**CGCTGGGCGCGTATATTTATTGCTGCTGCACCCGA
CCGCAGCGGCTGTCAGATATTAAGATACATGCAGGTTTCCTGAGGTTTGAAACTTCAAGTGGCCGTTAAGGACTCAA
AT**ATG**GAATTGATCCCGGTAATTTTATCCGGTGGCGTTGGTAGCCGTCTGTGGCCAGTATCAGAATTCATTAAAGAG
GAGAAAGGTACC**ATG**GCGAGTAGCGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGT
TAACGGTCACGAGTTCGAAATCGAAGGTGAAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAG
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>PP_4812-B
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GCTCCCACTGGGATTTGTGTAGGAGCGGGTTTACCCGCGAAAGGGCCGGCACTGCCAACATCACCGTCCAATTCAGC
CTCGATTAAGCCATTCATTGCTATCATCCCCGCCTCTCCAG**C**CACGAACTGCCCCGC**ATG**CCAGCCCTGCCCGACAG
CTTTTTCGACCGCGACGCCCAGACCCTGGCCAAGGCCCTGGAATTCATTAAAGAGGAGAAAGGTACC**ATG**___

>PP_3839-C
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ACCTGCAGGCTCTCCCTTCAAACGTCTTTTGCCCGCCTTCCATGGCGGGCTTTTTTATGAC**C**CTGCGCACTTCGTTG
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TTCTTGCAGCCCGCCGCATCCTTGCCGCGGGCTTTTCTTTTTTCCGCAAAGGCCAGCCAGGCATACGCAGGAATTTT
GTGGAAGCGCCCACCTTGACCATGACCTGAAGCAGTTTTGCCTGGGCCGGCAAGGTCTATGCTATCCCGACGATCAC
CCAAGCTCACATCGGATAGACACGGAGGCTCTC**ATG**AAAGCTGCTGTCGTTGCACCAGGCCGTCGCGTGGACGTGAT
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>PP_1992-D
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CGGGCATCCATGCCCGTCACCTGCCTCCGCAAGGCCTGCGTTCGGCCTCCTGAAGTCGCGAAGATCAAGATCAAGAT

CAAGATCAAGATCAAGATCAAGATCAACAGCAACAGCAACAGCAATAGCTACAGCAATAGGCAACGCTGGGGGAGGC
ACAGACAAAACCCTACAATCCGAGTAAAGTGCCGCCCCCGCCGTTTACAGCAAAAGGATCCCTTCC**ATG**ACACACCC
TTTGGATATCGCCGTCGTCGGCGCCACCGGCAGCGTCGGTGAAGCCTTGGAATTCATTAAAGAGGAGAAAGGTACC**A**
TG___

>PP_0786-E
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>PP_1972-F
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>PP_3668-G
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CGGCTGAAAGAACACTGGCATAGACCGGAAATCTGGATAACCGACCCAAAGGTACCCGCAG**ATG**TCGAACGAATCGA
AATGCCCGTTCCATCAAACCGCAGGTGGCGGCACCACCAACCGTGAATTCATTAAAGAGGAGAAAGGTACC**ATG**___

>PP_5046-H
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GCGACACGATCAAAAAACGCATCGTAAGGGTGCACTTTCACCCGCTAACCCAACGCCAAATGCACGCAAACGCCCCC
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Other sequences

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Anderson Promoters

RNAP recognition site (-35 and -10 elements) of the Anderson promoter series are underlined. TSSs are indicated in **bold** at the predicted site. TSSs of the Anderson promoter series were characterized in (Kosuri et al., 2013); most TSSs are located immediately after the 35 bp sequence as expected. Some weak promoters have undefined TSSs due to low RNAseq signal and some of the TSSs reported in (Kosuri et al., 2013) are shifted downstream by 1 base when **C** is the first nucleotide of the 5'-UTR. For all experiments reported in this manuscript, the first base of the 5'-UTR is **G** (see 5'-UTR sequence below) The promoters shown below appear in an order of ascending basal expression level in *P. putida* (weakest first).

>BBa_J23109-5'-UTR
TTTACAGCTAGCTCAGTCCTAGGGACTGTGCTAGC**G**AATTCATTAAAGAGGAGAAAGGTACC

>BBa_J23113
CTGATGGCTAGCTCAGTCCTAGGGATTATGCTAGC**G**

>BBa_J23117
TTGACAGCTAGCTCAGTCCTAGGGATTGTGCTAGC**G**

>BBa_J23114
TTTATGGCTAGCTCAGTCCTAGGTACAATGCTAGC**G**

>BBa_J23115
TTTATAGCTAGCTCAGCCCTTGGTACAATGCTAGC**G**

>BBa_J23107
TTTACGGCTAGCTCAGCCCTAGGTATTATGCTAGC**G**

>BBa_J23105
TTTACGGCTAGCTCAGTCCTAGGTACTATGCTAGC**G**

>BBa_J23106
TTTACGGCTAGCTCAGTCCTAGGTATAGTGCTAGC**G**

>BBa_J23108
CTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCG

>BBa_J23110
TTTACGGCTAGCTCAGTCCTAGGTACAATGCTAGCG

>BBa_J23111
TTGACGGCTAGCTCAGTCCTAGGTATAGTGCTAGCG

>BBa_J23119
TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCG

>5'-UTR (Ribosome-Binding-Site)
GAATTCATTAAAGAGGAGAAAGGTACC

5'-Proximal sequences

>PS1
CAATATGACGTGTTGTTAATTTGGTT

>PS2 (same as J1)
TTGGGTTCCACCGGATACCTCCGGAC

>PS3
GTCGTAAATAAGTAAGTCACTCCCAC

>PS4
GTTGTCCTTCTAGTCGCCCATGACTC

>PS5
ACACCGACTACCCCTGCTGGGCCCAG

sgRNA/scRNA sequences

>BBa_J23119(SpeI)-sgRNA-rrnBTerm
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GCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTC
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GCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACT

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