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Supplemental Information

High-Efficiency Multi-site Genomic Editing of *Pseudomonas putida* through Thermoinducible ssDNA Recombineering

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SUPPLEMENTARY INFORMATION

TRANSPARENT METHODS

Strains and media

The bacterial strains employed in this study are listed in Supplementary Table S2. *E. coli* and *P. putida* strains were grown in liquid LB with shaking (170 rpm) at 37 ºC and 30 ºC, respectively (Sambrook et al., 1989) with the exception of *E. coli* strains bearing SEVA plasmids endowed with the P_L/cI857 thermo-inducible expression system (cargo #14; i.e. pSEVA2514-*rec2-mutL*$_{E36K}^{PP}$ and derivatives), which were grown at 30 ºC to avoid promoter activation. After electroporation, recovery during recombineering experiments was performed in Terrific Broth without glycerol (TB: 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 2 g L^{-1} KH_2PO_4, 9.4 g L^{-1} K_2HPO_4). M9 minimal media was prepared according to (Sambrook et al., 1989). Solid media was prepared adding 15 g L^{-1} of agar to liquid media. M9 solid media was supplemented with 0.2% (w/v) citrate and appropriate antibiotics to select *P. putida* cells in mating experiments. Liquid and solid media were added, when necessary, with 50 µg ml^{-1} of kanamycin (Km), 15 µg ml^{-1} of gentamicin (Gm) for *P. putida* and 10 µg ml^{-1} of the same antibiotic for *E. coli*, 30 µg ml^{-1} of chloramphenicol (Cm), 100 µg ml^{-1} of streptomycin (Sm), 100 µg ml^{-1} of rifampicin (Rif), 50 µg ml^{-1} of nalidixic acid (Nal), 20 µg ml^{-1} of Uracil (Ura), 250 µg ml^{-1} of 5-fluoroorotic acid (5-FOA) and 5 mM of benzoic acid (pH 11). For screening of fluorescent colonies, LB solid media was prepared with 1 mg ml^{-1} of activated charcoal (Sigma-Aldrich Ref. C9157-500G) in order to better discriminate low-signal colonies. Activated charcoal was added to the LB-Agar prior autoclaving and the media poured into 150 mm Petri dishes after vigorous shaking to evenly distribute the insoluble charcoal particles.

General procedures, primers and bacterial transformation

Standard DNA manipulations were carried out following routine protocols (Sambrook et al., 1989) and according to manufacturer recommendations. Isothermal Assembly was performed with Gibson Assembly® Master Mix (New England Biolabs, Ipswich, MA, USA). Plasmidic DNA was

purified with the QIAprep® Spin Miniprep Kit, both purchased from Qiagen (Valencia, CA, USA). DNA Amplitools Master Mix (Biotools, Madrid, Spain) was used for diagnosis PCRs and amplification of DNA fragments for cloning purposes was done with Q5 polymerase (New England Biolabs, Ipswich, MA, USA). Synthetic oligonucleotides used in this study are listed in Supplementary Table S1 and were purchased from Sigma-Aldrich (St. Louis, MO, USA). PCR products were purified with the Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). DNA sequencing was performed in Macrogen (Spain). Transformation of *E. coli* strains was carried out with chemically competent cells using the $CaCl_2$ method (Sambrook et al., 1989). Plasmids were introduced in *P. putida* strains via tripartite mating as described in (Martinez-Garcia and de Lorenzo, 2012) and selected in solid M9 minimal media supplemented with 0.2% w/v citrate and appropriate antibiotics. Tetra-parental mating was used as described by (Choi et al., 2005) to insert the mini-transposon Tn*7*-M-P_{EM7}-*gfp* -RBS^{-} into the *att*Tn*7* site of *P. putida* EM42, using M9-citrate-Gm as selective media (see below for details).

Construction of plasmids and strains.

The medium-high copy number plasmid pSEVA2514-*rec2*-*mutL*$_{E36K}^{PP}$ (Supplementary Table S2) was used for the construction of two derivatives bearing low- and medium- copy number origins of replication. This plasmid was cut with PacI/SpeI and the 4.2 Kb DNA band, containing the *rec2* and *mutL*$_{E36K}^{PP}$ genes under the control of the thermo-inducible system P_L/*c*I857, was ligated to PacI/SpeI restricted plasmids pSEVA221 (low copy number) and pSEVA231 (medium copy number). Ligations were transformed into *E. coli* CC118 and selection was made in LB-Km plates, obtaining plasmids pSEVA2214-*rec2*-*mutL*$_{E36K}^{PP}$ and pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$. Both constructs were separately introduced in *P. putida* EM42 by tri-parental matings followed by selection in M9-citrate-Km solid media, obtaining the strains *P. putida* EM42 (pSEVA2214-*rec2*-*mutL*$_{E36K}^{PP}$) and *P. putida* EM42 (pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$). *P. putida* EM42 was also transformed by the same method with pSEVA2314, generating the control strain *P. putida* EM42 (pSEVA2314). A Tn*7* mini-transposon with the *gfp* gene under the control of the constitutive P_{EM7} promoter, but lacking the ribosome binding site (RBS) sequence, was constructed. To this end, first the *gfp* gene was placed under the control of the P_{EM7} promoter: plasmid pSEVA637 (Supplementary Table S2) was cut with HindIII/SpeI and the purified 0.7 Kb band (RBS + *gfp*

gene) was ligated to the pSEVA237R-PEM7 (Supplementary Table S2) backbone digested with HindIII/SpeI. Upon transformation in *E. coli* CC118 and selection on LB-Km plates, the resulting plasmid (pSEVA237-PEM7) was digested with PacI/SpeI and the purified 0.9 Kb band (containing the *gfp* gene under the control of the P_{EM7} and bearing a consensus 5´-AGGAGG-3´ RBS sequence) was ligated to a pTn7-M plasmid restricted with the same enzymes. Ligation mixture was used to transform *E. coli* competent cells and selection was done in LB-KmGm plates. The resulting plasmid, pTn7-M-PEM7-GFP, was used as a template to eliminate the RBS sequence. In order to achieve this, the plasmid was PCR amplified with primers Tn7-PEM7-F/ Tn7-PEM7-R (Tm= 58 ºC, 2 min. elongation, Q5 polymerase). The primers were designed to i) amplify the whole plasmid with the exception of the 7-nt Shine Dalgarno motif 5´-AGGAGGA-3´ located 7-nt away from the *gfp* start codon, ii) generate an PCR product sharing a 40-bp sequence at both sides of the molecule to allow isothermal assembly of the amplicon. The 3.9 Kb PCR product was purified and subjected to Gibson Assembly and the reaction was transformed into *E. coli*. Selection was made in LB-KmGm plates, thus obtaining the plasmid pTn7-M-PEM7-GFP-RBS^{-}. The region between the P_{EM7} promoter and the end of the *gfp* gene was fully sequenced with primers PS2 and PEM7-F to ensure the correct deletion of the RBS sequence. *E. coli* (pTn7-M-PEM7-GFP-RBS^{-}) was used as the donor strain to introduce the mini-transposon in the *att*Tn7 site of *P. putida* EM42. Both strains and the helper strains *E. coli* HB101 (pRK600) and *E. coli* (pTNS2) were used in a tetra-parental mating followed by selection in M9-citrate-Gm solid media. Colonies were streaked in the same media and subjected to two diagnostic PCRs to check the mini-transposon insertion. PCRs with primer pairs PS2/ PP5408-F (Tm= 60 ºC, 1 min. 30 seconds elongation) and PEM7-F/Tn7-GlmS (Tm= 60 ºC, 1 min. elongation) yielded bands of 2.2 Kb and 1.2 Kb, respectively, confirming the correct integration of the transposon in the *att*Tn7 locus. The resulting strain *P. putida* EM42::Tn7-M-P_{EM7}-*gfp*-RBS^{-} (referred as *P. putida* TA245 in Supplementary Table S2) was transformed by tripartite mating with pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$ plasmid. After selection on M9-citrate-KmGm plates, the strain *P. putida* TA245 (pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$) was obtained. Integrity of the constructs described above, either in *E. coli* or *P. putida*, was always checked by miniprep, restriction and agarose gel visualization.

Oligonucleotide design, recombineering protocol, cycling procedure and screening

The nine oligonucleotides used in this work for recombineering experiments (SR, NR, RR, PR, CR, RBS-C_6, RBS-Deg_6, RBS-C_9, RBS-Deg_9) were designed to introduce different allelic changes targeting the lagging strand of the *P. putida* chromosome. Supplementary Table S3 summarizes the main features of each oligonucleotide while complete sequence and additional details can be found in Supplementary Table S1. The recombineering protocol used here relies in the co-expression of the Rec2 recombinase and the $MutL_{E36K}^{PP}$ dominant negative allele from plasmids endowed with the thermo-inducible P_L/*c*I857 expression system (pSEVA2214-*rec2-mutL*$_{E36K}^{PP}$, pSEVA2314-*rec2-mutL*$_{E36K}^{PP}$ or pSEVA2514-*rec2-mutL*$_{E36K}^{PP}$). The protocol is basically identical to that described previously in (Aparicio et al., 2019b). Overnight cultures of *P. putida* strains harboring the proper plasmid were used to inoculate 20 ml of fresh LB-Km at OD_{600} = 0.1 in 100 ml Erlenmeyer flasks. Cultures were incubated at 30 ºC with vigorous shaking (170 rpm) until OD_{600} ~ 1.0 and flasks were then placed in a water bath at 42 ºC for 5 minutes to increase rapidly the temperature and induce the P_L promoter. Ten additional minutes of incubation at 42 ºC was performed in an air shaker at 250 rpm (induction total time at 42 ºC= 15 minutes) to trigger the expression of *rec2-mutL*$_{E36K}^{PP}$ genes, followed by 5 minutes in ice to cool down the bacterial culture and stop the induction. In non-induced cultures the heat-shock and cooling down steps were not performed. Competent cells were then prepared transferring 10 ml of each culture to 50-ml conical tubes and centrifuging the cells at 3,220 g/ 5 minutes. Cell pellets were resuspended in 10 ml of 300 mM sucrose and washed two additional times with 5 and 1 ml of the same solution. After centrifugation in a bench-top centrifuge (10,000 rpm, 1 minute), cellular pellets were finally resuspended in 200 μl of 300 mM sucrose and 100 μl of this suspension was added with the recombineering oligonucleotide. For single-oligonucleotide experiments, 1 μl from a 100 μM stock was used (1 μM final concentration). For multiplexed experiments, 10 μl of each oligonucleotide stock at 100 μM (SR, NR, RR, PR and CR) were mixed and 3 μl of this mixture were added to the competent cells (accounting for 0.6 μM of each oligo.). The cell suspension was mixed thoroughly by pipetting, placed in an electroporation cuvette (Bio-Rad, 2 mm-gap width) and electroporated at 2.5 kV in a Micropulser™ device (Bio-Rad Laboratories, Hercules, CA, USA). Cells were immediately inoculated in 5 ml of fresh TB in 100 ml Erlenmeyer flaks and recovered at 30 ºC/ 170 rpm. Before plating the cells for screening of allelic replacements, different recovery times and TB additions were used depending on the experiment. For one cycle recombineering experiments, overnight recovery was done in TB for assays with SR and NR

oligonucleotides while for experiments with oligonucleotides RBS-C_6, RBS-Deg_6, RBS-C_9 and RBS-Deg_9, TB supplemented with Km and Gm was preferred. Specifications for cycled recombineering assays (HEMSE) are depicted below.

High-efficiency multi-site genomic editing protocol

HEMSE is a cycled recombineering protocol run in a multiplexed fashion. The procedure involves a standard recombineering protocol in which, as explained before, cultures were subjected to electrotransformation with an equimolar mixture of several oligonucleotides. The recovery was performed in TB added with Km in order to maintain the plasmid along the cycles, and the incubation proceeded at 30 ºC with vigorous shaking (170 rpm) until an OD_{600} ~ 1.0 (Cycle-I). Culture aliquots were withdrawn for screening and the bacterial culture entered in the next round of recombineering by performing induction at 42 ºC/ 15 minutes, competent cell preparation, oligonucleotide mixture electroporation and recovery till reaching again a cell density around 1.0 at 600 nm (Cycle-II). Further cycles proceeded in the same way (Fig. 3 of main text). Each cycle took one day in average and recovery, when necessary, was performed overnight at room temperature without shaking to avoid culture overgrowth. When recovery step was completed at the end of the day, cultures were stored at 4 ºC overnight. A new cycle was started in the next morning incubating the culture 30 minutes at 30 ºC (170 rpm) before the induction step. Screening of allelic changes after recombineering was performed plating aliquots of recovered cultures in the appropriate selective and/or non-selective solid media, as follows:

• In single-olignucleotide experiments with SR and NR oligonucleotides (one cycle), overnight cultures were plated in LB-Sm (dilutions 10^{-4} and 10^{-5} for induced cultures and 10^{-2} and 10^{-3} for non-induced bacteria) and LB-Nal (dilutions 10^{-4} and 10^{-5} for induced cultures and 10^{-2} and 10^{-3} for non-induced bacteria), respectively, to estimate the allelic replacements, while dilutions 10^{-7} and 10^{-8} were done in LB without antibiotics to count viable cells. Plates were incubated 18 h. at 30 ºC and CFUs annotated.

• In single-oligonucleotide experiments with RBS-C_6, RBS-Deg_6, RBS-C_9, RBS-Deg_9 oligos (one cycle), cultures recovered overnight were plated on 150 mm width LB-KmGm-activated charcoal

plates using 10^{-6} dilutions. This allowed an average of 500 colonies per plate. To facilitate the identification of colonies displaying low levels of fluorescence, plates were incubated at 30 ºC for 5 days. Fluorescent colonies were streaked in the same media and insertion of putative ribosome binding sites upstream the *gfp* gene were checked by PCR amplifying this DNA region with primers PS2/ PP5408-F (Tm= 60 ºC, 1 min. 30 seconds elongation, 1.0 Kb product) and sequencing the amplicon with primer ME-I-Gm-ExtR. Non-redundant clones with different sequences inserted were selected and glycerol stocks made prior characterization by flow cytometry.

• Allelic replacements in HEMSE experiments were screened after recovery steps (OD_{600} ~ 1.0) of cycle-I, cycle-V and cycle-X. Viable cells were estimated plating dilutions 10^{-7} and 10^{-8} in LB plates. Single mutants coming from SR-, NR-, RR- and PR-mediated recombineering were analyzed by plating dilutions 10^{-4} and 10^{-5} in LB-Sm, LB-Nal, LB-Rif and LB-5FOA-Ura plates. Plates were incubated 24 h at 30 ºC and total CFUs of single mutants (Sm^R, Nal^R, Rif^R and $5FOA^R$) and viable cells were taken. Twenty $5FOA^R$ colonies were replicated on M9-citrate and M9-citrate-5FOA-Ura plates in order to discriminate authentic *pyrF* mutants ($5FOA^R/Ura^-$) from spontaneous $5FOA^R$ mutants ($5FOA^R/Ura^+$), as stated in (Galvao and de Lorenzo, 2005; Aparicio et al., 2016). Colonies grown on both media were discounted of the total $5FOA^R$ numbers as *pyrF*-unrelated, spontaneous mutants. Dilutions 10^{-6} in LB-benzoate plates allowed the estimation of *catA*-I^- mutants simply by counting the dark-brown colonies appeared after 10 days of incubation at 30 ºC. *catA*-I^- mutants accumulate catechol, which turns into brown intermediates after spontaneous oxidation and polymerization (Jimenez et al., 2014). In previous assays aimed to obtain *catA*-I- mutants through recombineering with CR oligo, it was noticed that long incubations were necessary to appreciate the colored phenotype in solid media (Fig. S2). The observed dark-brown colonies were always *catA*-I^- mutants, as was demonstrated by amplification of *catA*-I gene (primers catA-F/catA-R, Tm 55 ºC, 1 minute elongation) and sequencing of the 0.5 Kb amplicon with primer catA-F (data not shown) in 20 selected colonies. Multiple gene editions were also analyzed plating cultures from cycles I, V and X on LB solid media supplemented either with Sm+Nal+ Rif+5FOA+Ura (four editions mediated by SR, NR, RR and PR oligonucleotides), 24 incubation at 30 ºC, or with Sm+Nal+Rif+5FOA+Ura+benzoate (five editions mediated by the 5 oligonucleotides used in this study), 10 days incubation at 30 ºC. For

this last experiment, there were considered quintuple mutants those colonies displaying resistance to Sm, Nal, Rif and 5FOA and also showing the characteristic brown phenotype of *catA*-I$^-$ mutants. The recombineering frequency (RF) was calculated as the ratio between the number of colonies showing a given phenotype and the number of viable cells within the experiment, being this ratio normalized to 10^9 viable cells for graphic representation.

In order to check the accuracy of the allelic replacements, 18 colonies showing the quintuple mutant phenotype (Sm^R, Nal^R, Rif^R, 5FOA^R and catechol accumulation) were checked by PCR amplification and sequencing of the target genes. For each bacterial clone, five different PCRs were set up to amplify: *rpsL* (primers rpsL-Fw/ rpsL-Rv, Tm 57 ºC, 45 seconds elongation, 0.8 Kb product), *gyrA* (primers gyrA-Fw/ gyrA-Rv, Tm 57 ºC, 45 seconds elongation, 0.4 Kb product), *rpoB* (primers rpoB-F/rpoB-R, Tm 57 ºC, 45 seconds elongation, 0.4 Kb product), *pyrF* (primers pyrF-F/pyrF-R, Tm 52 ºC, 1 minute elongation, 1.2 Kb product) and *catA-I* (primers catA-F/catA-R, Tm 55 ºC, 1 minute elongation, 0.5 Kb product). The purified PCR products were sequenced with the putative forward primers and the sequence analysed for the expected changes mediated by recombineering. All clones analysed (n=18; 100%) showed the correct changes, demonstrating that the observed phenotypes corresponded to mutations mediated by the HEMSE procedure. Single allelic replacements in HEMSE experiments were not confirmed by PCR and sequencing since previous works showed that virtually 100% of Sm^R, Nal^R and *pyrF*$^-$ mutants obtained by recombineering with oligos SR, NR and LM (almost identical to RR oligo used in this work) harbored the expected changes in the target genes *rpsL*, *gyrA* and *pyrF* (Ricaurte et al., 2018; Aparicio et al., 2019a). Preliminary studies in this work showed, on the other hand, that single Rif^R mutants also displayed 100% accuracy in the expected mutations of the target gene *rpoB* (data not shown). As explained before, *catA*-I$^-$, dark-brown colonies were also analyzed by PCR and sequencing in previous test experiments (data not shown), with analogous results.

Flow cytometry

The visual selection of fluorescent colonies from recombineering experiments with oligos RBS-Deg$_6$ and RBS-Deg$_9$ gave rise to a collection of 31 RBS insertion mutants showing a wide variety of fluorescent signals. Together with the negative controls of *P. putida* TA245 (insertion of Tn*7*-M-

P_{EM7}-*gfp*-RBS^{-}) and *P. putida* EM42 (no *gfp* gene), a total of 33 strains were characterized for GFP production. Each strain was inoculated from glycerol stocks in 2 ml of LB-KmGm (*P. putida* EM42 in LB; *P. putida* TA245 in LB-Gm) and cultured at 30 ºC/ 170 rpm. 0.5 ml of overnight cultures (OD_{600} ~ 2-3) were centrifuged and resuspended in 1 ml of filtered Phosphate Buffered Saline (PBS) 1X (8 mM Na_2HPO_4, 1.5mM KH_2PO_4, 3 mM KCl, 137 mM NaCl, pH.7.0). Fifty μl of each suspension was added to 450 μl of PBS 1X to obtain cellular samples with OD_{600} ~ 0.1-0.15. Samples were analyzed in a MACSQuant^{TM} VYB cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) to quantify the emission of fluorescence as indicated in (Martinez-Garcia et al., 2014). GFP was excited at 488 nm and the fluorescence signal was recovered with a 525±40 nm band-pass filter. For each sample, at least 100,000 events were analyzed and the FlowJo v. 9.6.2 software (FlowJo LLC, Ashland, OR, USA) was used to process the results. Population was gated to eliminate background noise and the median of the GFP-A channel of two biological replicas was used for graphical representation.

Data and Software Availability

GenBank accession numbers for the plasmids used in this study are the following:

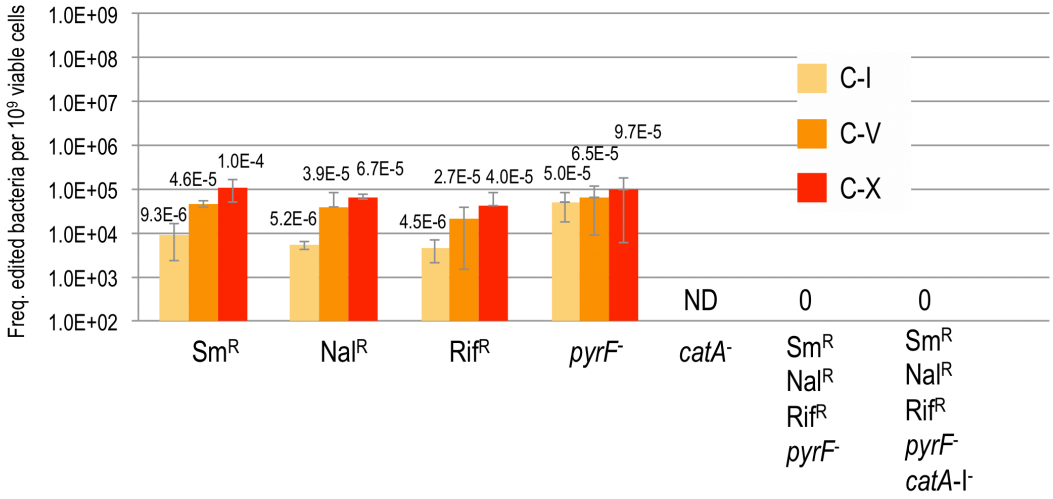
pSEVA2214-*rec2*-*mutL*$_{E36K}^{PP}$ (MN688223)

pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$ (MN688222)

pSEVA2514-*rec2*-*mutL*$_{E36K}^{PP}$ (MN180222)

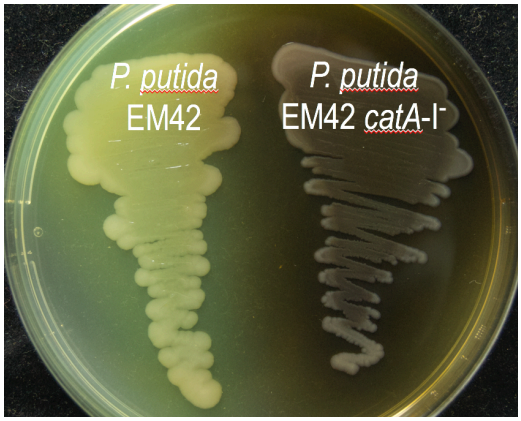
SUPPLEMENTARY FIGURES

Figure S1. Rec2-independent editing in HEMSE assays (related to Fig. 4 of main text)



Editing efficiencies of single and multiple changes in the control strain *P. putida* EM42 harboring the empty plasmid pSEVA2314 were assayed applying 10 cycles of HEMSE and an equimolar mixture of oligos SR, NR, RR, PR and CR, following the same procedure explained in Figure 4A and 4B. See more details in Transparent Methods section. Allelic replacements of *catA*-I gene were not determined in these assays (ND), while multiple editions could not be detected (0).

Figure S2. Phenotype of *P. putida* EM42 *catA*-I$^-$ strain (related to Fig. 4 of main text).



P. putida EM42 (pSEVA2314-*rec2*-*mutL*$_{E36K}^{PP}$) was subjected to recombineering with CR oligonucleotide (see Transparent Methods section). Three stop codons were inserted in the *catA*-I ORF, generating a mutant strain in which the metabolism of benzoic acid is impaired, leading to accumulation of catechol (Jimenez et al., 2014). Upon spontaneous oxidation and polymerization, catechol derivatives exhibit a characteristic dark-brown colour. *P. putida* EM42 and the *catA-I* mutant were grown in LB-Agar supplemented with benzoate 5 mM and incubated 10 days at 30 ºC to allow the visualization of the colored phenotype.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE S1. Oligonucleotides used in this study (related to Fig. 2 and Fig. 5 of main text).

Name	[(a)] Sequence (5' → 3')	Usage / Source
SR	G*T*C*A*GACGCACACGGCATACTTTACGCAG TGCCGAGTTAGGTTTT**G**TCGGCGTGGTGGTG TACACACGGGTGCACACGCCACGACGCTGC	Recombineering oligo for *rpsL* gene: AAA (K43) changed to ACA (T43), mismatch A:G, confers Sm resistance (Ricaurte et al., 2018)
rpsL-Fw	GACATGAAATGTTGCCGATG	To amplify and sequence part of *rpsL* gene of *P. putida* /(Ricaurte et al., 2018)
rpsL-Rv	CTGTTCTTGCGTGCTTTGAC	With rpsL-Fw, to amplify part of *rpsL* gene of *P. putida* (Ricaurte et al., 2018)
NR	AACGAGAACGGCTGGGCCATACGCACGATGG T**A**TT**T**GTAGACCGCAGTGTCGCCGTGCGGGTG GTA	Recombineering oligo for *gyrA* gene: GAC (D87) changed to AAT (N87), mismatches G:T and C:A, confers Nal resistance (Aparicio et al., 2019b)
gyrA-F	GGCCAAAGAAATCCTCCCGGTCAA	To amplify and sequence part of *gyrA* gene of *P. putida* (Aparicio et al., 2019b)
gyrA-R	AGCAGGTTGGGAATACGCGTCG	To amplify and sequence part of *gyrA* gene of *P. putida* (Aparicio et al., 2019b)
RR	TCCGAGAGAGGGTTGTTCTGGTCCATGAAC**A** GGGACAGCTGGCTGGAACCGAAGAACTCT	Recombineering oligo for *rpoB* gene: CAG (Q518) changed to CTG (L518), mismatch A:A, confers Rif resistance. This work
rpoB-F	CCTGGGTAACCGTCGCGTACGGTG	To amplify and sequence part of *rpoB* gene of *P. putida.* This work
rpoB-R	CGCCTTCCTTCACCACGCGGTACG	To amplify and sequence part of *rpoB* gene of *P. putida.* This work
PR	AGGTCCAGGAACACTTCGAAGCCCTTGTCACA CAGGGTTT**A**GACAATGCCCGAAGCGCTGCTG	Recombineering oligo for *pyrF* gene: GAA (E50) changed to TAA

	GTGAACAGCTCCTTGCCA	(Stop), mismatch A:G, confers 5FOA resistance and uracil auxotrophy. This work
pyrF-F	CGAGGGCTATGATGAGTATC	To amplify and sequence the *pyrF* gene of *P. putida* (Aparicio et al., 2016)
pyrF-R	GTCAGGTGAAGAGCAAAGAG	To amplify and sequence the *pyrF* gene of *P. putida* (Aparicio et al., 2016)
CR	GCAGCACGCGCAGAATGATCTGCTTGAAGCG CGGGTTTCCCTATTATCATTCGGCATGGTCCA GGCCAGCTACCCGGTTGAAGAAGGCT	Recombineering oligo for *catA-I* gene: insertion of three stop codons truncates the ORF, giving rise to brown colonies in presence of benzoate. This work
catA-F	AACTCGTCCTCGGTAATCTC	To amplify and sequence part of *catA-I* gene of *P. putida.* This work
catA-R	CAGCAATCAAGGAGATAACC	To amplify and sequence part of *catA-I* gene of *P. putida.* This work
Tn7-PEM7-F	AAAACATATGAGTAAAGGAGAAGAACTTTTCA	To remove RBS sequence from pTn7-M-PEM7-GFP by Gibson Assembly. This work
Tn7-PEM7-R	AACTCCAGTGAAAAGTTCTTCTCCTTTACTCAT ATGTTTTAAGCTTGCATGCCTGCAGGTCG	To remove RBS sequence from pTn7-M-PEM7-GFP by Gibson Assembly. This work
PEM7-F	AATACGACAAGGTGAGGAAC	To amplify and sequence from P_{EM7} promoter. This work
PP5408-F	CGATTCATCAGGTTGGATTCG	To amplify and sequence mini-Tn7 insertions from PP_5408 locus of *P. putida*. This work
Tn7-GlmS	AATCTGGCCAAGTCGGTGAC	To amplify and sequence mini-Tn7 insertions from *glmS* gene of *P. putida* (Lambertsen et al., 2004)
PS2	GCGGCAACCGAGCGTTC	To amplify and sequence from T_0 terminator (Silva-Rocha et al., 2013)
ME-I-Gm-ExtR	GTTCTGGACCAGTTGCGTGAG	To amplify and sequence from mini-Tn7 Gm resistance gene

		(Martinez-Garcia et al., 2014)
RBS-C$_6$	TCTAGAGTCGACCTGCAGGCATGCAAGCTTAGGAGGAAAAACATATGAGTAAAGGAGAAGAACTTTT	Recombineering oligo to insert a consensus 6 nucleotide RBS sequence upstream the *gfp* gene in strains Tn*7*-M-P$_{EM7}$-*gfp*-RBS^{-}. This work
RBS-Deg$_6$	TCTAGAGTCGACCTGCAGGCATGCAAGCTTRRRRRRAAAAACATATGAGTAAAGGAGAAGAACTTTT	Recombineering oligo to insert a degenerated (R=A,G) 6 nucleotide RBS sequence upstream the *gfp* gene in strains Tn*7*-M-P$_{EM7}$-*gfp*-RBS^{-}. This work
RBS-C$_9$	TCTAGAGTCGACCTGCAGGCATGCAAGCTTTAAGGAGGTAAAAACATATGAGTAAAGGAGAAGAACTTTT	Recombineering oligo to insert a consensus 9 nucleotide RBS sequence upstream the *gfp* gene in strains Tn*7*-M-P$_{EM7}$-*gfp*-RBS^{-}. This work
RBS-Deg$_9$	TCTAGAGTCGACCTGCAGGCATGCAAGCTTTARRRRRRTAAAAACATATGAGTAAAGGAGAAGAACTTTT	Recombineering oligo to insert a degenerated (R=A,G) 9 nucleotide RBS sequence upstream the *gfp* gene in strains *Tn7*-M-P$_{EM7}$-*gfp*-RBS^{-}. This work

[a] Asterisks denote phosphorothioate bonds. Single changes introduced by recombineering oligonucleotides SR, NR, RR and PR are highlighted in bold. The three stop codons inserted by oligo CR appear in blue. The sequences encompassing the stretch inserted by the four RBS-X oligos are shown in red color.

SUPPLEMENTARY TABLE S2. Bacterial strains and plasmids used in this work (related to Fig. 2 of main text)

Strain or plasmid	Relevant characteristics [a]	Reference or source
Escherichia coli		
CC118	Cloning host; Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thiE1 rpsE*(Sp^R) *rpoB*(Rif^R) *argE*(Am) *recA1*	(Manoil and Beckwith, 1985)
HB101	Helper strain used for conjugation; F$^-$ λ$^-$ *hsdS20*(rB$^-$ mB$^-$) *recA13 leuB6*(Am) *araC14* Δ(*gpt-proA*)62 *lacY1 galK2*(Oc) *xyl-5 mtl-1 thiE1 rpsL20*(Sm^R) *glnX44* (AS)	(Boyer and Roulland-Dussoix, 1969)
CC118λpir	CC118, λpir lysogen	(Herrero et al., 1990)
Pseudomonas putida		
EM42	KT2440 derivative; Δprophage1 Δprophage4 Δprophage3 Δprophage2 ΔTn7 Δ*endA-1* Δ*endA-2* Δ*hsdRMS* Δflagellum ΔTn*4652*	(Martinez-Garcia et al., 2014)
TA238	EM42 derivative; *rpsL*$^-$(Sm^R) *gyrA*$^-$(Nal^R) *rpoB*$^-$(Rif^R) *pyrF*$^-$(5FOA^R) *catA*-I$^-$	This work
TA245	EM42 derivative with mini-Tn*7*-M-P$_{EM7}$-*gfp*-RBS$^-$ transposon inserted in the *att*Tn*7* site	This work
Plasmids		
pSEVA2314	Inducible expression vector; *oriV*(pBBR1); cargo [P$_L$/cI857]; standard multiple cloning site; Km^R	(Aparicio et al., 2019a)
pSEVA2214-*rec2-mutL*$_{E36K}^{PP}$	pSEVA2214 derivative bearing the *rec2* recombinase and *mutL*$_{E36K}^{PP}$ allele ; *oriV*(RK2); cargo [*c*I857-P$_L$ → *rec2-mutL*$_{E36K}^{PP}$]; Km^R	This work GenBank nº MN688223
pSEVA2314-*rec2-mutL*$_{E36K}^{PP}$	pSEVA2314 derivative bearing the *rec2* recombinase and *mutL*$_{E36K}^{PP}$ allele ; *oriV*(pBBR1); cargo [P$_L$/cI857→ *rec2-mutL*$_{E36K}^{PP}$]; Km^R	This work GenBank nº MN688222

pSEVA2514-*rec2*-*mutL*$_{E36K}^{PP}$	pSEVA2514 derivative bearing the *rec2* recombinase and *mutL*$_{E36K}^{PP}$ allele ; *oriV*(RFS1010); cargo [*c*I857-P_L → *rec2*-*mutL*$_{E36K}^{PP}$]; Km^R	(Aparicio et al., 2019b) GenBank nº MN180222
pSEVA637	*oriV*(pBBR1); cargo [*gfp*]; Gm^R	(Silva-Rocha et al., 2013) (Martinez-Garcia et al., 2015)
pSEVA237R-PEM7	*oriV*(pBBR1); cargo [P_{EM7} → *mCherry*]; Km^R	(Silva-Rocha et al., 2013) (Martinez-Garcia et al., 2015)
pSEVA237-PEM7	*oriV*(pBBR1); cargo [P_{EM7} → *gfp*]; Km^R	This work
pTn7-M	*oriV*(R6K); mini-Tn7 transposon; standard multiple cloning site; Km^R Gm^R	(Zobel et al., 2015)
pTn7-M-PEM7-GFP	pTn7-M derivative with P_{EM7}-*gfp* in the mini-Tn7 transposon; *oriV*(R6K); Km^R Gm^R	This work
pTn7-M-PEM7-GFP-RBS$^-$	pTn7-M-PEM7-GFP derivative lacking the *gfp* RBS; *oriV*(R6K); Km^R Gm^R	This work
pRK600	Helper plasmid used for conjugation; *oriV*(ColE1), RK2 (mob$^+$ tra$^+$); Cm^R	(Kessler et al., 1992)
pTNS2	Helper plasmid for mini-Tn7 transposition; *oriV*(R6K), TnsABC+D specific transposition pathway; Ap^R	(Choi et al., 2005)

[a] Antibiotic markers: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; 5FOA, 5-fluoro-orotic acid; Gm, gentamicin; Nal, nalidixic acid; Rif, rifampicin; Sm, streptomycin; Sp, spectinomycin.

SUPPLEMENTARY TABLE S3. Main features of recombineering oligonucleotides used in this study (related to Fig. 2 of main text)

Name	P-thioate bonds	Length	Target gene	Change/mismatch	MMR sensitivity	ΔG (kcal/mol)	Phenotype
SR	Four at 5´-end	94	*rpsL*	A→C/A:G	Low	- 20.79	Sm^R
NR	None	65	*gyrA*	G→A/ G:T C→T/ C:A	High	- 11.84	Nal^R
RR	None	60	*rpoB*	A→T/ A:A	High	- 7.26	Rif^R
PR	None	81	*pyrF*	G→T/ G:A	Low	- 14.03	$5\text{-}FOA^R/Ura^-$
CR	None	89	*catA*-I	Insertion 3 Stops	Low	- 12.84	Catechol accumulation (Brown color)
RBS-C_6	None	67	*gfp* UTR	Insertion 7 nt	Low	- 4.94	Fluorescent
RBS-Deg_6	None	67	*gfp* UTR	Insertion 7 nt (deg.)	Low	Variable	Fluorescent
RBS-C_9	None	70	*gfp* UTR	Insertion 10 nt	Low	- 4.59	Fluorescent
RBS-Deg_9	None	70	*gfp* UTR	Insertion 10 nt (deg.)	Low	Variable	Fluorescent

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