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Recombineering-assisted linear CRISPR/Cas9-mediated multiplex genome editing (ReaL-MGE) for bacterial metabolic engineering

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Disclaimer

The authors declare no competing interests.

Abstract

Recombineering-assisted Linear CRISPR/Cas9-mediated Multiplex Genome Editing (ReaL-MGE) constitutes a significant advancement in bacterial genetic engineering. This technology synergizes the RNA-guided programmability of CRISPR/Cas9 with the 5′-3′ exonuclease and single-strand DNA annealing protein activities of phage recombinases, enabling precise kilobase-scale DNA manipulation at multiple genomic loci simultaneously. ReaL-MGE mitigates off-target effects, removes substrate restrictions, and circumvents the complexities associated with assembling multiple gRNAs on circular vectors. The development of successive ReaL-MGE iterations addresses bacterial intolerance to simultaneous multi-site genomic editing. ReaL-MGE enables the precise simultaneous integration of 22 kilobase-scale sequences (>1 kb each) into distinct genomic loci of non-model bacteria, thereby expanding the scope of bacterial genome engineering. Demonstrating cross-class applicability, ReaL-MGE facilitates multiplex genome editing in Gammaproteobacteria (*Escherichia coli* and *Pseudomonas putida*) and Betaproteobacteria (*Schlegelella brevitalea*), highlighting its potential for diverse synthetic biology applications in biotechnology, agriculture, and environmental science. Compared to alternative bacterial multiplex genome editing technologies, ReaL-MGE offers significant advantages, including unrestricted editing sites and positions, no substrate length limitation, and enhanced convenience and safety. This study provides a comprehensive protocol detailing ReaL-MGE's capabilities, demonstrating its superiority over prior multi-site editing techniques and its potential to transform multiplex genome engineering. The entire procedure entails approximately 9 days.

Image Attribution

No

Guidelines

Steps 1-10 (day 2), construction of expression and biosensor plasmids: 3 h
Steps 11-19 (day 2), electroporation of expression and biosensor plasmids: 3 h
Steps 20-22 (day 1), verification of expression plasmid and biosensor transformation: 4 h
Steps 23-29 (day 2), seamless modifications by ReaL-MGE: 12 h
Steps 30-40 (day 1), FACS sorting for GFP expression from the FapR biosensor: 5 h
Steps 41-48 (day 1), malonyl-CoA quantification: 2 h

Materials

Materials

Biological Materials

▲**CRITICAL** For a summary of all strains used in this protocol.

• *Escherichia coli* BL21 (DSM102052) is a Gram-negative bacterium classified within the domain Bacteria, phylum Pseudomonadota (synonym: Proteobacteria), class Gammaproteobacteria, order Enterobacterales, family Enterobacteriaceae, genus Escherichia, and species *Escherichia coli*. This strain is extensively utilized in biotechnological applications due to its robustness, rapid growth kinetics, and high recombinant protein expression capacity. *E. coli* BL21 possesses Redγβα homologous recombinases that enable high-fidelity genome editing.

• *Schlegelella brevitalea* (DSM7029) is a Gram-negative bacterium classified within the domain Bacteria, phylum Pseudomonadota (synonym: Proteobacteria), class Betaproteobacteria, order Burkholderiales, family Comamonadaceae, genus *Schlegelella*, and species *Schlegelella brevitalea*. In bioindustrial applications, *S. brevitalea* DSM7029 is notable for its potential in biosynthesis due to its metabolic versatility. *S. brevitalea* DSM7029 possesses Redβα$_{7029}$ homologous recombinases that enable high-fidelity genome editing.

• *Pseudomonas putida* KT2440 (DSM26250) is a Gram-negative bacterium classified within the domain Bacteria, phylum Pseudomonadota, class Gammaproteobacteria, order Pseudomonadales, family Pseudomonadaceae, genus *Pseudomonas*, and species *Pseudomonas putida*. Recognized as a preferred chassis organism for synthetic biology due to its genomic stability and genetic tractability, *P. putida* KT2440 has been extensively engineered for diverse applications including environmental bioremediation, biosynthesis of value-added compounds (e.g., bioplastics, fine chemicals), and plant growth promotion. *P. putida* KT2440 possesses BAS homologous recombinases that enable high-fidelity genome editing.

• *E. coli* strain GB05-dir (Gene Bridges) carries Rac recombinases RecET (ETγA) on the genome, which are regulated by arabinose promoters.

Reagents

Plasmids

▲**CRITICAL** For a summary of all plasmids used in this protocol.

• pBBR1-P$_{Rha}$-Redγβα-xseB$_{ecoli}$-P$_{BAD}$-Cas9-xseAi-Km.This plasmid with a broad-host-range pBBR1 origin. This construct encodes the arabinose-inducible *cas9*,rhamnose-inducible *redγβα* operon (*redα*, *redβ* and *redγ*) and *xseB$_{ecoli}$*from *E. coli*, cassette for xseA inactivation and conveys kanamycin resistance.

• pBBR1-P$_{Rha}$-Redγ-Redαβ7029-xseB$_{7029}$-xseAi-Km.This plasmid with a broad-host-range pBBR1 origin. This construct encodes the rhamnose-inducible *redγ-redαβ$_{7029}$*operon (*redα$_{7029}$*, *redβ$_{7029}$* and *redγ$_{ecoli}$*) and *xseB$_{7029}$*from *S. brevitalea*, cassette for xseA inactivation and conveys kanamycin resistance.

• pBBR1-P$_{Rha}$-BAS-xseB$_{kt2440}$-P$_{BAD}$-Cas9-xseAi-Km.This plasmid with a broad-host-range pBBR1 origin. This construct encodes the arabinose-inducible *cas9*,rhamnose-inducible *BAS* operon (*alpha*, *beta* and *SSB* from phage_AB31), and *xseB$_{kt2440}$* from *P. putida*, cassette for xseA inactivation and conveys kanamycin resistance.

• RK2-J233-GFP-genta-FapR$_{ecoli}$-amp. This biosensor plasmid is based on a RK2 origin and harbors the green fluorescent protein reporter gene (*gfp*) and gentamicin resistance gene (*genta*) under the control of the J233 promoter and codon optimized fapR gene for *E. coli*.

• RK2-J233-GFP-genta-FapR$_{7029}$-amp. This biosensor plasmid is based on a RK2 origin and harbors the *gfp* and *genta* genes under the control of the J233 promoter and codon optimized fapR gene for *S. brevitalea*.
• RK2-J233-GFP-genta-FapR$_{kt2440}$-amp. This biosensor plasmid is based on a RK2 origin and harbors the *gfp* and *genta* genes under the control of the J233 promoter and codon optimized *fapR* gene for *P. putida*.
• p15A-cm-Cas9-J23119. This plasmid with a p15A origin. This construct encodes the *cas9*,J23119 promoter, and conveys chloramphenicol resistance.
• QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
• Qiagen Plasmid Mini Kit (Qiagen, cat. no. 12123)
• Tryptone (Oxoid, cat. no. LP0042)
• Yeast extract (Oxoid, cat. no. LP0021)
• NaCl (Sangon Biotec, cat. no. SB0476)
• NaOH (Sangon Biotec, cat. no. A100583)
• K_2HPO_4 (Sangon Biotec cat. no. A501212)
• $MgSO_4$ (Sangon Biotec cat. no. A500864)
• DNA 6 × loading buffer (Takara, cat. no. 9156)
• Agar (Solarbio, cat. no. A8190)
• Autoclaved ddH_2O kept at room temperature (RT, 18–22 °C) and on ice
• Chloramphenicol (Sigma-Aldrich, cat. no. C1919)
• Kanamycin sulfate (Sigma-Aldrich, cat. no. K4000)
• Gentamicin solution (50 mg mL^{-1}; Sigma-Aldrich, cat. no. G1397)
• Isopropanol (Sinopharm, cat. no. 80109218)
• Ethanol, absolute (Sinopharm, cat. no. 10009218)
• 70% (vol/vol) ethanol (Qiagen, cat. no. AM1091)
• RNase A (10 mg mL^{-1}; DNase and protease free; Thermo Scientific, cat. no. EN0531)
• $_L$-Arabinose (Sigma-Aldrich, cat. no. A3256)
• $_L$-Rhamnose (Sigma-Aldrich, cat. no. 83650)
• Sucrose (Sangon Biotec, cat. no. A502792)
• Glycerol (Sangon Biotec, cat. no. A100854)
• 1-kb DNA ladder (New England BioLabs, cat. no. N3232)
• PrimeSTAR Max DNA Polymerase (Takara, cat. no. R045B)
• 10 × Tris/boric acid/EDTA (TBE) buffer (Bio-Rad, cat. no. 161-070)
• Ethidium bromide solution (10 mg mL^{-1}; Dingguo, cat. no. NEP028-1)
• Agarose (Takara, cat. no. 5261)
• Buffer P1 (Qiagen, cat. no. 19051)
• Buffer P2 (Qiagen, cat. no. 19052)
• Buffer P3 (Qiagen, cat. no. 19053)
• Primers and oligonucleotides (Sangon Biotec): oligomers required for constructing the mutation and detecting the mutation in the *Pseudomonas* chromosome (see **Table 1** for examples)

Equipment

• Thermomixer (Eppendorf, model F1.5, cat. no. 5384000.071)
• MixMate (Eppendorf, cat. no. 022674226)

- Benchtop centrifuge, kept at RT (Eppendorf, model 5424R, cat. no. 5424000.010)
- Benchtop centrifuge, kept at 4°C (Eppendorf, model 5424R, cat. no. 5404000.014)
- Vortex (Scientific Industries, cat. no. G560E)
- Electroporator (Eppendorf, model 2510, cat. no. 940000009)
- Electroporation cuvettes with 1-mm gap, kept on ice (Eppendorf, cat. no. 940001005)
- Petri dishes, 94 mm × 16 mm (Greiner Bio-One, cat. no. 633180)
- Digital gel imaging system (GelDoc XR+, Bio-Rad)
- UV spectrophotometer (NanoDrop 2000c, Thermo Scientific)
- UV-visible spectrophotometer (T6 New Century, Purkinje General Instrument)
- Gel electrophoresis apparatus (Beijing Junyi, cat. no. JY300C)
- Flow cell sorter (BD, cat. no. FACSAria™ Fusion)
- Multifunctional microplate reader (Agilent, cat. no. BioTek Synergy H1)
- Incubators kept at 30°C and 37°C (Ningbo Jiangnan, cat. no. HWS-0288)
- pH detector (Sartorius, cat. no. PB-10)
- Sterile 10-µL inoculation loops (Sangon Biotec, cat. no. IL311-10-S-Q)
- Sterile 1-µL inoculation loops (Sangon Biotec, cat. no. IL311-1-S)
- 0.22-µm syringe filters (Pall, cat. no. PN4612)
- Millipore membrane filters (Merck-Millipore, cat. no. VSWP01300)
- 1-mL cuvettes (Fisher Scientific, cat. no. 14955127)
- PCR tube strips, 200 µL (Sangon Biotec, cat. no. F601550-0001)
- 1.5-mL microcentrifuge tubes (Sangon Biotec, cat. no. F600620-9001)
- 2.0-mL microcentrifuge tubes (Sangon Biotec, cat. no. F600619-9001)
- 50-mL centrifuge tubes (sterile, DNase/RNase-Free; Sangon Biotec, cat. no. CT788-GS)
- Syringe needles, 25-gauge 5/8, 0.5 mm × 16 mm (BD Medical, cat. no. 301805)
- Multipette (Eppendorf, cat. no. 4981000.019)

Reagent setup

LB broth

Prepare the solution by dissolving 10 g tryptone, 5 g yeast extract, and 1 g NaCl in ~900 mL ultrapure water. Following pH adjustment to 8.0 with 10% NaOH, adjust the volume to 1 L. Sterilize the medium via autoclaving (121°C, 20 min). After allowing it to cool, add antibiotics prior to use.

▲**CRITICAL** The broth can be kept at room temperature (RT) for several months.

LB agar plates

Prepare the solution by dissolving 10 g tryptone, 5 g yeast extract, 1 g NaCl and 12 g agar in ~900 mL ultrapure water. Following pH adjustment to 8.0 with 10% NaOH, adjust the volume to 1 L. Sterilize the medium via autoclaving (121°C, 20 min). After allowing it to cool, add antibiotics prior to use. Pour 20~25 mL medium into Petri dishes and allow the agar to solidify in a sterile hood.

▲**CRITICAL** To prevent photodegradation of tetracycline, agar plates supplemented with this antibiotic should be shielded from light during storage, typically by wrapping in aluminum foil.

▲**CRITICAL** Agar plates are typically prepared aseptically for experimental use. Any unused plates can be stored at 4°C for up to one week.

CYMG broth

Prepare the solution by dissolving 8 g tryptone, 4 g yeast extract, 4.06 g $MgCl_2 \cdot 2H_2O$ and 5 mL glycerol in ~900 mL ultrapure water. Following pH adjustment to 8.0 with 10% NaOH, adjust the volume to 1 L. Sterilize the medium via autoclaving (121°C, 20 min). After allowing it to cool, add antibiotics prior to use.
▲**CRITICAL** The broth can be kept at RT for several months.

CYMG agar plates

Prepare the solution by dissolving 8 g tryptone, 4 g yeast extract, 4.06 g $MgCl_2 \cdot 2H_2O$, 5 mL glycerol and 12 g agar in ~900 mL ultrapure water. Following pH adjustment to 8.0 with 10% NaOH, adjust the volume to 1 L. Sterilize the medium via autoclaving (121°C, 20 min). After allowing it to cool, add antibiotics prior to use. Pour 20~25 mL medium into Petri dishes and allow the agar to solidify in a sterile hood.

0.8% (wt/vol) agarose gels

Dissolve 0.56 g of agarose powder in 70 mL of 1× TBE buffer. Heat the mixture in a microwave oven until the agarose is completely dissolved. Allow the molten agarose solution to cool to approximately 60°C. Add 6 µL of ethidium bromide solution (10 mg mL^{-1}) and mix thoroughly to ensure homogeneity. Cast the solution into a gel tray fitted with an appropriate comb and allow polymerization to occur at ambient temperature until solidified.

Antibiotic stock solutions

Prepare 30 mg mL^{-1} chloramphenicol solution in 100% (vol/vol) ethanol. Divide 1-mL aliquots into 1.5-mL tubes in a sterile hood and store them at -20°C until needed. Prepare stock solution of gentamycin (5 mg mL^{-1}) and kanamycin (30 mg mL^{-1}) in autoclaved ddH_2O. Sterilize the solution by filtration in a sterile hood. Divide 1-mL aliquots into 1.5-mL tubes and store them at −20°C until further use.

10% (wt/vol) $_L$-arabinose and 10% (wt/vol) $_L$-rhamnose

Dissolve 5.0 g of sucrose in approximately 40 mL of sterile, deionized, distilled water (ddH_2O). Quantitatively transfer the solution to a 50 mL volumetric flask and bring the final volume to 50.0 mL with additional sterile ddH_2O. Sterilize the solution by filtration through a 0.22 µm membrane filter under aseptic conditions within a laminar flow cabinet. Aseptically aliquot 1.0 mL volumes of the sterile solution into pre-sterilized 1.5 mL microcentrifuge tubes. Store aliquots at -20°C for long-term preservation until required.

1 × TBE electrophoresis buffer

Dilute 50 × TBE buffer in ddH_2O to a 1 × solution and store it at RT until further use.

Troubleshooting

Safety warnings

No

Ethics statement

No

Before start

Experimental design

Overview of the Procedure

The utilization of ReaL-MGE systems can be divided into four parts (a total of 57 Procedure steps):

- Part 1, preparation of plasmids, strains and reagents
- Part 2, establishment of ReaL-MGE
- Part 3, multiplex malonyl-CoA metabolic and genome engineering in *E. coli*
- Part 4, application to different hosts

Part 1

1 **Preparation of plasmids, strains and reagents**

To establish plasmid systems with recombinase and Cas9 controlled by separate inducible promoters and produce linearized guide RNA (gRNA) cassettes, execute these precisely ordered procedures.

1.1 Select bacterial strains based on experimental requirements (e.g., *E. coli* BL21, *S. brevitalea* DSM7029, and *P. putida* KT2440). Verify that candidate strains possess authenticated genetic backgrounds, including well-characterized genome sequences and validated compatibility with specific recombination or transformation experiments.

1.2 Select broad-host-range vector systems with verified stability and compatible antibiotic markers. For designated strains (*E. coli* BL21, *S. brevitalea* DSM7029, and *P. putida* KT2440), employ strain-adapted recombination plasmids: pBBR1-P_{Rha}-Redγβα-Km-amp-ccdB, pBBR1-P_{Rha}-Redγ-Redαβ7029-Km-amp-ccdB, and pBBR1-P_{Rha}-BAS-Km-amp-ccdB respectively. Perform ScaI digestion followed by 37°C incubation (3 h) to generate linearized vectors containing rhamnose-inducible promoter systems, recombinases, and antibiotic resistance gene, flanked by homology sequences for precise integration of *cas9* gene, *xseB* gene, and *xseA* knockout cassette.

1.3 Perform PCR amplification using plasmid RK2-P_{BAD}-Cas9-genta as a template with high-fidelity polymerases to generate pBAD-regulated Cas9 expression cassettes. Amplify the PCR products using primers with complementary overhangs and regulatory sequences such as terminators to ensure robust expression. Validate amplification fidelity through 1.2% agarose gel electrophoresis and sequencing.

1.4 Amplify *xseB* gene using strain-specific primers: xseB-ecoli-1/2 for *E. coli*., xseB-7029-1/2 for *S. brevitalea* DSM7029, and xseB-kt2440-1/2 for *P. putida* KT2440. Confirm amplicon sizes using 1.2% agarose gel electrophoresis and validate sequence via sequencing with original PCR primers.

1.5 To prepare electrocompetent *E. coli* GB05-dir (Gene Bridges) for co-transformation with linearized vectors and plasmid elements while inducing RecET recombinase expression, single colonies were streaked onto LB agar plates and inoculated into 1.3 mL LB broth. After culturing overnight at 37°C with 950 rpm shaking, 50 µL aliquots were transferred to fresh 1.3 mL LB broth and incubated at 37°C for 2 hours. The cultures received 35 µL 10% $_{L}$-arabinose solution to induce RecET recombinase expression for 40 minutes. Cells underwent centrifugation at 9,600 *g* for 1 min at room temperature (RT), with removal of supernatant followed by two successive washes using 1 mL sterile distilled water. After the final centrifugation, 970 µL supernatant was

removed and discarded, leaving concentrated cells for subsequent transformation procedures.

1.6 To construct plasmid pBBR1-P_{Rha}-Redγβα-xseB-P_{BAD}-Cas9-xseAi-Km via RecET-mediated linear plus linear homologous recombination (LLHR), electroporate competent *E. coli* GB05-dir into a 1 mm electroporation cuvette using a mixture containing SacI-digested pBBR1-P_{Rha}-Redγβα-Km-amp-ccdB fragment, *xseB* gene, BGI-synthesized xseAi cassette, and BAD-Cas9 cassette. Apply a 1,350 V pulse for electroporation. Immediately add 1 mL antibiotic-free LB broth to the cuvette, transfer the suspension to a sterile 1.5 mL microcentrifuge tube, and incubate at 37 °C for 1 h. Centrifuge the cells at 9600 *g* for 30 s at RT, and discard 950 µL supernatant. Resuspend pelleted cells in the remaining 30 µL medium and plated onto LB agar containing 15 µg mL^{-1} kanamycin using a sterile loop. Incubate the plates at 37 °C for 24 hours. Construct plasmids pBBR1-P_{Rha}-BAS-xseBkt2440-P_{BAD}-Cas9-xseAi-Km and pBBR1-P_{Rha}-Redγ-Redαβ7029-xseB7029-Km following identical LLHR protocols, substituting strain-specific components.

1.7 After colony growth, select 12 colonies from the plate and streak onto fresh plates for preservation, then inoculate into 1.8 ml of antibiotic-supplemented LB broth and incubate at 37°C for 12 hours. Plasmid extraction followed standard protocols. Isolated expression plasmids underwent verification through restriction enzyme digestion and sequencing. Correct plasmids were preserved in water at −20°C.

1.8 The biosensor plasmid uses an RK2 origin of replication (oriV), with *gfp* and gentamicin resistance genes, regulated by the J233 promoter and an *E. coli* codon-optimized *fapR* gene. For plasmid assembly, amplify oriV from plasmid RK2-apra-cm using primers rk2-1/2; amplify the J233-GFP cassette from plasmid pBBR1-Rha-GFP-kan with primers J233-GFP-1/2. Extract the gentamicin resistance gene from plasmid R6K-loxM-genta with primers genta-1/2; amplify the ampicillin resistance gene from plasmid R6K-amp-ccdB with primers amp-1/2. The $fapR_{ecoli}$ gene was synthesized by BGI. Co-transform these five fragments into *E. coli* GB05-dir for LLHR according to **Steps 1.5-1.6**, generating plasmid RK2-P_{fapO}-GFP-genta-$FapR_{ecoli}$-amp. Isolate plasmid as described in **Step 1.7**, is validated through restriction enzyme digestion and sequencing. Construct plasmids RK2-P_{fapO}-GFP-genta-$FapR_{7029}$-amp and RK2-P_{fapO}-GFP-genta-$FapR_{kt2440}$-amp using identical methods, replacing $fapR_{ecoli}$ with fapR genes for *S. brevitalea* DSM7029- and *P. putida* KT2440-optimized fapR variants respectively.

1.9 Generate the gRNA cassette by mutually primed PCR synthesis using phosphorothioate-modified primers. One primer contains the universal tracrRNA sequence, and the other incorporates the target-specific gene sequence.

1.10 Generate recombineering substrates via PCR amplification with phosphorylated/phosphorothioate-modified 5′ terminal primers to enhance dsDNA

stability, improve ReaL-MGE efficiency, and facilitate homology arm integration for precise gene targeting.

Part 2

2 **Establishment of ReaL-MGE**

Electroporation of expression plasmids into the *E. coli* BL21, *S. brevitalea* DSM7029 and *P. putida* KT2440

2.1 Puncture the lid of a 1.5 mL microcentrifuge tube using a syringe needle (25-gauge 5/8, 0.5 mm × 16 mm). Add 1.3 mL of antibiotic-free LB medium into the tube. Transfer a single *E. coli* BL21 colony into the medium and culture the suspension overnight at 37°C with shaking at 950 rpm in an Eppendorf thermomixer.

2.2 Puncture the lid of a 1.5 mL microcentrifuge tube. Inoculate 1.3 mL of fresh LB medium with 50 µL of overnight culture. Incubate the culture at 37°C with shaking at 950 rpm in an Eppendorf thermomixer, monitoring OD_{600} until it reaches 0.8.

2.3 Centrifuge the culture at 9,600 *g* for 1 min at RT. Discarded the supernatant by decantation and resuspend the pellet in 1 ml of autoclaved ddH_2O. Repeated the entire process twice.

2.4 Gently remove the supernatant by pipetting and discard, leaving 30 µL of ddH_2O for cell resuspension.

2.5 Pipette 4 µL (500 ng) of the expression plasmid (pBBR1-P_{Rha}-Redγβα-xseB-P_{BAD}-Cas9-xseAi-Km) and the biosensor plasmid (RK2-P_{fapO}-GFP-genta-$FapR_{ecoli}$-amp) into the tube. Mix the plasmids and bacterial cells by gentle pipetting, then transfer the mixture into a 1 mm electroporation cuvette. Perform electroporation at 1350 V using an Eppendorf 2510 electroporator.

2.6 Immediately after electroporation, aseptically add 1 mL of antibiotic-free LB medium to the electroporation cuvette. Resuspend cells by gently pipetting, then transfer the mixture to a 1.5 mL microcentrifuge tube. Incubate at 37°C for 1 hour with shaking at 950 rpm in an Eppendorf thermomixer.

2.7 Spread 50 µL of recovery culture onto an LB agar plate containing kanamycin and ampicillin with a sterile 10 µL loop. Air-dry the plate in a sterile hood.

2.8 Incubate plates at 37°C for 12 hours until colony visibility.

2.9 The transfer of expression and biosensor plasmids into *P. putida* and *S. brevitalea* follow the the procedure described in **Steps 2.1-2.8**, with two differences: incubate

plates at 30°C for 48 hours in CYMG medium for *S. brevitalea*, and incubate plates at 30°C for 12 hours for *P. putida*.

2.10 Prepare 1.5 mL microcentrifuge tubes with vented caps and add 1.3 mL of LB or CYMG medium supplemented with kanamycin and ampicillin.

2.11 Transfer single colonies from the plates into individual tubes with sterile 200 µL pipette tips, then shake cultures overnight at 30°C or 37°C at 950 rpm in an Eppendorf thermomixer.

2.12 Confirm expression and biosensor plasmids integration in selected colonies by colony PCR screening.

Part 3

3 **multiplex malonyl-CoA metabolic and genome engineering in *E. coli***

ReaL-MGE 1.0 functions optimally in bacterial strains tolerating the CRISPR/Cas9 system cytotoxicity, including model organisms like *E. coli* and *P. putida*. Perform two sequential electroporations: deliver genome-editing elements in the first step, then apply counter-selection in the second step to enhance editing accuracy and efficiency.

3.1 Puncture a 1.5 mL microcentrifuge tube cap with a syringe needle (25-gauge 5/8, 0.5 mm × 16 mm). Add 1.3 mL of LB medium containing kanamycin and ampicillin. Inoculate with a single *E. coli* BL21 colony (harboring pBBR1-P_{Rha}-Redγβα-xseB-P_{BAD}-Cas9-Km and RK2-J233-GFP-genta-FapRe$_{coli}$-amp) and incubate overnight at 37°C with 950 rpm shaking in an Eppendorf thermomixer.

3.2 Puncture a 1.5 mL microcentrifuge tube lid. Transfer 50 µL overnight culture in 1.3 mL of LB medium containing kanamycin and ampicillin and incubate at 37°C with 950 rpm shaking in an Eppendorf thermomixer. At 40 min before optimal transformation, add 35 µL of 10% $_L$-rhamnose to induce recombinase and XseB overexpression. Continue incubation until cells reach optimal transformation. Harvest mid-log-phase cells, wash twice with sterile water to remove residual salts, and concentrate.

3.3 Generate the gRNA cassette and dsDNA donor substrates (HA_L-T7-HA_R, HA_L-LVA-HA_R, and HA_L-noncoding-HA_R) following **Steps 1.9-1.10**. Amplify tandem PCR products using the gRNA cassette and dsDNA donor substrate as templates with primers containing the gRNA target sequences as partial homology arms.

3.4 Mix *E. coli* BL21 electrocompetent cells with the ligated gRNA cassette and dsDNA donor substrates (gRNA cassette-T7, gRNA cassette-LVA, gRNA cassette-nocoding). Electroporate the mixture in 1 mm gap width cuvettes at 1350 V using an Eppendorf 2510 electroporator.

3.5 Following electrotransformation, incubate the cells in 1 mL of antibiotic-free LB broth supplemented with 10 nM dNTPs (GC content is 50%) at 30°C, 950 rpm shaking for 4 hours. Introduce 35 µL of 10% L-arabinose during recovery to induce Cas9 expression.

3.6 Prepare competent cells following the first round of electroporation. Mix the cells with the gRNA cassette and perform the second electroporation.

3.7 Incubate electroporated cells in 1 mL of antibiotic-free LB broth at 30°C with 950 rpm shaking for 8 hours. Add 35 µL of 10% L-arabinose during the second recovery phase to induce Cas9 counter-selection. Adjust recovery phase parameters to balance repair efficiency and cell viability.

Part 4

4 **FACS sorting for GFP expression from the FapR biosensor in *E. coli* BL21 wild type and recombinants**

Develop a screening strategy for mutants exhibiting enhanced malonyl-CoA production. Construct a malonyl-CoA biosensor system using green fluorescent protein (GFP). Integrate a malonyl-CoA-responsive regulatory element into the biosensor to directly correlate intracellular malonyl-CoA levels with GFP fluorescence intensity. Analyze fluorescence of single cells using flow cytometry to quantify GFP intensity. Select candidates exhibiting the highest GFP signals, indicative of elevated malonyl-CoA levels. Set detection thresholds using control samples to reduce false positives.

4.1 Centrifuge the cultured cells from **Step 3.7** at 9000 *g* for 1 minute at RT and remove the supernatant with a pipette carefully. Resuspend the pellet in 1 mL of sterile PBS through gentle pipetting. Repeat centrifugation and washing were to eliminate residual media components and reduce fluorescence background interference. Resuspend cells in 500 uL sterile PBS and adjust to optimal density.

4.2 Activate low-flow-rate mode to optimize sorting efficiency. Configure the FITC channel (488 nm excitation, 530/30 nm emission) for sorting GFP expression cells in *E. coli* BL21 wild type and recombinants using the FapR biosensor.

4.3 Load the prepared bacterial suspension into a flow cytometer-compatible tube. Analyze the sample in setup mode and optimize Forward Scatter (FSC) and Side Scatter (SSC) gain voltages to encompass all bacterial events.

4.4 Resolve bacterial populations by plotting FSC area (FSC-A) versus FSC height (FSC-H) toexclude duplicates. Generate collection plots with GFP fluorescence histograms for high GFP-expressing mutants and wild-type strains. Acquire the sample in setup mode and calibrate the acquisition channel voltage.

4.5 Acquire data from a minimum of 10,000 events and record full datasets. Define sort gate boundaries and acquisition modality for cell sorting. Load the collection tube and commence sorting.

4.6 Isolate highly efficient candidates sorted via flow cytometry and plate onto gentamicin-supplemented LB agar plates to obtain single colonies.

4.7 Prepare 1.5 mL microcentrifuge tubes with pierced caps and add 1.3 mL of LB broth supplemented with kanamycin, ampicillin, and gentamicin.

4.8 Inoculate colonies from plates into tubes using a 200 µL pipette tip (one colony per tube) and incubate overnight at 37°C with 950 rpm shaking in an Eppendorf thermomixer.

4.9 Puncture a 1.5 mL microcentrifuge tube lids and inoculate 50 µL of overnight culture into 1.3 mL of LB broth with kanamycin, ampicillin, and gentamicin. Incubate at 37°C with 950 rpm shaking in an Eppendorf thermomixer until OD_{600} reaches 0.8.

4.10 Transfer 200 µL bacterial culture into a black-walled clear-bottomed 96-well fluorescence microplate and include at least 3 replicates.

4.11 Perform the assay using a fluorescence enzyme marker with excitation at 488 nm and emission at 510 nm. Measure OD_{600} using the same sample to normalize fluorescence intensity. Calculate the GFP fluorescence/OD_{600} ratio to eliminate cell density effects.

4.12 Puncture 2 mL microcentrifuge tube lids and inoculate 50 µL overnight culture from **Step 4.8** into 1.75 mL of LB broth with kanamycin, ampicillin, and gentamicin. Incubate at 37°C with 950 rpm shaking in an Eppendorf thermomixer until OD_{600} reaches 0.4.

4.13 Chill the culture on ice and centrifuged at 9000 *g* for 15min at 4°C. Resuspend the pellet in PBS after washing, add 120 µL of lysis buffer (45:45:10 acetonitrile: methanol: water containing 0.1 M formic acid) on ice, and vortex vigorously. Incubate the extract on ice with intermittent vortexing for 15 min.

4.14 Add ammonium hydroxide to neutralize the acetic acid and centrifuge at 15000 *g* for 3 min at 4°C. Analyze the supernatant by LCMS. Perform standard analysis of prepared samples using an LCDAD system coupled to a Bruker Impact HD microTOF Q III ESI-MS ion trap instrument in positive ionization mode.

4.15 Use a Thermo™ Acclaim™ RSLC 120 C18 column (100 × 2.1 mm, 2.2 µm particle size). Apply a solvent gradient with solvent A (10 mM tributylamine, 15 mM acetic acid, and 5% methanol in distilled water) and solvent B (isopropyl alcohol). Record detection using both the diode array and ESI-MS.

4.16 Prepare a series of malonyl-CoA standard solutions at varying concentrations. Dilute each solution with an equal volume of lysis buffer (45:45:10=acetonitrile: methanol: water containing 0.1 M formic acid). Inject 5 µL standard solution and detect the target ion (m/z = 854.12 $[M+H]^+$). Record the peak area and generate a standard curve by plotting concentration versus peak area.

4.17 Calculate the sample peak area, substitute it into the standard curve equation to determine the malonyl-CoA concentration of the samples, and normalize it to bacterial solution concentration per unit OD_{600}.

4.18 Confirm the correlation between fluorescence intensity and intracellular malonyl-CoA concentration by measuring GFP fluorescence intensity and malonyl-CoA content in the same strain.

4.19 Detect genotypes of mutants with the highest intracellular malonyl-CoA concentrations and identify key genes and genome reduction strategies using colony PCR. Perform whole-genome sequencing on the top candidates to verify intended mutations and exclude off-target effects. Correlate specific genomic changes with phenotypic outcomes through sequencing data analysis.

Part 5

5 **application to different hosts**

ReaL-MGE 2.0 employs a linearized CRISPR/Cas9 system to regulate virulence in Cas9-intolerant bacteria, such as *S. brevitalea* DSM7029. Apply ReaL-MGE 2.0 systematically to target genes related to malonyl-CoA metabolism-related genes in *S. brevitalea* DSM7029. The procedure involves the following detailed steps.

5.1 Prepare *S. brevitalea* DSM7029Δglb strains harboring pBBR1-P_{Rha}-Redγ-Redαβ7029-xseB7029-Km and RK2-P_{fapO}-GFP-genta-$FapR_{7029}$-amp following **Steps 3.1-3.2**. Culture at 30°C in CYMG broth containing kanamycin and ampicillin with 950 rpm shaking in an Eppendorf thermomixer.

5.2 Prepare recombineering substrates by PCR using phosphorylated or phosphorothioate-modified primers.

5.3 The linearized CRISPR/Cas9 system, comprising Cas9 and single-gRNA, was prepared by PCR. The system featured Cas9 under the P_{genta} promoter and the gRNA cassette driven by the P_{J23119} promoter. The amplification from the p15A-P_{J23119}-Cas9-cm plasmid template employs primer set Pgenta-Cas9-1/tracrRNA-Cas9-2, incorporating phosphorothioate modifications.

5.4 Mix competent *S. brevitalea* DSM7029Δglb cells with the Cas9+gRNA mixture and dsDNA donor substrates for targeted gene editing. Perform the first electroporation with 1 mm gap-width cuvettes at 1350 V in an Eppendorf 2510 electroporator.

5.5 Following electrotransformation, incubate cells in 1.3 mL of antibiotic-free CYMG broth supplemented with 10 nM dNTPs (GC content is 50%) at 22°C, 950 rpm shaking for 8 hours.

5.6 Prepare competent cells following the first round of electroporation. Mix the cells with the Cas9+gRNA mixture and perform the second electroporation.

5.7 After the second electroporation, cells were cultured in 1 mL of antibiotic-free CYMG broth at 22°C, 950 rpm shaking for 12 hours.

5.8 Malonyl-CoA detection and key gene identification follow the same procedures established for *E. coli* BL21.

5.9 The mutant library for the malonyl-CoA metabolic network in *P. putida* KT2440 adopted methodologies
analogous to those in *E. coli* BL21, with a difference: after the first electroporation, incubate electroporated cells in 1.3 mL of antibiotic-free LB broth supplemented with 10 nM dNTPs (GC content is 50%) at 22°C, 950 rpm shaking for 4 hours. Following the second round of electroporation, incubate cells for 8 hours. Malonyl-CoA detection and key gene identification follow the same approach as in *E. coli* BL21.

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