Protocol
Chromosomal integration of complex DNA constructs using CRAGE and CRAGE-Duet systems

Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering. Our recent development of the CRAGE (chassis-independent recombinase-assisted genome engineering) system enables single-step integration of large, complex DNA constructs directly into bacteria genomes across multiple phyla. This protocol describes the details of the experimental design and procedures of CRAGE and extended CRAGE-Duet systems. It also describes a strategy that combines CRISPR with CRAGE, which allows implementation of CRISPR-Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Zhiying Zhao, Jian-Fang Cheng, Yasuo Yoshikuni
yyoshikuni@lbl.gov (Y.Y.)
zyzhao@lbl.gov (Z.Z.)

Highlights
Domestication of non-model bacteria using CRAGE and CRAGE-Duet systems

Single-step chromosomal integration of complex DNA constructs (payloads)

Use of CRISPR-Cas9, CRISPRa, and CRISPRi tools in non-model bacteria

Zhao et al., STAR Protocols 3, 101546
September 16, 2022 © 2022 The Author(s).
https://doi.org/10.1016/j.xpro.2022.101546
Protocol

Chromosomal integration of complex DNA constructs using CRAGE and CRAGE-Duet systems

Zhiying Zhao,1,6,* Jan-Fang Cheng,1 and Yasuo Yoshikuni1,2,3,4,5,7,*

1The US Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
2Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
3Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
4Center for Advanced Bioenergy and Bioproducts Innovation, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
5Global Center for Food, Land, and Water Resources, Hokkaido University, Hokkaido 060-8589, Japan
6Technical contact
7Lead contact
*Correspondence: yyoshikuni@lbl.gov (Y.Y.), zyzhao@lbl.gov (Z.Z.)

https://doi.org/10.1016/j.xpro.2022.101546

SUMMARY

Our recent development of the CRAGE (chassis-independent recombinase-assisted genome engineering) system enables single-step integration of large, complex DNA constructs directly into bacteria genomes across multiple phyla. This protocol describes the details of the experimental design and procedures of CRAGE and extended CRAGE-Duet systems. It also describes a strategy that combines CRISPR with CRAGE, which allows implementation of CRISPR-Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering. For complete details on the use and execution of this protocol, please refer to Wang et al. (2019), Wang et al. (2020), and Liu et al. (2020).

BEFORE YOU BEGIN

We recently reported the development of the chassis-independent recombinase-assisted genome engineering (CRAGE) system (Wang et al., 2019). CRAGE enables single-step integration of large complex DNA constructs (payloads) directly into the chromosomes of diverse non-model bacteria. In this technology, we first integrate a landing pad (LP) containing a Cre recombinase gene flanked by two mutually exclusive lox sites into the chromosome via an available integration method (e.g., a transposon and a suicide plasmid). We then replace the LP with the payloads (also flanked by the same lox sites), mediated by Cre recombinase; the payloads are inserted with high accuracy and efficiency (Wang et al., 2019). We further extended CRAGE to CRAGE-Duet by introducing a third mutually exclusive lox site, allowing use of any applications a dual plasmid system can offer at the genome level (Wang et al., 2020; Liu et al., 2020).

These CRAGE systems can be ideal platforms for implementing clustered regularly interspaced short palindromic repeat (CRISPR) systems, overcoming a major limitation to using CRISPR in non-model bacteria. CRISPR systems are important tools for functional genomics studies, as gene function can be characterized effectively by deletion and transcriptional repression and activation mediated by CRISPR/Cas9 (CRISPRi), CRISPRd, and CRISPRa, respectively (Gilbert et al., 2013; Peng et al., 2018; Zalatan et al., 2015). Our group implemented CRISPR on CRAGE-Duet and used this approach to characterize secondary metabolite biosynthetic gene clusters (BGCs) using Photobacterium luminescens as a model system (Ke et al. (2021). The results demonstrate that the CRAGE-CRISPR system is a simple yet compelling approach to BGC characterization.
Here, we describe general protocols for various uses of CRAGE, CRAGE-Duet, and CRAGE-CRISPR systems. These protocols include all the steps necessary to select appropriate CRAGE systems, domesticate non-model bacteria using CRAGE systems, and implement CRISPR systems. We do not include the construction of the LP plasmids, but the plasmids can be requested through our laboratory or purchased through Addgene. While we used a CRAGE-CRISPR system to characterize bacterial secondary metabolite BGCs, we do not include such characterizations in these protocols.

Select an appropriate CRAGE platform for your applications

© Timing: ~2 h (user-dependent)

We have developed two different CRAGE systems, CRAGE and CRAGE-Duet. CRAGE enables integration of a single payload, while CRAGE-Duet enables integration of two payloads. To help users select the most appropriate CRAGE system for their applications, this section describes the differences between the two systems and their possible applications (Table 1).

1. CRAGE system.

CRAGE is useful for any applications requiring heterologous gene expression in bacteria. This system uses a plasmid, pW17, to integrate the LP into the recipient bacterial genome. This plasmid comprises a gene coding a mariner transposase and a transposon containing the LP flanked by inverted repeat (IR) sequences. The LP includes a Cre recombinase gene and a kanamycin (Km)-resistant gene (KmR) flanked by two mutually exclusive lox sites (loxP and lox5171). It also contains a T7 RNA polymerase (T7RP) gene under the control of the lacUV5 regulon outside the lox sites. This plasmid can be transformed into recipient microbes using conjugation, electroporation, or other methods. Upon transforming pW17, the LP transposons...
integrate at random locations in the genomes of the bacterial population. Users can select a bacterial strain whose phenotype has been least affected by integration for subsequent use.

2. CRAGE-Duet system.

The CRAGE-Duet system offers dual integration sites, allowing any applications that dual plasmid systems enable at the genome level. For example, it allows modular assembly of pathways and introduction of new regulations to orthogonal expression systems (e.g., the T7 expression system). CRAGE-Duet uses the pW37 plasmid to integrate the 3-lox LP into the recipient bacterial genome. This LP contains the KmR and Cre recombinase gene, flanked by loxP and lox5171 (the first integration site) and lox2272 and loxP (the second integration site), respectively. As with the CRAGE system, this plasmid can be transformed into recipient microbes using conjugation, electroporation, or other methods. Upon transformation, the LP transposons integrate at random locations in the genomes of the bacterial population. Users can select a bacterial strain whose phenotype has been least affected by a particular random integration for subsequent use.

Select appropriate CRAGE accessory plasmids

© Timing: ~2 h (user-dependent)

We have developed a variety of accessory plasmids to help integrate payloads into the genomes of target non-model bacteria. This section helps users choose and request suitable accessory plasmids.

We designed the accessory plasmids pW34 and pWSY to target the CRAGE LP and the first integration site of the CRAGE-Duet LP. The payloads on these plasmids, flanked by two mutually exclusive lox sites (loxP and lox5171), are integrated into the LPs with Cre-mediated cassette exchange.

pW34 can be used to confirm that CRAGE or CRAGE-Duet has been implemented successfully. This accessory plasmid carries the luxCDABE operon under the control of the T7 promoter between two lox sites. Upon transformation of pW34, the luxCDABE operon is integrated into the genome of the recipient strain via Cre-mediated cassette exchange. Because the payload also contains the apramycin (Apr) resistance gene (AprR), simple selection with Apr resistance followed by counterselection with Km sensitivity allows users to identify successful integrants. Detection of luminescence also confirms integration of this payload. After this simple counterselection, integration efficiency is usually 100%. The backbone of the pW34 plasmid can be used as a template for the integration of other payloads. Because pW34 uses R6Kγ as an origin of replication, we recommend using this plasmid to integrate constructs of up to 12 kb.

pWSY is used to integrate payloads larger than 10 kb. Because this plasmid is a derivative of a BAC-based plasmid, it is cumbersome to use. We recommend using it only when payloads are so large that pW34 cannot stably maintain them. pWSY also contains yeast CEN/ARS and is compatible with yeast transformation-associated recombination (TAR) cloning to assemble large complex DNA constructs. We found pWSY tends to replicate in bacterial species (mainly species in Enterobacteriaceae) genetically closer to E. coli. See potential solutions for problem 4 for troubleshooting.

The accessory plasmids pW38 and pW39 are derivatives of pW34 but are designed to target the second integration site of the CRAGE-Duet system. These plasmids contain T7RP under the control of the lacUV5 promoter and lacUV5 regulon. The payloads between two mutually exclusive lox sites (lox2272 and loxP) on these plasmids are integrated into the second integration site of the CRAGE-Duet LPs with Cre-mediated cassette exchange.

Design the CRAGE-CRISPR system

© Timing: 1–2 days (user-dependent)
The versatility of the CRAGE-Duet system allows users to combine it with the powerful CRISPR systems in non-model bacteria for functional genomics studies. This section describes the accessory plasmids required for implementation of the CRAGE-CRISPR system.

The accessory plasmid pR6K-2L-SpCas9 implements CRISPRi and CRISPR/Cas9, and pR6K-2L-dCas9-RNAPu implements CRISPRa. pR6K-2L-SpCas9 contains the Cas9 gene and the recET genes, and pR6K-2L-dCas9-RNAPu contains the dCas9-RNAPu gene. In both plasmids, these genes are flanked by loxP and lox5171 and are targeted to the first integration site of the CRAGE-Duet system.

The accessory plasmid pR6K-lox2272WT integrates the custom sgRNA and/or repair arm into the second integration site. For the sgRNA and repair arm, users design 20 bp and 500 bp sequences based on the target sequence in the recipient bacterial genome. pR6K-2L-SpCas9, pR6K-2L-dCas9-RNAPu, and pR6K-lox2272WT are derivatives of pW34.

In addition to working with CRAGE-Duet, which has a 3-lox LP, CRISPR might also work with the 2-lox-LP CRAGE, in which Cas9, recombinases, sgRNA, and repair DNA template are all included in a single accessory vector.

**Choose conjugal donor strain**

We used the conjugal donor strain *E. coli* BW29427 to transform all LPs and accessory plasmids. Users can easily select out this strain on LB agar plates without diaminopimelic acid (DAP). All the plasmids we created are compatible with other commonly used *E. coli* conjugal donor strains, including S17-1, SM10 (Simon et al., 1983), and ET12567(pUB307) (Flett et al., 1997), if the user prefers a different strain.

**Test antibiotic resistance of target bacteria**

© Timing: 2–3 days

To facilitate selection of integrants, users must determine the minimal inhibitory concentration (MIC) of several antibiotics for the target bacteria. Our studies found many bacteria cannot grow with Km and Apr concentrations of more than 500 mg/mL or 200 mg/mL, respectively. Therefore, we recommend that users test 50, 200, and 500 μg/L of Km, and 50, 100, and 200 μg/mL of Apr. After overnight liquid culture, users will spot 1 μL of each cell on plates containing antibiotics with different concentrations. Alternatively, they can use antibiotic test strips to determine the MICs.

*Note:* For strains with a natural resistance of more than 1 mg/mL, users may consider different antibiotic markers to replace the Km in the landing pad and Apr in the accessory plasmids as alternatives.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| TransforMax™ EC100D™ pir+ Electrocompetent *E. coli* | Lucigen/Epicenter | Cat# ECP09500 |
| *E. coli* BW29427 | University of Illinois | #aka WM3064 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 2-log DNA ladder | New England Biolabs | Cat# N0550S |
| 96-well black/clear flat bottom polystyrene NBS microplate | Corning | Cat# 3651 |
| Agarose UltraPure | Invitrogen | Cat# 16500-500 |
| Amino acids mix | Sunrise Science Products Inc. | Cat# 1001 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Apramycin sulfate salt | Sigma-Aldrich | Cat# A2024-1G |
| Betaine | Sigma-Aldrich | Cat# B2629-100G |
| CaCl₂ solution, 1 M | Sigma-Aldrich | Cat# 21115 |
| Citric acid monohydrate | Sigma-Aldrich | Cat# 1909 |
| D-glucose | Sigma-Aldrich | Cat# G8270 |
| Costar 96-well clear round well plate, sterile with lid | Fisher | Cat# 07-200-760 |
| Diaminopimelic acid | Sigma-Aldrich | Cat# 33240-5G |
| Filter membranes, nitrocellulose | Sigma-Aldrich | Cat# N8395-100EA |
| Gel loading dye, purple (6X), no SDS | New England Biolabs | Cat# B702SS |
| Gene Pulser®/MicroPulser™ electroporation cuvettes, 0.1 cm gap | Bio-Rad | Cat# 1652089 |
| Gibson assembly HiFi HC 1-step kit | SGI-DNA | Cat# GA1100-4X10 |
| Glass balls (5 mm) | VWR | Cat# 26396-596 |
| LB agar plate | Teknova | Cat# L1066 |
| LB agar plate with 50 μg/mL apramycin | Teknova | Cat# L1066 |
| LB agar plate with 50 μg/mL kanamycin | Teknova | Cat# L1025 |
| LB agar plate with 200 μg/mL kanamycin | Teknova | Cat# L1077 |
| LB (Miller’s) broth | Growcells | Cat# MBL-7030 |
| Kanamycin sulfate salt | Sigma-Aldrich | Cat# 60615-5G |
| M9 minimal salts (2X) | Fisher | Cat# A1374401 |
| MgSO₄ solution, 1 M | Sigma-Aldrich | Cat# M3409 |
| Phusion High-Fidelity PCR Master Mix (2X) | New England Biolabs | Cat# M0513S |
| SOC medium | New England Biolabs | Cat# B9020S |
| Sybr Safe | Invitrogen | Cat# S33102 |
| TAE, 50× | Growcells | Cat# MRGF-4210 |
| Trace mineral solution | ATCC | Cat# MD-TMS |
| Vitamin supplement | ATCC | Cat# MD-VS |
| Yeast extract | Sigma-Aldrich | Cat# Y1625 |

Critical commercial assays

| Gel extraction kit | Promega | Cat# A9282 |
| Plasmid midi kit | Promega | Cat# A2495 |
| Quant-it dsDNA HS assay kit | Invitrogen | Cat# Q32851 |
| DNeasy blood & tissue Kits | QIAGEN | Cat# 69506 |

Experimental models: Organisms/strains

| Photorhabdus luminescens subsp. laumondii TT01 | DSMZ | Cat# DSM15139 |
| Pseudomonas simiae WCS417r | JGI | SB119 |
| Serratia odorifera | DSMZ | Cat# DSM4582 |

Oligonucleotides

| CCACCTTCGTAAAGCTGTAGTG (forward primer for CRAGE LP region) | IDT | SBP572 |
| TCCAGATCTCAAACTGGAACACTC (reverse primer for CRAGE LP region) | IDT | 99W |
| GAAAAGCTGGCGGTAGTAAGCC | IDT | SBP202 |
| AGGCCGACACCCCG (forward primer for CRAGE backbone region) | IDT | SBP203 |
| GGGGCTGCTCTTAACCGCGCAGCGCT (forward primer for CRAGE backbone region) | IDT | 197_Dt |
| GCTACAGAGCATAAGGTGGTGG ATCC (forward primer for CRAGE-Duet LP and backbone region) | IDT | 190_Dt |
| GTAATGCTCCTACCTGTCGCG (reverse primer for CRAGE-Duet LP region) | IDT | 44W |
| CGCTGATAACCTCGTCCGG (reverse primer for CRAGE-Duet backbone region) | IDT | 27F |

(Continued on next page)
MATERIALS AND EQUIPMENT

Prepare buffers, plates, and media in advance and store them at 4°C to extend their shelf life. Store all antibiotics at –20°C to extend their shelf life. We recommend handling all bacterial cultures in a laminar flow cabinet to maintain sterilized conditions. Users should follow their institutions’ protocols for waste disposal, and should properly bleach or autoclave all biological wastes before disposal.

**M9-based media**

| Reagent                | Final concentration | Amount |
|------------------------|---------------------|--------|
| Glucose                | 4 g/L               | 4 g    |
| Yeast extract          | 5 g/L               | 5 g    |
| 2x M9 minimal salts    | 1 x                 | 500 mL |
| Citric acid monohydrate| 3 g/L               | 3 g    |
| 1 M MgSO<sub>4</sub> solution | 2 mL/L         | 2 mM   |
| 1 M CaCl<sub>2</sub> solution | 100 µL/L       | 0.1 mM |
| Trace mineral solution | 2.5 mL/L            | 2.5 mL |
| Vitamin supplement     | 2.5 mL/L            | 2.5 mL |
| ddH<sub>2</sub>O       | n/a                 | 493 mL |
| **Total:**             |                     | 1000 mL|
Note: Sterilize the media using a 0.22 μm filter, and store it at 4°C for up to 1 year.

### Agarose gel

| Reagent                      | Final concentration | Amount  |
|------------------------------|---------------------|---------|
| Agarose                      | 1%                  | 1 g     |
| 1× TAE                       | 1×                  | 100 mL  |
| Sybrsafe (10,000×)           | 1×                  | 10 μL   |
| **Total:**                   |                     | 100 mL  |

⚠ CRITICAL: EDTA in TAE may cause respiratory tract irritation. Use a mask to avoid breathing dust. Wear protective gloves, eye shields, and clothing. If there is any skin or eye contact, wash with water for several minutes.

### Betaine

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| Betaine       | 5 M                 | 29.275 g |
| ddH₂O         | n/a                 | 50 mL    |
| **Total:**    |                      | 50 mL    |

Note: Prepare the stock solution at 5 M, sterilize it using a 0.22 μm filter, store it at –20°C, and use it at 0.5 M final concentration in PCR reaction.

**Alternatives:** Users may use 5% DMSO as an alternative to Betaine.

### Diaminopimelic acid (DAP)

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| Diaminopimelic acid | 60 mM              | 114 mg   |
| ddH₂O            | n/a                 | 10 mL    |
| **Total:**       |                      | 10 mL    |

Note: Prepare stock solution at 60 mM, sterilize it using a 0.22 μm filter, store it at –20°C, and use it at 0.3 mM final concentration in culture media.

### Kanamycin

| Reagent | Final concentration | Amount   |
|---------|---------------------|----------|
| Kanamycin | 50 mg/mL           | 0.5 g    |
| ddH₂O   | n/a                 | 10 mL    |
| **Total:** |                  | 10 mL    |

Note: Prepare stock solution at 50 mg/mL, sterilize it using a 0.22 μm filter, store it at –20°C, and use it at the appropriate final concentration in growth media.

⚠ CRITICAL: Kanamycin causes allergic respiratory and skin reactions; it may also cause difficulty breathing and damage fertility or a fetus. Wear protective gloves and avoid breathing dust.
Note: Prepare stock solution at 50 mg/mL, sterilize it using a 0.22 μm filter, store it at −20°C, and use it at the appropriate final concentration in growth media.

△ CRITICAL: Apramycin may irritate skin or eyes; it may also cause difficulty breathing and damage to fertility and fetuses. Wear protective gloves and avoid breathing dust.

Alternatives: Teknova supplies 50 and 200 μg/mL Km LB plates, as well as 50 and 100 μg/mL Apr LB plates.

STEP-BY-STEP METHOD DETAILS
Integrating CRAGE and CRAGE-Duet LPs

Timing: 5–10 days (user-dependent)

This section describes step-by-step methods for conjugal transformation of pW17 and pW37, as well as integration of the CRAGE and CRAGE-Duet LPs into the genomes of recipient bacteria. The step numbers correspond to those of the Graphical Abstract and Figure 1.

1. Preparation of donor and recipient strains.
   a. Culture donor strain BW29427 harboring pW17 or pW37 LP plasmid in LB medium supplemented with 0.3 mM diaminopimelic acid (DAP) and 50 μg/mL Km at 37°C in an incubation shaker at 200 rpm overnight.
   b. Culture the recipient bacteria using their optimal growth conditions.

   Note: With improper handling, bacteria can contaminate media, and cross-contamination can occur between cultures. Use proper sterilization techniques. If working in a laminar flow hood, make sure not to block airflow inside it. Use filter tips and change them often.

2. Conjugal transformation of the LP plasmid.
   a. Measure optical density at 600 nm (OD600) for the donor and recipient cultures and mix with a 4:1 donor to recipient ratio in a 2 mL tube (see notes below). Centrifuge this mixture (18,000 × g, 1 min) and wash three times with 1 mL LB to remove residual antibiotics from the culture.

   Note: For landing pad integration, we typically mix 400 μL to 1 mL of OD=2 donor strain with 100–250 μL of OD=2 recipient strains. Users can increase conjugation efficiency by changing the donor to recipient ratio in either direction. Some recipient strains can be conjugated in less than 6 h, while other strains may take more than 24 h. For strains with lower conjugation efficiency, consider increasing donor and/or recipient strains by 2- to 5-fold as well as increasing conjugation incubation time.

   b. Resuspend the resulting pellet in 50 μL LB with 0.3 mM DAP media and transfer onto a nitrocellulose filter membrane on top of an LB agar plate containing 0.3 mM DAP (Figure 2A).
Incubate this plate at 28°C (or at an optimal temperature for the target strain) for 12–24 h (Figure 2B).

Note: Use strain-specific temperatures for conjugation. We used 28°C because it was the optimal temperature for many bacteria with which we worked.

Researchers can use LB with 0.3 mM DAP instead of LB throughout steps 2a–2c to simplify the procedure. Include a blank control to confirm that the procedure is contamination-free. To do this, pipette 25 μL LB and/or LB with DAP that was used for wash and resuspension steps (steps 2a–2c) onto an empty LB with DAP agar plate and incubate with other experimental samples. Although multiple conjugations can be done on the same plate, we strongly recommend using separate or multi-well plates to prevent contamination (Figure 2C).

c. Scrape off the bacterial mixture grown on the membrane using an inoculation loop and resuspend it into 500 μL LB media. Then centrifuge it (18,000 × g, 1 min), wash it with 1 mL LB three times, and resuspend it in 1 mL LB. Plate 100 μL of the mixture on an LB plate containing the appropriate concentration of Km.

Note: Single colonies can also be streaked out simply on the selection plate using an inoculation loop.

Depending on the conjugation efficiency, users might consider plating the mixture with 10⁻¹, 10⁻², or 10⁻³-fold dilution onto selection plates. Although freezing will compromise conjugation efficiency, users can freeze the conjugation mixture and store it by adding glycerol to a final concentration of 15%.

d. Incubate the plate at an optimal temperature for the growth of the recipient bacteria until single colonies appear (Figure 2D, 1–3 days, strain-dependent); then restreak single colonies on selection media.
3. Screening and verifying LP integration.
   a. Inoculate single colonies onto 96-well plates containing 150 µL of LB media with 7.5% glycerol and an appropriate concentration of Km, determined using the MIC test for the recipient strain.
   b. Use 0.5 µL of each overnight culture in a 20 µL PCR reaction to determine whether the LP has integrated into the genome or has remained as a plasmid (Figure 3).
      i. For the CRAGE system, primers SBP572 (CCACCTTCGTAAGACTGTAGTG) and 99W (TCCCAGATCTCAAACGGAACACACTC) amplify a 374 bp LP region, and primers SBP202 (GAAAAGCTGGGCGCGTTAAGCCAGCCCCCGACACCCG) and SBP203 (GGGGCTGCTTAAACGCGCCAGCTTTTCAATTC) amplify a 645 bp plasmid backbone region.
      ii. For the CRAGE-Duet system, primers 197 DT (GCTATCAGGACATAGCGTTGGCTACC) and 190 DT (GAATGCTCTGCAGTGTCG) amplify a 423 bp LP region, and primers 197 DT (GCTATCAGGACATAGCGTTGGCTACC) and 44W (CGCTGCATAACCGCTCTGG) amplify a 859 bp backbone region. If the LPs are integrated into the genome of the recipient bacteria, only the LP region is amplified.
   c. To identify the integration location of the LP, isolate genomic DNA from 2 mL overnight culture using QIAGEN blood and tissue kit followed by Illumina or PacBio sequencing, with 10–500 ng or 2–5 µg input DNA, respectively. Users can map the raw reads to the LP using Geneious Prime or Integrative Genomics Viewer (IGV). This allows the user to identify the reads that map both 3\'- and 5\'-ends of the LP as well as the LP integration site. Users can then map the flanking sequences that correspond to the genome to identify the integration location. Alternatively, users can use inverse PCR (Ochman et al., 1988) at the junctions of LP and genomic DNA combined with Sanger sequencing to determine the LP integration site (Liu et al., 2020).

   **Note:** Typically, we pick 8–16 colonies from each conjugal transformation plate.

   Some of the conjugation steps (e.g., mixing and washing) are contamination prone; therefore, we recommend using 16S rRNA sequencing to ensure the integrity of the strains. The amount of input DNA for sequencing may vary depending on the requirement of each sequencing facility. Follow the guidelines from each facility.
4. Testing the growth phenotype to evaluate the impact of the LP integration.
   a. Culture the LP-integrated strains and their corresponding wild-type strain in 1 mL LB with appropriate antibiotics at the strain-specific optimal growth temperature to reach an OD$_{600}$ of 2–6.
   b. Centrifuge cells (18,000 g, 1 min), wash them with M9 minimal media three times and resuspend them into a fresh medium. Dilute this culture to an OD$_{600}$ of 0.1.
   c. Add 150 µL of cultures to each well of a 96-well black/clear flat-bottom plate (Corning). Perform the growth assay in triplicate for each strain.
   d. Use a Synergy H1 microplate reader (BioTek) to measure the growth of the LP strains at intervals of 15 min for 20–48 h at a strain-specific optimal growth temperature with constant shaking (Figure 4).
   e. Select the LP strains with growth phenotypes more similar to those of the wild-type strain for subsequent study. Information about the LP insertion location identified in the previous section also supports the selection of the target strain.

   **Note:** In our experience, almost all strains grow well. However, we sometimes see obvious growth differences between WT and LP strains. Strains whose growth is not compromised will be carried over to the downstream experiment.

**Integrating the payload into the LPs**

- **Timing:** 5–10 days (user-dependent)

5. Integrating a lux operon to confirm LP function.
   a. Conjugally transform pW34 containing the luxCDABE operon into the LP strain. Because of the high conjugation and integration efficiency, we recommend streaking out a single colony directly from the conjugation mixture (Figure 5A).
   b. Screen 8 to 10 single colonies from each conjugation for their resistance to Apr and sensitivity to Km through counterselection (Figure 5B).
   c. Inoculate the positive integrants of the luxCDABE operon in 1 mL of LB medium with appropriate antibiotics and grow them at optimal temperature overnight.
d. Dilute these cultures to an OD600 of 0.1 with fresh LB medium containing appropriate antibiotics.

e. Transfer 4 x 150 µL aliquots of each culture to a 96-well plate with a clear bottom. Add IPTG to these cultures at final concentrations of 0, 0.01, 0.1, and 1.0 mM. Incubate the cultures in the Synergy H1 microplate reader at the optimal growth temperature for 24 h. Measure the OD and bioluminescence at intervals of 15 min (Figures 5C and 5D).

**Note:** Once successful integration of the luxCDABE operon and luminescence activity is confirmed, the LP strain is ready for the downstream payloads integration steps.

Single constructs or libraries of constructs can be transformed and directly integrated into the set of both CRAGE and CRAGE-Duet LP strains. Multi-well plates can help improve throughput.

Because the transposon is randomly integrated into the genomes, the LP may be accidentally integrated into a location essential to the growth of the recipient strains. In our experience, an optimal LP strain, in which the LP is inserted in the non-polycistronic intergenic region, is usually found if 10 transconjugants are selected at step 2. In addition to identifying the LP integration location and evaluating the growth phenotype, we recommend that users perform assays for other phenotypes that are important in downstream scientific applications.

**Figure 3. CRAGE landing pad integration screening**
(A and B) Agarose gel electrophoresis (1% agarose) of PCR products using (A) the CRAGE LP or (B) backbone-specific PCR primer sets. Lanes 1–8 were from LP integrants. Lane 9 was from pW17 conjugation plasmid DNA used as a template. Lane M is an NEB 2-log ladder.

**Figure 4. Growth curve comparison between wild-type and CRAGE LP-integrated strains**
One wild-type (WT) *Serratia odorifera* and its seven CRAGE variants were incubated in a Synergy H1 microplate reader with shaking for 20 h at 28°C. The optical density (OD) was measured at intervals of 15 min. No obvious differences were seen between the CRAGE LP strain and its WT.
Integrating the second payload into the LPs (CRAGE-Duet only)

© Timing: 5–10 days (user-dependent)

6. Integrating the inducible or constitutive promoter.

For the CRAGE-Duet system, the second payloads are delivered to the second integration site. As a proof-of-principle experiment, use donor strains with T7RP under IPTG-inducible promoter pW38 and/or constitutive promoter pW39 for the conjugation at the second integration site (lox2272 and loxP). Select strains in which T7RP has been integrated by their resistance to both Km and Apr (indicating successful cassette exchange), followed by a luminescence assay (refer to step 5 for details).

Implementing the CRAGE-CRISPR system

© Timing: 5–10 days (user-dependent)

Users can use 3-lox LP strains created from steps 2 and 3 (described above) for all the downstream conjugation and verification steps.

7. Once the LP for the CRAGE-Duet system is successfully integrated into the genome, implement the CRAGE-CRISPR system in these strains. Use the plasmids pR6K-2L-SpCas9 and pR6K-2L-dCas9-RNAPω to implement CRISPRi and CRISPRa, respectively. These plasmids are targeted...
to the first integration site between loxP and lox5171. Follow the step 5 protocol to integrate the payloads on these plasmids into the LP site.

8. Use pR6K-lox2272WT as a base plasmid to integrate sgRNA constructs for the CRISPR systems. Modify these plasmids to target sequences of interest. Follow the step 6 protocol to integrate the payloads into the second integration site of the CRAGE-Duet LP.

Once the LP is integrated into the genome, integrating payloads using the Cre-mediated cassette exchange is generally more efficient than mariner-transposase-mediated transposon integration. Although we generally use a 1 mL culture of recipient strain, 100 μL is often sufficient for conjugal integration of payloads into the recipient LP strain. If the efficiency of the conjugal recipient strain is very high, users can shorten the conjugation incubation time to optimize the protocol.

EXPECTED OUTCOMES

The main outcome of this protocol is the rapid integration of simple to large complex payloads into non-model bacteria. Successful implementation of CRAGE and/or CRAGE-Duet allows functional genomics studies of non-model bacteria, engineering of members of microbial communities, and acceleration of the Design–Build–Test–Learn (DBTL) cycle (Liu et al., 2015) for industrial strain development. All plasmids described in the protocol are available to the scientific community upon request or purchased through Addgene.

LIMITATIONS

We have demonstrated that the CRAGE technology can be used to engineer more than 40 species including α-, β- and γ-Proteobacteria and some Actinobacteria (Wang et al., 2019). We are currently expanding this portfolio. We have tested payload constructs ranging from < 1 kbp to 60 kbp. The integration efficiency remained constant regardless of payload size. The integration limit likely depends on the size of the DNA fragment the BAC-based accessory plasmid can carry, which is about 300 kbp.

TROUBLESHOOTING

Problem 1
Conjugation tends to introduce cross-contamination.

Potential solution
At any step in which multiple recipient strains are handled in parallel, cross-contamination tends to occur. Even minor contamination in one strain can cause cross-contamination in strains that have high conjugation efficiency (1–10^{10} colony-forming units/conjugation). Because conjugation efficiency changes dramatically depending on the strain, perform every procedure with extra caution to prevent any contamination. Always include blank culture media as controls in all culture and conjugation steps. We also highly recommend checking the identity of strains using 16S rRNA sequencing to confirm the correct strain at each critical step.

Problem 2
LP colony PCR not working.

Potential solution
Do not allow the volume of overnight culture used in the colony PCR to exceed 2.5% of total PCR volume (0.5 μL cells in 20 μL PCR reaction). Depending on the strain and the cell density of its culture, we found that diluting the overnight culture by 20- to 40-fold facilitates the colony PCR. Betaine is a well-known PCR additive that helps reduce the formation of secondary structures caused by GC-rich regions. 0.5 mM betaine is the best solution for all PCRs in our experience. The addition of betaine also helps the PCR amplification of genomes with high GC content.
Problem 3

The LP transposon does not jump into the genome.

**Potential solution**

If pW17 and pW37 can replicate in the recipient strains (which is sometimes the case in species that are genetically closer to *E. coli*), the LP is sometimes not integrated into the genome, resulting in no positive clones at step 3. We cure the plasmid as the solution to this problem. To do this, we pass transformants serially in fresh LB medium without antibiotics for 10–30 days. We streak out the culture on LB containing appropriate antibiotics periodically for PCR screening to identify the successful integrants, as outlined in Figure 6. The curation period is greatly strain-dependent.

Problem 4

The LP is rearranged upon transposition into the genome.

**Potential solution**

We recommend sequencing the whole landing pad fragment to make sure that this region was inserted accurately. We often found that species of β-Proteobacteria are troublesome upon transposition. The lox site might be missing, or the LP cassette might get rearranged. For some β-Proteobacteria strains, we have overcome this problem by using a minimal CRAGE vector without Cre and then transiently expressing it using a delivery vector for the downstream cassette exchange steps. We will make the modified vector available to the scientific community upon request.

Problem 5

Payloads are not integrated into the genome.

**Potential solution**

When using pWSY-based accessory vectors, the constructs tend to remain in the plasmid format in the bacteria, especially for species of *Enterobacteriaceae* (Figure 7). In this case, use simple curation procedures similar to the problem 3 solution (which explains how to cure the plasmid) to screen the strains in which the cassette exchange was successful (those strains will be resistant to Apr and susceptible to Km).
We also recommend using pW34, which is based on the R6Kγ origin of replication and cannot replicate in recipient microbes.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Yasuo Yoshikuni (yyoshikuni@lbl.gov).

Materials availability
Some plasmids generated in this study have been deposited to Addgene: pW17 (Cat# 158207), pW34 (Cat# 158210), and pW5Y (Cat# 158211). Other plasmids and strains can be requested from the lead contact upon completing a Materials Transfer Agreement.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

ACKNOWLEDGMENTS

The work conducted by the US Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported under contract DE-AC02-05CH11231. We thank Dr. A. Deutschbauer for providing the pKMW2 transposase plasmid and Dr. William W. Metcalf for providing E. coli donor strain BW29427. We thank Dr. Ben Cole for providing Pseudomonas simiae WCS417r strain. We thank Anita Wahler for her professional editing.

AUTHOR CONTRIBUTIONS

Z.Z., Y.Y., and J.F.C. designed the study; Z.Z. performed the experiments; Z.Z. and Y.Y. wrote, reviewed, and approved the manuscript. J.F.C. reviewed and approved the manuscript.

DECLARATION OF INTERESTS

Lawrence Berkeley National Laboratory filed a United States patent application for CRAGE technology (US patent 20190048354). The application lists Y.Y., G.W., Z. Zhao, J.F.C., and D.R. as inventors.

REFERENCES

Flett, F., Mersinias, V., and Smith, C.P. (1997). High efficiency intergeneric conjugal transfer of plasmid DNA from Escherichia coli to methyl DNA-restricting streptomycetes. FEMS Microbiol. Lett. 155, 223–229. https://doi.org/10.1111/j.1574-6968.1997.tb13882.x.

Gilbert, L.A., Larson, M., Morsut, L., Liu, Z., Brar, G., Torres, S., Stern-Ginossar, N., Brandman, O., Whitehead, E., Doudna, J., et al. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442–451. https://doi.org/10.1016/j.cell.2013.06.044.

Ke, J., Robinson, D., Wu, Z.Y., Kuftin, A., Louie, K., Kosina, S., Northen, T., Cheng, J.F., and Yoshikuni, Y. (2021). CRAGE-CRISPR facilitates rapid activation of secondary metabolite biosynthetic gene clusters in bacteria. Cell Chem. Biol. S2451945621003986. https://doi.org/10.1016/j.chembiol.2021.08.009.

Liu, H., Robinson, D.S., Wu, Z.Y., Kuo, R., Yoshikuni, Y., Blaby, I.K., and Cheng, J.F. (2020). Bacterial genome editing by coupling Cre-lox and CRISPR-Cas9 systems. PLoS One 15, e0241867. https://doi.org/10.1371/journal.pone.0241867.

Liu, R., Bassalo, M.C., Zeitoun, R.I., and Gill, R.T. (2015). Genome scale engineering techniques for metabolic engineering. Metab. Eng. 32, 143–154. https://doi.org/10.1016/j.ymben.2015.09.013.

Ochman, H., Gerber, A.S., and Hartl, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. Genetics 120, 621–623. https://doi.org/10.1093/genetics/120.3.621.

Peng, R., Wang, Y., Feng, W., Yue, X., Chen, J., Hu, X., Li, Z., Sheng, D., Zhang, Y., and Li, Y. (2018). CRISPR/dCas9-mediated transcriptional
improvement of the biosynthetic gene cluster for the epothilone production in Myxococcus xanthus. Microb. Cell Factories 17. https://doi.org/10.1186/s12934-018-0867-1.

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. BioTechnol. 1, 784–791. https://doi.org/10.1038/nbt1183-784.

Wang, B., Zhao, Z., Jabusch, L.K., Chiniquy, D.M., Ono, K., Conway, J.M., Zhang, Z., Wang, G., Robinson, D., Cheng, J.F., et al. (2020). CRAGE-duet facilitates modular assembly of biological systems for studying plant–microbe interactions. ACS Synth. Biol. 9, 2610–2615. https://doi.org/10.1021/acssynbio.0c00280.

Wang, G., Zhao, Z., Ke, J., Engel, Y., Shi, Y.M., Robinson, D., Bingol, K., Zhang, Z., Bowen, B., Louie, K., et al. (2019). CRAGE enables rapid activation of biosynthetic gene clusters in undomesticated bacteria. Nat. Microbiol. 4, 2498–2510. https://doi.org/10.1038/s41564-019-0573-8.

Zalatan, J.G., Lee, M., Almeida, R., Gilbert, L., Whitehead, E., La Russa, M., Tsai, J., Weissman, J., Dueber, J., Qi, L., and Lim, W. (2015). Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. Cell 160, 339–350. https://doi.org/10.1016/j.cell.2014.11.052.