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Synthetic Biology to Engineer Bacteriophage Genomes

Ana Rita Costa, Joana Azeredo, and Diana Priscila Pires

Abstract

Recent advances in the synthetic biology field have enabled the development of new molecular biology techniques used to build specialized bacteriophages with new functionalities. Bacteriophages have been engineered toward a wide range of applications, including pathogen control and detection, targeted drug delivery, or even assembly of new materials.

In this chapter, two strategies that have been successfully used to genetically engineer bacteriophage genomes will be addressed: the bacteriophage recombineering of electroporated DNA (BRED) and the yeast-based phage-engineering platform.

Key words Bacteriophage, Bacteriophage engineering, YAC, BRED

1 Introduction

Bacteriophages (or phages) have long been recognized for their major role in the evolution of molecular biology and bacterial genetics [1]. However, only more recently has the scientific community become aware of their extraordinary potential for various biotechnological applications. Bacteriophages are now considered as one of the most promising alternatives to antibiotics in areas from healthcare to food processing, agriculture, and veterinary [2–5]. Besides, bacteriophages have been modified to be used as tools for bacterial detection, as vehicles for targeted drug delivery, and to display specific peptides or proteins on the surface of their capsid (phage display) [6–10].

The ever-expanding collection of bacteriophage genomes deposited in the National Center for Biotechnology Information, either fully or partially sequenced, has revealed that a vast number of genes encoded in bacteriophage genomes have yet to be assigned a function. This suggests that further understanding of their basic biology is required. In this context, the ability to build bacteriophage mutants is pivotal for the assessment of gene/protein
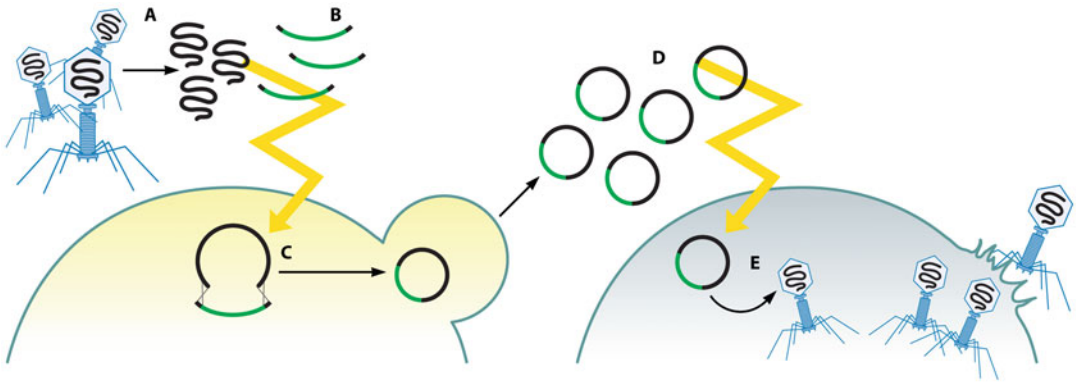
Fig. 1 Bacteriophage recombineering of electroporated DNA. Purified phage DNA (**a**) and dsDNA recombineering substrates (**b**) are co-electroporated into cells (**c**). Recombination between their homologous regions (in orange) (**d**) results in recombinant phage particles (containing DNA fragments in green) (**e**). (Figure reprinted from Pires et al. [11])

function. As a consequence, some recombination-based methods have already been successfully implemented for the purpose of engineering virulent bacteriophages [11, 12].

One of the first strategies reported for the genetic manipulation of phage genomes is the Bacteriophage Recombineering of Electroporated DNA (BRED, Fig. 1). This technique was originally created to generate point mutations, insertions, deletions, and gene replacements in lytic mycobacteriophages [13, 14]. In the BRED method, bacteriophage DNA and the DNA of interest (target substitution, deletion, or insertion) are simultaneously introduced by electroporation into bacterial cells that have been equipped with a recombination system (typically the λ Red or Rac systems), which enhances the frequency of homologous recombination [13]. BRED has also been used to genetically engineer *Escherichia coli* bacteriophages [15, 16], and it has been suggested that with slight modifications to the protocols and appropriate recombineering systems, this approach can be applied to many other bacteriophages targeting different bacterial species. One of the major issues associated with BRED is the screening for mutant phages. However, counterselection techniques can be used to further improve the selection of mutant phages. In recent years, multiple CRISPR-Cas-based methods have been developed for this purpose, in which the wild-type phages are targeted by a programmed CRISPR-Cas system. Both DNA- and RNA-targeting CRISPR-Cas systems have been successfully employed [17–23].

To genetically manipulate bacteriophages without inflicting toxic effects on the host cell, a yeast-based platform for the assembly of bacteriophage genomes (Fig. 2) was developed [24]. In this method, *Saccharomyces cerevisiae* is used as a surrogate for genetic manipulation, which requires a yeast artificial chromosome (YAC).
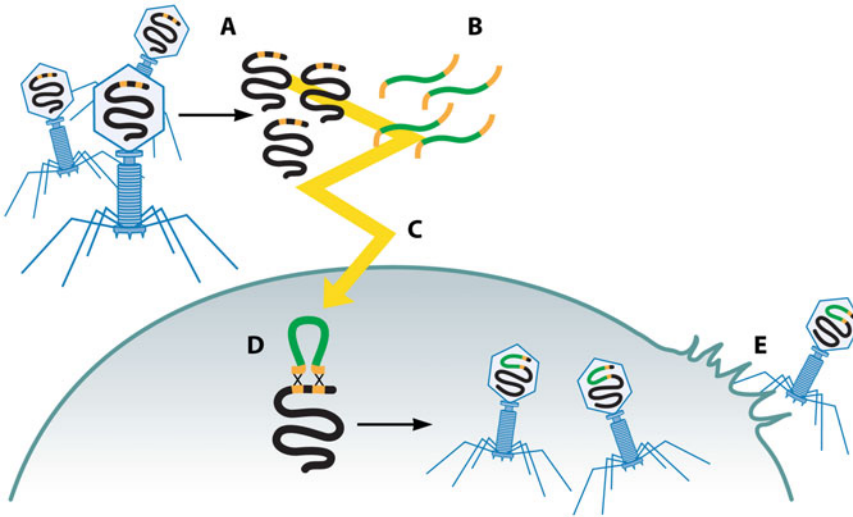
Fig. 2 Yeast-based assembly of phage genomes. Purified phage DNA (**a**) is electroporated into *S. cerevisiae* together with linear YAC molecules with overhangs (in black) homologous to the 5′ and 3′ ends of the linear phage genome (**b**). Recombination in the yeast cell enables genomic subcloning (YAC backbone in green) (**c**), which upon YAC purification and electroporation (**d**) allows the recovery of phage particles (**e**). (Figure reprinted from Pires et al. [11])

The bacteriophage genome needs first to be PCR-amplified in multiple, overlapping large amplicons. To these, any of the desired mutations can be performed, or heterologous DNA can also be added. The first (5′) and last (3′) bacteriophage amplicons and the YAC must share regions of homology, which can be added by polymerase chain reaction (PCR), using primers with shared overhangs. The bacteriophage amplicons are then co-transformed with the linear YAC into *S. cerevisiae*. The native recombination machinery will recognize the regions of homology and assemble the YAC and bacteriophage in the proper order, determined by the regions of homology. This results in a complete bacteriophage genome cloned into a replicative vector (YAC). The construct is then extracted from yeast cells, transformed into the bacterial host cells, and plated to check for bacteriophage plaques. The bacteriophage plaques formed are then picked, amplified, and sequenced to confirm the introduction of the desired mutations. Several bacteriophages targeting different bacterial species, including *E. coli*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae*, have been genetically modified using this method [24–26]. Although the rebooting of the assembled phage genomes is usually done in the host cells through electroporation, it can also be accomplished using L-forms [27, 28] or cell-free methods [29–33]. L-forms are particularly useful for rebooting phages in Gram-positive cell hosts, which are difficult to transform. L-forms are wall-deficient, metabolically active cells that can be transformed with phage genomes,

which are efficiently rebooted and recovered by disruption of the osmotically stabilized L-form cells. Cell-free methods use the native bacterial transcription-translation machinery in a tube and escape the constraints of working with a living cell. These have been successfully used to reboot phages from Gram-negative and Gram-positive hosts.

2 Materials

Prepare all solutions using distilled water. All solutions are sterilized (autoclaved at 121 °C for 15 min) and stored at room temperature unless indicated otherwise. The growth medium used in the procedures described herein is Lysogeny Broth (LB), but other rich media can also be used, according to the requirements of the host bacterium.

2.1 Bacteriophage Recombineering of Electroporated DNA (BRED)

2.1.1 Preparation of Recombineering Cells

1. Electrocompetent cells of the bacterial host (*see* **Note 1**).

2. Plasmid encoding recombineering functions, e.g., pKD46 (*see* **Note 2** and **Note 3**).

3. Sterile electroporation cuvettes (*see* **Note 4**).

4. SOC medium (*see* **Note 5**): prepare according to the manufacturer's instructions.

5. Ampicillin 1000× stock solution at 100 mg/mL (*see* **Note 3**). Sterilize by filtration using a 0.22 μm filter.

6. LBA plates containing 100 μg/mL ampicillin: prepare LBA (LB broth prepared according to the manufacturer's instructions with 1.2–1.5% (wt/vol)) of agar (*see* **Note 6**), and let it cool to about 55 °C. Add ampicillin stock solution to obtain a final concentration of 100 μg/mL. Pour plates under aseptic conditions and let dry. Store at 4 °C.

7. LB broth prepared according to the manufacturer's instructions (*see* **Note 7**).

8. LB containing 100 μg/mL ampicillin (prepare LB broth according to the manufacturer's instructions, let it cool to about 55 °C, and add ampicillin stock solution to obtain a concentration of 100 μg/mL).

9. Sterile spreaders.

10. Sterile 15 mL centrifuge tubes.

11. Plasmid extraction kit, commercially available.

12. Restriction enzyme that cuts pKD46 only once, e.g., BamHI, SacI, or NcoI.

13. Agarose.

14. 1× Tris-acetate-EDTA (TAE) buffer: dilute 50 times the 50× TAE solution (*see* **Note 8**).

15. DNA gel stain, e.g., SYBR Safe.

16. DNA gel loading dye (e.g., 6× concentrated).

17. Molecular weight DNA ladder, e.g., 1-Kb DNA ladder.

18. Sterile glycerol.

19. Sterile 1.5 mL cryogenic vials.

2.1.2 Bred

1. Overnight culture of the recombineering-competent bacterial host cells grown in LB with 100 μg/mL ampicillin.

2. Sterile 250 mL flasks.

3. LB broth prepared according to the manufacturer's instructions (*see* **Note 7**).

4. Sterile 10% (wt/vol) L-arabinose: sterilize the solution using a 0.22 μm filter and store at room temperature.

5. Purified bacteriophage solution (*see* **Note 9**).

6. Sterile 10% (wt/vol) glycerol.

7. Sterile 1.5 mL microcentrifuge tubes.

8. Recombineering DNA substrate (*see* **Note 10**).

9. Sterile electroporation cuvettes (*see* **Note 4**).

10. SOC medium.

11. LB soft agar: LB broth prepared according to the manufacturer's instructions with 0.4–0.7% (wt/vol) of agar (*see* **Note 11**). After autoclaving, store accordingly (*see* **Note 12**).

2.1.3 Recovery and Confirmation of Mutant Bacteriophages

1. Overnight culture of the bacterial host.

2. Sterile 1.5 mL microcentrifuge tubes.

3. LB broth prepared according to the manufacturer's instructions (*see* **Note 7**).

4. Chloroform.

5. Confirmation primer sets (*see* **Note 13**).

6. PCR tubes.

7. Sterile SM buffer: 100 mM NaCl, 8 mM $MgSO_4.7H_2O$, and 50 mM Tris-HCl, pH 7.5.

8. LB soft agar (*see* **Notes 11 and 12**).

9. LBA plates (LB broth prepared according to the manufacturer's instructions with 1.2–1.5% (wt/vol) of agar (*see* **Note 6**).

2.2 Yeast-Based Assembly of Bacteriophage Genomes

2.2.1 Bacteriophage DNA Isolation

1. Bacteriophage lysate (150 mL) filtered through 0.22 μm filters (*see* **Note 14**).
2. Buffer L1: 20 mg/mL of RNase A, 6 mg/mL of DNase I, 0.2 mg/mL of BSA, 10 mM EDTA, 100 mM Tris-HCl, and 300 mM NaCl. Use sterile distilled water. Adjust the pH to 7.5 and store at 4 °C. Do not autoclave.
3. Buffer L2: 30% (wt/vol) of polyethylene glycol (PEG) 6000 and 3 M NaCl. Store at 4 °C.
4. Buffer L3: 100 mM Tris-HCl, 100 mM NaCl, and 25 mM EDTA. Adjust pH to 7.5.
5. Buffer L4: 4% (wt/vol) of sodium dodecyl sulfate (SDS).
6. Buffer L5: 2.55 M potassium acetate. Adjust the pH to 4.8.
7. QIAGEN-tip100 columns.
8. Isopropanol (100% (v/v)).
9. Ethanol 70% (vol/vol).
10. Ethanol 95% (vol/vol).
11. Sterile 50 mL centrifuge tubes.
12. Sterile 15 mL centrifuge tubes.
13. Sterile 1.5 mL microcentrifuge tubes.
14. Sterile ultrapure water.

2.2.2 Preparation of Yeast Competent Cells

1. *Saccharomyces cerevisiae* BY4741, or other.
2. Yeast Extract-Peptone-Dextrose (YPD) Broth: prepare commercially available YPD according to the manufacturer's instructions (*see* **Note 15**).
3. Sterile 50 mL centrifuge tubes.
4. Sterile 1.5 mL microcentrifuge tubes.
5. Sterile 250 mL flasks.
6. Sterile distilled water.

2.2.3 Yeast Transformation

1. Vector: 100–200 ng of linearized YAC obtained via PCR (*see* **Note 16**).
2. Bacteriophage DNA amplicons: molar ratio of 5:1 or 3:1 (insert:vector) of each DNA fragment (*see* **Note 17**).
3. 50% (wt/vol) of PEG 3350.
4. 1 M Lithium acetate (LiAc).
5. Salmon sperm DNA (2 mg/mL), commercially available.
6. Sterile 1.5 mL microcentrifuge tubes.
7. Sterile spreaders.
8. Agar plates prepared with synthetic defined medium (SD) with the appropriate dropout supplement (*see* **Note 18**).

2.2.4 Yeast Colony PCR	1. Yeast colonies growing on appropriate agar plates.

2. Confirmation primer sets: a set of primers to amplify all the connections between adjacent fragments should be used to confirm the correct assembly of the construct.

3. PCR tubes.

4. 0.02 M sodium hydroxide (NaOH).

2.2.5 Plaque Formation Assays

1. LBA plates (LB broth prepared according to the manufacturer's instructions with 1.2–1.5% (wt/vol) of agar (*see* **Note 6**)).

2. Overnight culture of the bacterial host.

3. Electrocompetent cells of the bacterial host (*see* **Note 1**).

4. Sterile electroporation cuvettes (*see* **Note 4**).

5. Sterile Super Optimal broth with Catabolite repression (SOC) medium (*see* **Note 5**): prepare according to the manufacturer's instructions.

6. LB soft agar: LB broth prepared according to the manufacturer's instructions with 0.4–0.7% (wt/vol) of agar (*see* **Notes 11** and **12**).

7. Sterile 15 mL centrifuge tubes.

3   Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1   Bacteriophage Recombineering of Electroporated DNA

This protocol was adapted from Marinelli et al. [13, 14] with some minor modifications. Plasmid pKD46 will be used as an example to provide recombineering functions to the cells.

3.1.1 Preparation of Recombineering Competent Cells

1. Add 100–500 ng (up to 5 μL) of pKD46 to 20–100 μL of bacterial host electrocompetent cells.

2. Carefully transfer the mixture into a chilled 0.1- or 0.2 cm electroporation cuvette and transform the cells via electroporation at appropriate settings (*see* **Note 19**).

3. Add 1 mL of SOC to the electroporated cells immediately after the pulse.

4. Transfer the suspension to a sterile 1.5 mL microcentrifuge tube and incubate for 1–2 h at the appropriate host temperature under agitation (120–150 rpm).

5. Spread 100–200 μL onto prewarmed LBA plates containing ampicillin. Incubate overnight at 30 °C. Cells are cultured at the permissive temperature of 30 °C to maintain the electroporated temperature-sensitive plasmid pKD46.

6. Select a few colonies and grow each in separate sterile 15 mL culture tubes containing 5 mL of LB with ampicillin for a few hours or overnight.

7. Extract plasmid from each culture using a commercial plasmid extraction kit.

8. Digest the extracted DNA using an adequate restriction enzyme following the manufacturer's instruction. Include the initial plasmid DNA prep as a positive control.

9. Prepare a 1% (wt/vol) agarose gel in 1× TAE. Microwave for 1–3 min until the agarose is completely dissolved. Let the agarose solution cool to about 50 °C and add a DNA gel stain (e.g., SYBR Safe). Pour the agarose into a gel tray with the well comb in place. Let the gel sit at room temperature for about 20 min or until solid. Place the agarose gel into the electrophoresis unit and fill with 1× TAE until the gel is covered.

10. Add loading dye to each of the digested samples. Load the samples and a molecular weight DNA ladder into separate lanes and run the gel at 80–120 V until the gel front is approximately 20–25% of the end of the gel. Using a device with the appropriate light source (blue light if SYBR Safe is used), visualize the DNA fragments and confirm the correct size of the plasmid with the positive-control digestion lane (6329 bp).

11. After confirming positive recombineering competent cells, inoculate 5 mL of LB containing ampicillin with a single colony of the positive cells. Grow overnight at 30 °C, 200 rpm.

12. Prepare a cell stock: add 850 μL of the overnight grown culture and 150 μL of sterile 100% (vol/vol) glycerol to cryogenic vials. Mix well and store at −80 °C.

3.1.2 Recombineering of Bacteriophage DNA

1. Inoculate 100 mL of LB containing ampicillin with the overnight grown recombineering competent bacteria in a sterile 250 mL flask. Grow the cells at 30 °C to the early-log phase (corresponding to an optical density at 600 nm of approximately 0.3, measured in a microtiter plate reader).

2. Induce the expression of the recombineering system of pKD46 by adding 1 mL of sterile 10% (wt/vol) L-arabinose (*see* **Note 20**) to the medium and incubate for an additional 30 min.

3. Infect the cells with the purified bacteriophage solution at a multiplicity of infection (MOI) of 1–3 to guarantee that all cells get infected and let the infection occur for the duration of the eclipse time of the bacteriophage growth curve (*see* **Note 21**).

4. Prepare electrocompetent cells according to the bacterial species used.

5. Add 100–500 ng of the recombineering DNA substrate to 20–100 μL of the electrocompetent cells.

6. Pipette the mixture into an electroporation cuvette and transform the cells via electroporation at appropriate settings.

7. Immediately after the pulse, add 1 mL of SOC to the electroporated cells, and transfer to a sterile 1.5 mL microcentrifuge tubes.

8. Incubate for 1–2 h at the appropriate temperature under agitation (120–150 rpm).

9. Mix the cells with approximately 3 mL of soft agar and 100 μL of a fresh host bacteria culture, and pour onto LBA plates.

10. Incubate overnight at the appropriate temperature. Since the plasmid pKD46 is no longer needed, the cells can be incubated at 42 °C to be cured of the plasmid.

11. Check for the presence of bacteriophage plaques.

3.1.3 Recovery and Confirmation of Mutant Bacteriophages

1. Add 100 μL of an overnight culture of the bacterial host to 5 mL of LB. Distribute 100 μL by 1.5 mL microcentrifuge tubes.

2. Pick about 10 bacteriophage plaques and place one in each of the prepared microcentrifuge tubes.

3. Grow for 2 h at the appropriate temperature and under agitation (120–150 rpm).

4. Add 30 μL of chloroform, vortex, and centrifuge at $9000 \times g$ for 15 min. Collect the supernatant into sterile 1.5 mL microcentrifuge tubes.

5. Use 1–2 μL of the collected bacteriophage supernatant to confirm the mutation by PCR using an appropriate confirmation primer set (*see* **Note 22**).

6. Add 100 μL of a host bacterial culture and 100 μL of serial dilutions of the positive bacteriophage mixture (made in SM buffer or LB) to about 3 mL of LB soft agar and pour onto an LBA plate.

7. Grow overnight at the appropriate temperature.

8. Repeat **steps 1–5** to screen the secondary plaques by PCR. This should be performed at least three times to guarantee a purified mutant bacteriophage.

3.2 Yeast-Based Assembly of Bacteriophage Genomes

This protocol was adapted from Ando et al. [24], with some minor modifications.

3.2.1 Bacteriophage DNA Isolation (See Note 23)

1. Add 216 μL of buffer L1 to 150 mL of bacteriophage lysate and incubate at 37 °C for 30 min with gentle shaking (50–90 rpm).

2. Add 30 mL of ice-cold buffer L2 and incubate on ice under agitation (50–90 rpm) for at least 1 h.

3. Transfer the suspension to sterile 50 mL centrifuge tubes.

4. Centrifuge the suspension (10,000× g, 4 °C, 30 min) and discard the supernatant.

5. Resuspend the pellets in a total of 9 mL buffer L3 in a 50 mL centrifuge tube.

6. Add 9 mL of buffer L4 and incubate the tube at 70 °C for 20 min. Cool on ice.

7. Add 9 mL of buffer L5 and mix gently by inverting the tube.

8. Centrifuge the sample (10,000× g, 4 °C, 30 min) and load the supernatant onto the QIAGEN-tip 100 system according to the manufacturer's instructions.

9. Precipitate the eluted DNA by adding 0.7 volumes of isopropanol and centrifuging the samples (10,000× g, 4 °C, 30 min) in 50 mL centrifuge tubes.

10. Wash the pellet with 1 mL of 70% (vol/vol) ethanol and transfer the sample to a clean 1.5 mL microcentrifuge tube.

11. Centrifuge the sample (10,000× g, 4 °C, 5 min).

12. Discard the supernatant and wash the pellet with 1 mL of 95% (vol/vol) ethanol.

13. Centrifuge the sample (10,000× g, 4 °C, 5 min) and discard the supernatant.

14. Invert the tube and air-dry the pellet for a few minutes; do not overdry as this results in loss of recoverable DNA.

15. After being completely air-dried, resuspend the pellet in 100 μL of sterile water and store at −20 °C.

3.2.2 Preparation of Yeast-Competent Cells

1. Grow the yeast in 5 mL of YPD (in 15 mL culture tubes) at 30 °C for 16–24 h under agitation (200 rpm).

2. Transfer the culture into 50 mL of YPD in a 250 mL flask and incubate at 30 °C for 4 h under agitation (200 rpm).

3. Transfer the culture to 50 mL centrifuge tubes and harvest the cells by centrifugation (5000× g, RT, 5 min).

4. Resuspend the pellet in 25 mL of sterile water.

5. Repeat the **steps 3** and **4**: harvest the cells by centrifugation (5000× g, RT, 5 min) and resuspend the cell pellet in 25 mL of sterile water.

6. Harvest the cells by centrifugation (5000× *g*, RT, 5 min) and resuspend the cell pellet in 1 mL of sterile water.

7. Transfer the cellular suspension to 1.5 mL microcentrifuge tubes and centrifuge again (13,000× *g*, RT, 30 s).

8. Discard the supernatant and resuspend the cells in 1 mL of sterile water.

9. Use 100 μL of this cellular suspension for each transformation.

3.2.3 Yeast Transformation

1. Combine all DNA samples (bacteriophage DNA amplicons and linearized YAC amplicon) in a 1.5 mL microcentrifuge tube (up to 34 μL total volume).

2. Mix the DNA samples with a transformation mixture composed of 100 μL of yeast competent cells, 240 μL of 50% (wt/vol) PEG 3350, 36 μL of 1 M LiAc, and 50 μL of 2 mg/mL salmon sperm DNA previously denatured in a boiling water bath for 5 min.

3. Incubate the mixture at 42 °C for 45 min in a water bath.

4. Centrifuge the mixture (13,000× *g*, RT, 30 s) and resuspend the cells in 200 μL of YPD.

5. Incubate for 2–3 h at 30 °C.

6. Spread the cells on the appropriate agar plates (*see* **Note 18**).

7. Incubate the plates at 30 °C for 3 days and check for yeast transformants.

3.2.4 Yeast Colony PCR to Check for the Correct DNA Assembly

1. Add 10 μL of 0.02 M NaOH to PCR tubes.

2. Pick a single colony (transformants) with a clean pipet tip to each PCR tube.

3. Place the tubes in a thermocycler at 99 °C for 10 min.

4. Spin down the cell debris.

5. Use 3 μL of each supernatant as template for each 50 μL PCR reaction (higher supernatant volumes may interfere with the PCR reaction).

3.2.5 Plaque Formation Assays

Before plaque formation assays, extraction of captured bacteriophage genomes (YAC-bacteriophage DNA) from yeast cells needs to be performed using commercially available Yeast Genomic DNA Purification Kits according to the manufacturer's instructions.

1. Prepare electrocompetent cells of the host bacterium.

2. Electroporate 100–500 ng of YAC-bacteriophage DNA into 50–100 μL of electrocompetent bacterial cells (*see* **Note 24**) in a 0.1–0.2 cm gap electroporation cuvette (*see* **Note 4**) and transform the cells via electroporation at the appropriate settings (*see* **Note 19**).

3. Add 1 mL of SOC immediately after the pulse.

4. Transfer to a sterile 15 mL culture tube and incubate for 1–3 h at the appropriate host temperature under agitation (120–150 rpm).

5. Mix 200–500 μL of the suspension with 3 mL of LB soft agar and pour onto a LBA plate.

6. Incubate the plates overnight at the proper growth temperature.

7. Check for bacteriophage plaques (*see* **Note 25**).

4   Notes

1. Commercial electrocompetent cells are available for some hosts and can be used for this procedure. Otherwise, prepare your own electrocompetent cells using an appropriate protocol.

2. Plasmid pKD46 is an ampicillin-resistant and temperature-sensitive plasmid that encodes the lambda Red genes *exo*, *beta*, and *gam*. The product Exo degrades one strand of double-stranded DNA (dsDNA), generating a single-stranded DNA (ssDNA) that is annealed to the target DNA by the DNA-pairing enzyme Beta. Gam prevents the degradation of the dsDNA by inhibiting the *E. coli* RecBCD and SbcD enzymes [34, 35]. Plasmid pKD46 has the recombineering functions under control of the arabinose promoter pBAD and carries a temperature-sensitive origin of replication to be cured from the cells after recombination [36]. It should be incubated at the permissive temperature of 30 °C and cured at 42 °C. Plasmids other than pKD46 are currently available that contain recombineering functions from other bacteriophages and bacteria. However, the existing recombineering systems and plasmids have been optimized for Gram-negative bacteria and may not give optimal results in Gram-positive bacteria.

3. Plasmid pKD46 and other recombineering plasmids typically confer resistance to ampicillin or kanamycin. If using ampicillin/kanamycin-resistant bacteria, one should replace the selection marker as appropriate.

4. According to the bacterial host and cell volume, 0.1- or 0.2 cm gap electroporation cuvettes can be used.

5. SOC is a nutrient-rich bacterial growth medium used for microbiological cultures. It was developed by Douglas Hanahan in 1983 [37] and is an adjusted version of the commonly used LB. The growth of bacteria in SOC results in higher transformation efficiencies. SOC is commercially available, but it may also be prepared as follows: 20 g/L of tryptone,

5 g/L of yeast extract, 0.584 g/L of sodium chloride, 0.186 g/L of potassium chloride, 0.952 g/L of anhydrous magnesium chloride, 2.467 g/L of heptahydrate magnesium sulfate, and 3.603 g/L of glucose.

6. Alternatively, commercially available LBA, which corresponds to LB plus agar, can be used according to the manufacturer's instructions.

7. LB is commercially available, but it may also be prepared as follows: 10 g/L of tryptone, 10 g/L of sodium chloride, and 5 g/L of yeast extract. Adjust the pH to 7.0 with 5 N NaOH.

8. TAE buffer is used both as running buffer and to prepare the agarose gel for electrophoresis. TAE buffer is commonly prepared as a 50× stock solution, which can be prepared as follows: 2 M Tris base, 1 M acetic acid, and 50 mM EDTA. The diluted 1× TAE working solution will contain 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.

9. Use a bacteriophage solution purified with PEG. Add 1 μg/mL DNAse I and RNAse to a bacteriophage lysate and incubate the suspension for 30 min at room temperature. Add 58.4 g/L of NaCl and incubate on ice for 1 h under agitation (50–90 rpm). Centrifuge the samples (9000× g, 4 °C, 10 min), recover supernatant, and add 100 g/L of PEG 8000. Place the samples for 5 h to overnight at 4 °C under agitation (50–90 rpm). Centrifuge the samples (9000× g, 4 °C, 10 min) and discard the supernatant. Invert the tubes for 5 min and resuspend the pellet containing the precipitated bacteriophage particles in SM buffer (6 mL of SM buffer for each 50 mL of the centrifuged sample). Add chloroform in a proportion of 1:4 (vol/vol), vortex briefly, and centrifuge the samples (3500× g, 4 °C, for 10 min). Recover and filter the aqueous phase (upper phase) containing the purified bacteriophage.

10. The recombineering DNA substrate includes regions homologous to the bacteriophage to modify. It has been reported that ≤35-bp homology are enough for recombineering purposes [38]. However, since the length can influence the efficacy of recombination, a minimal 50–100-bp homology on each side of the substrate is recommended to improve the results.

11. LB soft agar is typically prepared with 0.6% (wt/vol) of agar. However, agar percentages ranging from 0.4 to 0.7% (wt/vol) can be used.

12. Soft agar can be stored at 50–60 °C if used within 1–2 days or at 4–21 °C if stored longer. Solid soft agar can be melted using a water bath or a microwave but should be allowed to cool before being mixed with cells.

13. To confirm the mutation, order two 25–30-bp flanking primers, with a melting temperature of at least 60 °C, that anneal upstream and downstream of the deletion, insertion, or replacement locus in the bacteriophage genome. The mutant product must be easily distinguished from the wild type. For insertions and gene replacements, it is also possible to order a primer that anneals within the introduced region to be used with one of the flanking primers. Point mutations may be detected using Mismatch Amplification Mutation Assay (MAMA)-PCR.

14. Bacteriophage lysates can be obtained by infecting 150 mL of exponentially growing cells with the appropriate bacteriophage at a MOI of 0.1–0.01 and incubating the cultures overnight. Centrifuge the samples (9000× *g*, 10 min, 4 °C) and filter through 0.22-μm filters.

15. YPD is commercially available, but it can also be prepared as follows: 10 g/L of yeast extract, 20 g/L of bacteriological peptone, and 20 g/L of dextrose (glucose).

16. The YAC amplicon is amplified by PCR and gel-extracted before yeast transformation.

17. The viral genome can be amplified by PCR so that each adjacent fragment shares a homology of at least 30 bp at their 5′ and 3′ ends. The first and last fragments of the bacteriophage genome are amplified with primers that carry homologous overhangs with the YAC fragment, which is also obtained by PCR. When the yeast transformation is performed using DNA fragments of the bacteriophage genome, other genes of interest can be cloned into the bacteriophage genome: the target gene to be cloned should be amplified by PCR using primers with overhangs homologous to the bacteriophage genome; these homologous regions determine where in the bacteriophage genome the foreign gene will be incorporated; all DNA fragments are then co-transformed and assembled in the yeast along with the YAC DNA.

18. Transformants are selected on synthetic defined medium (SD) dropout according to the YAC being used. For example, when using the pRS415 yeast centromere vector with LEU2 marker (ATCC 87520), transformants are selected on SD leucine dropout (SD-Leu) agar plates (0.67% (wt/vol) of Yeast Nitrogen Base (YNB), 0.069% (wt/vol) of CSM-Leu, 2% (wt/vol) of dextrose, 2% (wt/vol) of agar, yeast culture grade).

19. The settings used for electroporation should be adjusted according to the bacterial host used.

20. The induction of the recombineering functions depends on the plasmid used. For pKD46, L-arabinose is used to drive the

pBAD promoter and, thus, the expression of the recombineering proteins. Other plasmids may require different inductions methods.

21. BRED explores the process of bacteriophage infection to seize the bacteriophage DNA while inside the bacterium, allowing it to be treated as a plasmid during transformation with a DNA substrate. It is thus necessary that bacteriophage infection occurs only for the duration of the eclipse period of the bacteriophage, i.e., the span of time from bacteriophage DNA ejection into the bacterial cytoplasm and to the maturation of the first bacteriophage particle. This requires prior knowledge of the bacteriophage growth parameters, which can be obtained by performing one-step growth curves.

22. The selection process can be facilitated if a marker is added during substrate construction, e.g., a myc epitope or a gene encoding a luminescent or fluorescent protein. In the first case, the mutant bacteriophages may be selected using an appropriate antibody coupled, for example, to magnetic beads. For the second, mutant bacteriophages may be detected by the emission of luminescence or fluorescence light. In some cases, it is possible to take advantage of the phenotypic modifications caused by the mutation itself, e.g., the modification of the lytic spectra of a bacteriophage by mutation of receptor binding proteins.

23. Commercially available kits or alternative protocols for bacteriophage DNA isolation can be used.

24. The concentration of DNA needed to generate bacteriophage plaques is variable and depends on the bacterial host and transformation efficiencies achieved.

25. After electroporation of the YAC-bacteriophage DNA into bacterial host cells, the bacteriophage genes can be transcribed and generate bacteriophage particles, which can be detected after plating. Bacteriophage plaques, if formed, are picked, checked by plaque PCR, and sequenced to verify if the construct is correct.

Acknowledgments

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