Chapter 13

Phage Transduction

Shan Goh

Abstract

Bacteriophages mediate horizontal gene transfer through a mechanism known as transduction. Phage transduction carried out in the laboratory involves a bacterial donor and a recipient, both of which are susceptible to infection by the phage of interest. Phage is propagated in the donor, concentrated, and exposed transiently to recipient at different multiplicity of infection ratios. Transductants are selected for the desired phenotype by culture on selective medium. Here we describe transduction of *ermB* conferring resistance to erythromycin by the *C. difficile* phage ϕC2.

Key words Phage, Transduction, Antibiotic resistance genes

1 Introduction

Transduction refers to phage-mediated transfer of DNA from a donor bacterial cell to a recipient bacterial cell. Since the discovery of transduction in the Salmonella phage, P22 [1], other phages were also shown capable of either generalized or specialized transduction. Generalized transduction occurs when phage transfers random bacterial DNA, usually not associated with phage DNA [2]. Specialized transduction occurs when phage transfers specific pieces of bacterial DNA together with phage DNA [2].

Transduction was used in early molecular investigations to map genes in *Escherichia coli* and *Salmonella* spp. These experiments used very high titers of phage ($\geq 10^9$ PFU/mL) propagated in a donor strain, and phage suspensions were mixed directly with recipient cells on selective agar plates [3]. Modifications of this protocol included mixing of cells in liquid media/buffers before plating, and the inclusion of controls for sterility of phage suspensions and for spontaneous mutations of the recipients [4]. Transduction of antibiotic resistance genes was measured using similar protocols. Otherwise, phages were induced at high titers of $\geq 10^8$ PFU/mL from a donor lysogen, either with mitomycin C or UV irradiation, and used directly for transduction with recipient

Adam P. Roberts and Peter Mullany (eds.), *Clostridium difficile: Methods and Protocols*, Methods in Molecular Biology, vol. 1476, DOI 10.1007/978-1-4939-6361-4_13,

strains [5]. Phage that was not induced at high titers has been assayed for transduction using larger volumes of induced phage [6]. Although this has not been attempted in *C. difficile*, it appears feasible provided only one phage type is induced from the donor strain, and presence of the phage can be validated in some way (i.e., plaque assay or transmission electron microscopy). This may be challenging as many *C. difficile* lysogens contain more than one phage type [7, 8], it is difficult to find *C. difficile* hosts for *C. difficile* phage to plaque on [9], and access to/availability of a transmission electron microscopy is not always easy.

The methods presented here are based on *C. difficile* phage ϕC2 transduction of *ermB* between *C. difficile* isolates [10]. They are similar to early methods used in terms of propagating the phage in donor cells, then mixed with recipient cells in liquid media, and plated onto selective plates. The main changes pertain to concentration of the phage so as to obtain a high titer of $\geq 10^8$ PFU/mL, and maintaining viability of *C. difficile* cells under anaerobic conditions.

2 Materials

2.1 Bacterial Growth and Phage Propagation Media

Anaerobe basal agar (ABA): dehydrated powder (Oxoid Thermo Scientific).

Brain heart infusion agar (BHIA): dehydrated powder (Oxoid Thermo Scientific).

Brain heart infusion broth (BHIB): dehydrated powder (Oxoid Thermo Scientific).

Blood agar: 5% defibrinated horse blood, dehydrated BHIA.

Soft agar (SA): anaerobe basal broth dehydrated powder (Oxoid Thermo Scientific), 0.74% Bacteriological agar no. 1 (Oxoid Thermo Scientific), 0.01 M $CaCl_2$, 0.4 M $MgCl_2 \cdot 6H_2O$.

Petri dishes 90 and 150 mm.

2.2 Broth Microdilution MIC Susceptibility Assays

0.22 μm syringe filters.

Schandlers broth (SB): dehydrated powder (Oxoid Thermo Scientific).

Clindamycin: dissolve clindamycin powder in 70% ethanol to obtain 50 mg/mL concentration, filter sterilize stock through 0.22 μm syringe filter.

Chloramphenicol: dissolve chloramphenicol powder in 95% ethanol to obtain 50 mg/mL concentration.

Erythromycin: dissolve erythromycin powder in 95% ethanol to obtain 50 mg/mL concentration.

Tetracycline: dissolve tetracycline powder in 70 % ethanol to obtain 10 mg/mL concentration, filter sterilize stock through 0.22 μm syringe filter.

96-well plates.

Multichannel pipette.

Multichannel reagent reservoirs.

2.3 Phage Purification

Phage buffer: dissolve the following in water to indicated concentrations 0.15 M NaCl, 10 mM Tris–HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, adjust pH to 7 with HCl and make up to a final volume of 1 L, autoclave.

0.22 μm syringe filters.

5–10 mL syringes.

1 M NaCl: dissolve NaCl in water to 1 M, autoclave.

PEG 8000.

Chloroform.

1 M KCl: dissolve KCl in water to 1 M, autoclave.

2.4 PCR

PCR grade water.

Phusion High-Fidelity PCR Master mix (Thermo Scientific).

Forward primer E5 (specific for *ermB*): CTCAAAACT TTTTAACGAGTG.

Reverse primer E6 (specific for *ermB*): CCTCCCGTTA AATAATAGATA.

Genomic DNA extracted with Qiagen Puregene yeast kit.

2.5 Transduction

BHIA.

BHIS-T (taurocholate) agar: dehydrated BHIA, 5 mg/mL yeast extract, 0.1% taurocholate, 0.1% l-cysteine, dissolve all in water and autoclave.

BHIA + Tet (10 μg/mL): dehydrated BHIA, 1/1000 tetracycline stock solution.

BHIA + Erm (50 μg/mL): dehydrated BHIA, 1/1000 erythromycin stock solution.

Disposable spreaders.

Phage buffer.

BHIB.

3 Methods

3.1 Finding Donors and Recipients of Antibiotic Resistant Genes (ARG) for Phage Transduction

C. difficile isolates that are hosts for plaque formation by phage must be tested for antibiotic susceptibility in order to determine appropriate donors and recipients for transduction of ARGs. Antibiotic susceptibility may be tested in a number of ways using standard guidelines provided by a number of organizations such

as CLSI [11], BSAC [12], and EUCAST [13]. Here, a broth microdilution method following CLSI guidelines [11] is used.

1. Prepare a fresh 48 h culture of *C. difficile* on BA.
2. Pre-reduce SB for overnight (10 mL per *C. difficile* isolate to be tested).
3. For each *C. difficile* isolate, anaerobically subculture five colonies of similar sizes into 10 mL pre-reduced SB and incubate for 18–20 h.
4. Prepare 2× stock solutions of antibiotics in SB (2–256 mg/L). Dispense 50 μl of each antibiotic concentration per well in a 96-well plate, and include a no antibiotic control (i.e., 50 μl of SB) per antibiotic for each *C. difficile* isolate.
5. Dilute 18–20 h *C. difficile* culture in SB to obtain approximately 2×10^6 CFU/mL (*see* **Note 1**).
6. Within 15 min of preparing the standardized bacterial culture, inoculate 50 μl to wells containing antibiotics. For each antibiotic, leave a series of dilutions uninoculated with bacteria and add SB instead.
7. Incubate anaerobically for 46–48 h.
8. Score growth visually and record the minimum antibiotic concentration that prevents growth (MIC).
9. Compare MIC values with known MIC breakpoints from CLSI M11-A7 [11] to determine whether *C. difficile* test isolate is antibiotic resistant (a donor) or sensitive (a recipient).

3.2 PCR of Antibiotic Resistant Donor Isolates

Many mobilizable antibiotic resistant genes in *C. difficile* are sequenced. Presence of a resistance gene conferring an antibiotic resistant phenotype identified above should be confirmed by carrying out a PCR on the donor isolates, and the amplicon should be sequenced. For best results, the PCR template should be genomic DNA (gDNA) extracted from an overnight culture grown in BHIB. We use the Gentra Puregene Yeast/Bacteria kit (Qiagen) for gDNA extraction, and the Phusion High-Fidelity PCR Master Mix with HF Buffer (Life Technologies).

1. Prepare Phusion Master Mix on ice as follows: 1× Phusion Master Mix, 0.5 μM E5 primer, 0.5 μM E6 primer, and 10–50 ng gDNA in a final volume of 25 μl.
2. Mix by vortexing and spin down briefly. Run PCR using the following conditions (*see* **Note 2**): 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 56 °C for 15 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.
3. Analyze PCR products in a 1% agarose gel and an appropriate DNA ladder (e.g., 2-log ladder, New England Biolabs). The expected amplicon is 750 bp.

3.3 Propagate Phage in Donor (Agar Overlay Method)

Phage may be propagated either in liquid medium or on agar plates, and with overnight or log phase host cultures. This depends on phage preferences, which can be determined by methods described elsewhere. For example, ϕC2 propagates equally well in overnight and log phase cultures of CD062 (a recipient strain for transduction) but prefers log phase cultures of CD80 (a donor strain for transduction). ϕC2 does not propagate well in liquid medium, hence the method outlined is for propagation on agar plates in log phase cultures of CD80. Large agar plates (150 mm diameter) are preferable, otherwise standard agar plates (90 mm diameter) will work but yield lower volumes of harvested phage.

1. Inoculate 1–2 *C. difficile* colonies into 10 mL of pre-reduced BHIB and incubate for 18–20 h.
2. If phage prefers log phase cultures for propagation, prepare a log phase culture of the donor by inoculating 500 μl of an overnight culture into 5 mL pre-reduced BHIB. Incubate for 3–4 h, with occasional shaking by hand (*see* **Note 3**).
3. Prewarm labeled ABA plates to 37 °C. Melt SA thoroughly, ensuring there are no lumps and keep molten in a 50 °C water bath (*see* **Note 4**).
4. Inoculate a sterile universal bottle with 1 mL of phage suspension (~10^5 PFU/mL).
5. Remove log phase donor culture (tightly sealed in tube) from the anaerobic workstation.
6. Add 4 mL of donor, followed by 9 mL of SA into the universal bottle containing phage, and immediately overlay onto ABA plate by swirling gently but quickly to cover the entire surface.
7. Immediately transfer the plate into the anaerobic workstation. Lift off the lid slightly to let out trapped oxygen, then replace the lid and incubate overnight (*see* **Note 5**).
8. Include a no-phage control plate, which is a positive control for donor growth.
9. Semi-confluent lysis of bacteria should be obvious when compared to the no-phage control (*see* **Note 6**).
10. Harvest the phage by scrapping the layer of SA containing phage into a tube containing the same volume of phage buffer (i.e., 14 mL per plate).
11. Vortex vigorously to obtain a homogenous suspension.
12. Centrifuge at 11,000 × *g* for 15 min at 4 °C.
13. Filter the supernatant using a syringe and a 0.45 or 0.22 μm syringe filter to obtain a crude phage suspension. Store at 4 °C.

3.4 Semi-purify Crude Phage Suspension

Phage should be concentrated by PEG precipitation if the titer is less than 10^8 PFU/mL. Dialyzed phage suspensions are ideal for use in transduction, but require particle separation through a pre-formed density gradient using an ultracentrifuge, followed by several rounds of dialysis. The use of semi-purified phage suspensions for transduction gives satisfactory results, provided some controls are included.

1. To remove bacterial nucleic acid present in the phage suspension, treat with DNase I (10 μg/mL) and RNase A (10 μg/mL) for 30 min at 37 °C.
2. Add 1 M NaCl and dissolve by swirling.
3. Add 10% w/v PEG 8000 and dissolve by stirring.
4. Place suspension on ice and inside a fridge for overnight.
5. Centrifuge suspension at 5,000 × *g* for 20 min at 4 °C.
6. Remove the supernatant and resuspend the pellet in 1/50–1/100 the original volume with phage buffer and a transfer pipette. Pipette up and down to break up the PEG pellet.
7. Add the same volume of chloroform and vortex to mix well (*see* **Note 7**).
8. Centrifuge at 5,000 × *g* for 20 min at room temperature.
9. Recover the aqueous phase (or supernatant if using KCl, *see* **Note 7**) and determine phage titer by plaque assays.

3.5 Determine Phage Titer

1. Prepare overnight or log phase cultures of an appropriate host strain as described in "Propagate phage in donor (agar overlay method)".
2. Make tenfold serial dilutions of the phage suspension in 1 mL phage buffer.
3. Dispense 100 μl of appropriate serial dilutions into sterile bijou bottles, and include a no phage control bottle. Routine dilutions used are 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.
4. Pre-warm five labeled ABA plates (standard 90 mm size) to 37 °C. Melt SA thoroughly, ensuring there are no lumps and keep molten in a 50 °C water bath.
5. Remove *C. difficile* culture from the anaerobic workstation and add 600 μl to each bijou bottle.
6. Add 1.5 mL of SA and immediately overlay onto ABA plate by swirling gently but quickly to cover the entire surface (*see* **Note 5**).
7. Incubate plates overnight.
8. Select the plate with 30–300 plaques for counting and calculation of phage titer.

3.6 Transduction

Unless otherwise stated, the procedure is carried out in an anaerobic workstation. This protocol is based on ϕC2 transduction between donor CD80 and recipient CD062.

1. Prepare log phase culture of CD062 (3–4 h).
2. Bring materials needed for transduction into anaerobic cabinet. These are: semi-purified ϕC2/80 of a known titer, 1.5 mL microfuge tubes, P1000 pipette, tips, waste bin, four plates of each antibiotic type, two BHIA plates, phage buffer, disposable spreaders, tube rack.
3. Dilute log phase CD062 culture 1/10 (to achieve approx. 10^7 CFU/mL) with pre-reduced BHIB (*see* **Note 8**).
4. Add volumes of phage and bacteria in microfuge tubes to obtain expected MOI of 0.125, 0.25, and 0.5. i.e., 125 µl phage + 1000 µl bacteria for 0.125 MOI, 250 µl phage + 1000 µl bacteria for 0.25 MOI, 250 µl phage + 500 µl bacteria for 0.5 MOI. The actual multiplicity of infection (MOI) should be calculated the following day after viable counts of the recipient are carried out (*see* **Note 9**). Include a negative control tube with 250 µl phage buffer and 1000 µl bacteria.
5. Mix tubes by inverting several times and incubate for 1 h
6. Meanwhile, serially dilute log CD062 used for transduction tenfold, 5×.
7. Spread plate 100 µl of 10^{-4}, 10^{-5} dilutions onto BHIA plates for viable count.
8. Spread plate 100 µl of 10^{-4}, 10^{-5} dilutions onto BHIA + Tet (10 µg/mL) and BHIA + Erm (50 µg/mL) plates to detect spontaneous mutants.
9. Carrying on from **step 6**. Move tubes out of the anaerobic workstation and spin in a microfuge for 1 min, 10,000 × *g* to pellet cells.
10. Transfer tubes back into cabinet, remove S/N and resuspend cells in 1 mL BHIB.
11. Repeat wash (**steps 9** and **10**).
12. Resuspend cells in 200 µl BHIB.
13. Plate 100 µl of each tube containing phage and bacteria onto BHIA + Tet (10 µg/mL) and BHIA + Erm (50 µg/mL) (*see* **Note 10**).
14. Plate 100 µl of phage suspension used for transduction onto BHIS-T plate for sterility (free of contaminating donor spores).
15. Check control plates from **steps 8** and **14**, which should be free of growth.
16. Count number of transductant colonies on antibiotic plates after 48 and 72 h.
17. Calculate actual CFU/mL of CD062 (using viable count plates from **step 7**) used for transduction and calculate actual MOI for each tube (*see* **Note 11**)
18. Transduction frequency was calculated as transductants per input PFU.

3.7 Validation of Transductants

Transductants should be validated by PCR detection of the antibiotic resistance gene, followed by sequencing of the PCR product. Colonies of transductants can be boiled and used for PCR following standard protocols.

1. Lightly touch a colony of erythromycin resistant transductant with a sterile toothpick.
2. Resuspend cells in 10 μl of PCR grade water.
3. Boil cells at 95 °C for 15 min.
4. Meanwhile prepare Phusion Master Mix on ice as follows: 1× Phusion Master Mix, 0.5 μM E5 primer, and 0.5 μM E6 primer in a final volume of 15 μl per reaction.
5. Mix by vortexing and spin down briefly.
6. Add 15 μl of the Phusion Master Mix to each tube of boiled cells to obtain a final volume of 25 μl.
7. Run PCR with conditions shown above for the donor.

4 Notes

1. We usually find a 1/50 dilution to be sufficient, but one should carry out viable counts on BHIA plates to be sure. This can be done by removing 10 μl of the diluted culture into 20 mL SB and then plating 100 μl onto a BHIA plate. After an overnight incubation, 100 colonies are expected from a 2×10^6 CFU/mL culture.
2. The melting temperatures of PCR primers used with Phusion Master Mix should be calculated using a Tm calculator specific for the enzyme: https://www.lifetechnologies.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html.
3. It may be necessary to determine the growth curve of your *C. difficile* isolate under your experimental conditions if cultures are not turbid after 3–4 h of incubation.
4. Repeated melting of solidified SA will lead to caramelizing (darkening) of the agar, reducing performance. It is advisable to prepare SA in small aliquots of 10–50 mL, which can be discarded after it has been melted several times or has darkened.
5. Log phase cultures are extremely sensitive to oxygen and will not produce good lawns if exposed to oxygen for more than 10 min. **Steps 5** and **6** must be carried out within 10 min.
6. Titrations of the phage suspension may be included to aid in visualizing single plaques, which will be difficult to see in a semi-confluent lysis plate.

7. If phage is sensitive to chloroform, add 1 M KCl to PEG suspension instead and dissolve by inverting, then leave on ice for 30 min for phage particles to dissociate from PEG.
8. CD062 4 h culture is approx. 10^8 CFU/mL. This should be tested for each isolate prior to transduction.
9. These MOI ratios are a good starting point but they can be increased or decreased according to needs. MOI > 0.5 tended to lead to low or no recovery of transductants, likely due to lysis of CD062 by ϕC2, as a bacterial cell pellet was not visible after **step 9** of transdcution. This observation was noted in early transduction studies involving *E. coli* transducing phages [4].
10. Volumes of recipient, phage, PB, and BHIB should be scaled up if screening for more than two antibiotic genes.
11. MOI = Plaque forming units (PFU) of phage used for infection / number of cells. For example, if 2×10^7 cells is infected by 0.5 mL of phage with a titer of 107 PFU/mL. The MOI will be $0.5 \times 10^7 / 2 \times 10^7 = 0.25$.

References

1. Zinder ND, Lederberg J (1952) Genetic exchange in Salmonella. J Bacteriol 64:679–699
2. Birge EA (2000) Bacterial and bacteriophage genetics, 4th edn. Springer, New York
3. Eisenstark A (1965) Transduction of *Escherichia coli* genetic material by Phage P22 in *Salmonella typhimurium* × *E. coli* hybrids. Proc Natl Acad Sci U S A 54:1557–1560
4. Mise K (1971) Isolation and characterization of a new generalized transducing bacteriophage different from P1 in *Escherichia coli*. J Virol 7:168–175
5. Minshew BH, Rosenblum ED (1972) Transduction of tetracycline resistance in *Staphylococcus epidermidis*. Antimicrob Agents Chemother 1:508–511
6. Stanton TB, Humphrey SB, Sharma VK, Zuerner RL (2008) Collateral effects of antibiotics: carbadox and metronidazole induce VSH-1 and facilitate gene transfer among *Brachyspira hyodysenteriae* strains. Appl Environ Microbiol 74:2950–2956
7. Nale JY, Shan J, Hickenbotham PT, Fawley WN, Wilcox MH, Clokie MR (2012) Diverse temperate bacteriophage carriage in *Clostridium difficile* 027 strains. PLoS One 7:e37263
8. Hargreaves KR, Colvin HV, Patel KV, Clokie J, Clokie MR (2013) Genetically diverse *Clostridium difficile* strains harbouring abundant prophages in an estuarine environment. Appl Environ Microbiol 80:2644
9. Goh S, Riley TV, Chang BJ (2005) Isolation and characterization of temperate bacteriophages of *Clostridium difficile*. Appl Environ Microbiol 71:1079–1083
10. Goh S, Hussain H, Chang BJ, Emmett W, Riley TV, Mullany P (2013) Phage phiC2 mediates transduction of Tn6215, encoding erythromycin resistance, between *Clostridium difficile* strains. mBio 4:e00840-00813
11. CLSI (2009) Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard, 7th edn. CLSI, Wayne, PA
12. BSAC (2015) BSAC methods for antimicrobial susceptibility testing, version 14, January 2015. BSAC, UK
13. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical M, Infectious D (2003) Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 9:ix–xv