Chapter 12

Induction and Purification of *C. difficile* Phage Tail-Like Particles

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Abstract

Due to the inherent limitations of conventional antibiotics for the treatment of *C. difficile* infection (CDI), there is a growing interest in the development of alternative treatment strategies. Both bacteriophages and R-type bacteriocins, also known as phage tail-like particles (PTLPs), show promise as potential antibacterial alternatives for treating CDI. Similar to bacteriophages, but lacking a viral capsid and genome, PTLPs remain capable of killing target bacteria. Here we describe our experience in the induction and purification of *C. difficile* PTLPs. These methods have been optimized to allow production of concentrated, non-contractile, and non-aggregated samples for both sensitivity testing and structural electron microscopy studies.

Key words *Clostridium difficile*, Phage tail-like particles, Induction, Purification, Microscopy

1 Introduction

The most important risk determinant for the development of CDI beyond exposure to the organism itself is the use of antibiotics, due to their widespread effects which result in both the intended killing of their target organism and the unintended killing of a significant number of other host bacteria. The resultant perturbation to the bacterial component of the gut microbial community not only produces an environment conducive to the development of CDI, but it also promotes recurrences of CDI after initial treatment, which affects as many as 20–50 % of patients following their first episode of this infection [1, 2].

Bacteriophages and phage endolysins both show promise as potential antibacterial alternatives for treating CDI [3, 4]. In addition, many bacteria produce non-propagating PTLPs, often referred to as "defective prophages." Characterized by phage structural components with an absence of a phage capsid and phage genome, PTLPs can retain selective phage-like lytic activity by creating a channel in the wall of their target cell that leads to a rapid dissipation of

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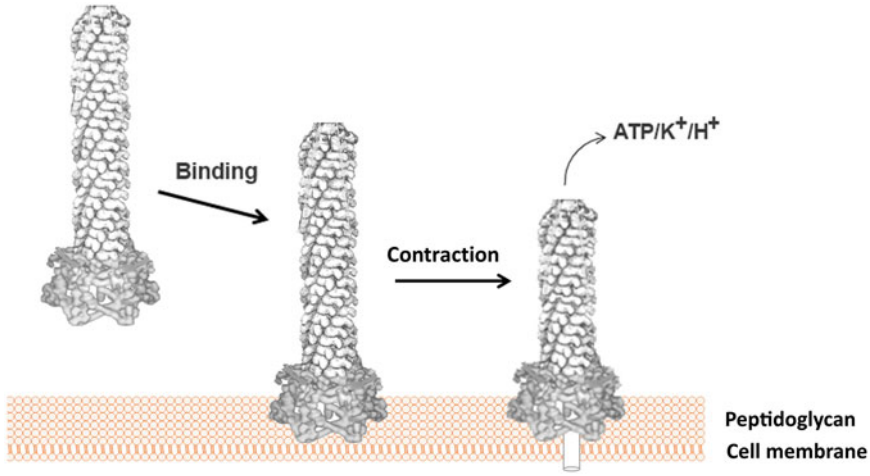

Fig. 1 PTLP bactericidal mechanism. Attachment is mediated by specific binding of the receptor-binding protein to a bacterial surface receptor. Subsequent sheath contraction and penetration of the PTLP core through the bacterial cell wall produce an open channel that results in cell death

membrane proton potential and cell death (Fig. 1). Since PTLPs are a form of bacteriocins, they can be broadly categorized into one of the two categories, either as contractile (R-type) or as non-contractile (F-type) forms. The former family is structurally similar to tails of T-even phages (*Myoviridae*), while the morphology of the latter family resembles the flexible tails of lambda phage (*Siphoviridae*) [5].

Studies have shown that PTLPs can, with a narrow specificity, effectively reduce target bacterial numbers both in vitro and in vivo. This may offer a means for construction of new therapeutic agents with directed action against specific bacteria. *C. difficile* is known to produce PTLPs ,[6, 7] and recently seminal data have demonstrated the efficacy of both natural and modified PTLPs to efficiently kill specific strains of *C. difficile* both in vitro [8, 9] and in vivo [10].

The structure of the contractile mechanisms of PTLPs produced by *P. aeruginosa* has recently been described [11]. Our group aimed to extend similar transmission and cryo-electron microscopy (cryo-EM) structural studies to *C. difficile* PTLPs, and in particular its tail-subunit which has a large, novel, receptor-binding protein. Cryo-EM studies necessitate high-quality samples at a high concentration. The initial PTLP purification work in our laboratory using ultracentrifugation pelleting (90,000 × *g*, 2 h) and fast (14,000 × *g*, 100 k MWCO) ultrafiltration concentration approaches resulted in non-ideal samples for cryo-EM study (Fig. 2). These samples typically contained (1) excessive cellular debris, (2) significant numbers of contracted and tail-sheared particles, and/or (3) extensive clumping of PTLPs due to aggregation of their "flower-like" receptor-binding proteins, especially at higher concentrations. Here we detail an alternative method for preparation of *C. difficile* PTLP samples that largely removes these contaminants and artifacts.

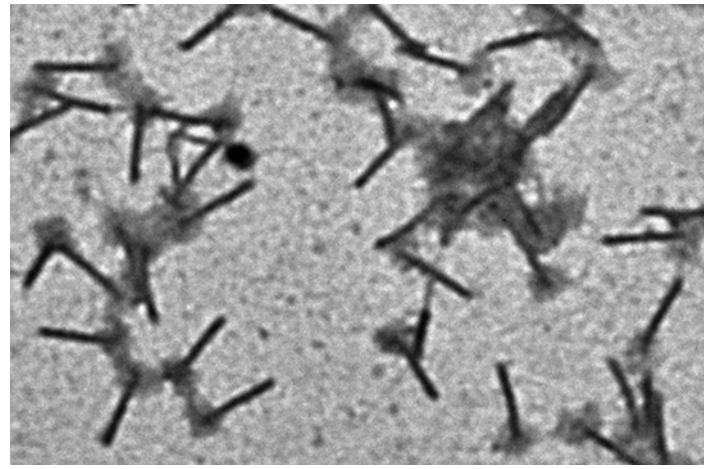

Fig. 2 Aggregation of PTLP receptor-binding proteins following concentration

2 Materials

2.1 C. difficile Growth and PTLP Induction

1. Sheep blood (5%) agar plates (TSA II—BBL 221261, BD Diagnostics, USA).
2. Pre-reduced brain–heart infusion supplemented with l-cysteine (BHIS) broth (AS-872, Anaerobe Systems, USA).
3. 50× Norfloxacin (150 μg/ml solution): Add 10 ml Milli-Q water to 6 mg norfloxacin (N9890 Sigma Aldrich, USA). Acidify with two drops 1 N HCL and vortex vigorously to dissolve. Add 30 ml DPBS and then 0.2 μm filter. Store at 4 °C.
4. Sterile cotton swab applicators.

2.2 PTLP Precipitation

1. SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCL, pH 7.5), 0.2 μm filter.
2. 5 M NaCl (146 g NaCl in 500 ml water).
3. 0.2 μm Surfactant-free cellulose acetate syringe filters (190-9920, Nalgene).
4. Magnetic stirrer and stir bar.
5. 250 ml GSA centrifuge tubes.
6. Balance/scale.
7. PEG-8000/$MgSO_4$ solution: To 500 ml H_2O in a 1 L bottle add 1.8 g $MgSO_4 \cdot 7H_2O$.

   Slowly add 120 g polyethylene glycol 8000 (PEG8000; P5413 Sigma-Aldrich) with stirring. Cap and shake bottle until largely dissolved. Heat bottle in 50 °C water bath for 20 min to completely dissolve. 0.45 μm filter. Store at 4 °C.
8. DNase I (2000 U/ml, M0303L, New England Biolabs, USA).
9. RNase A (20 μg/ml, Life Technologies).

2.3 Electron Microscopy

1. 400-Mesh continuous-carbon copper grids (Electron Microscopy Sciences, PA, USA).
2. Dumont Tweezer L5 (72882-D); Dumont self-closing N4-style forceps (72870-D, Electron Microscopy Sciences, USA).
3. 1.0% Uranyl acetate: Weigh 4 g of uranyl acetate (UA) under the fume hood (wear protection) and add to the 100 ml volumetric flask. Pipette 96 ml of near-boiling CO_2-free double-distilled water into the flask. Stir until uranyl acetate dihydrate crystals are dissolved (several hours). Allow solution cool down to room temperature (pH 4.5). Filter through a Whatman #1 filter into a lightproof bottle and cap tightly. Store at 4 °C.
4. 0.75% Uranyl formate: Weigh 0.0044 g of uranyl formate (UF) powder and mix into 0.587 ml of 12.5 mM NaOH in a 1.5 ml Eppendorf tube. Place capped Eppendorf tube in boiling water bath to solubilize UF. Vortex and repeat boiling until UF is dissolved. Pass through 0.22 μm syringe filter and adjust to pH to 4.5–5.5. Storage at room temperature away from light for up to 1 week.
5. Ultra-pure Milli-Q water.
6. Petri dishes.
7. Whatman #1 filter paper cut into wedges.
8. Timer.

2.4 CsCl Purification

1. 62.5% CsCl solution: 25 g CsCl + 15 ml deionized water, 0.2 μm filtered.
2. TE buffer: 10 mM Tris–HCl at a pH of 8.0, and 1 mM EDTA.
3. Thin-wall ultraclear centrifuge tubes (#344059, Beckman Coulter).
4. 9″ Glass borosilicate Pasteur pipettes.
5. Balance/scale.
6. 21-G disposable needle/syringes.
7. Slide-A-Lyzer Dialysis Cassettes 10,000 MWCO 3 ml capacity (#66380 Thermo Scientific, USA).

2.5 Concentration

1. 300 kDa MWCO spin filters (OD300C33; NanoSep Omega, Pall Corp., USA).
2. SM buffer.
3. 1.7 ml Sterile microfuge tubes with locking caps.

3 Methods

3.1 C. difficile Growth

1. Swab-culture *C. difficile* isolate HMC114 (*see* **Note 1**) under anaerobic conditions using an Anoxomat, or similar anaerobic system, at 37 °C for 48 h onto pre-reduced blood agar plates.

3.2 PTLP Induction

Although PTLPs are spontaneously produced by *C. difficile* in lower numbers, they cannot be propagated in the manner of bacteriophage. Production can raised by 2 orders of magnitude through induction of an SOS response using sub-inhibitory amounts of antibiotics or UV irradiation [12, 13] (*see* **Note 2**).

1. Resuspend bacteria from one half of plate into 3 ml BHIS broth and use to swab (*see* **Note 3**) 12–15 plates pre-swabbed (*see* **Note 4**) with 50× norfloxacin solution.
2. Incubate plates under anaerobic incubation for 16 h.

3.3 PTLP Precipitation

1. Collect induced cells from plate surfaces by flooding each with 5 ml SM buffer, pooling into a single tube, and thoroughly suspend by pipetting and vortexing.
2. Add 1/5 volume of 5 M NaCl.
3. Incubate at room temperature for 30 min with shaking to promote disaggregation of PTLPs from the cells.
4. Pellet cells and large debris at 12,000 × *g* for 15 min at 4 °C.
5. Gently pass supernatant through 0.2 μm surfactant-free cellulose acetate filter.
6. Precipitate PTLPs by adding a half volume of PEG-8000/$MgSO_4$ solution for final concentrations of 8%/5 mM, respectively.
7. Slowly stir on ice for 30 min.
8. Precipitate PTLPs at 4 °C, overnight.
9. Split PTLP sample into 250 ml GSA tubes and balance.
10. Pellet the PTLPs by centrifugation at 3400 × *g* for 40 min at 4 °C (*see* **Note 5**).
11. Decant supernatant, draining excess PEG onto paper towels.
12. Suspended pellet (*see* **Note 6**) in 3 ml SM buffer.
13. Allow PTLPs to solubilize in buffer for 1 h with gentle shaking at room temperature.
14. Digest with DNase I (8 μl; plus 300 μl 10× reaction buffer) and RNase A (10 μl) for 1 h at 37 °C.
15. Extract PEG with repeat equal volumes of $CHCl_3$ (*see* **Note 7**) carefully collecting the upper aqueous phase each time, until the interface is clear.
16. Store samples at 4 °C.

3.4 Cesium Chloride Gradient Purification

1. Prepare CsCl step-gradients in ultracentrifuge tubes using 62.5% CsCl diluted with TE buffer.
2. Use the following ratios (volumes) of CsCl to TE per tube: 1:2 (3 ml), 1:1 (3 ml), 2:1 (3 ml), 1:0 (1.5 ml). Layer sequentially denser CsCl solutions to the bottom of each tube using a Pasteur pipette.

3. Layer the PTLP sample (approximately 3 ml each) on top of step gradients.
4. Adjust and balance final tube volumes to 1 mm below rim using TE buffer.
5. Ultracentrifuge tubes in a Beckman SW41 rotor at 35,000 × *g* at 4 °C for 2 h.
6. Two opaque bands are typically visible near the middle of the tube. Carefully collect the second band from the top (Fig. 3) in a minimal volume by gentle side puncture of tube with a 21-G needle/syringe.
7. Transfer collected PLTP sample to cassette and dialyze against 500× volume SM buffer at 4 °C, with two buffer changes.

3.5 Concentration of PTLP Samples

1. Pre-rinse 300 kDa MWCO filters with three washes of SM buffer (*see* **Note 8**).
2. Gently concentrate PTLPs by centrifuging 1 ml samples at 4000 × *g* at 4 °C through 300 kDa MWCO filters until approximately 100 µl volume remains (*see* **Note 9**).
3. Wash PTLPs with two sequential 1 ml volumes of sterile-filtered SM buffer, again reducing the volume to 100 µl with each 1000 × *g* spin.
4. Transfer concentrated PTLP samples to microfuge tubes. Seal lids with parafilm and store at 4 °C. Samples are ready for sensitivity testing (Fig. 4) and microscopy studies (Fig. 5).

3.6 Electron Microscopy

1. Hold the glow-discharged grid in forceps above filter paper-lined Petri dish and apply a 3 µl sample onto a copper grid (*see* **Note 10**). Allow PTLPs to absorb onto grid surface for

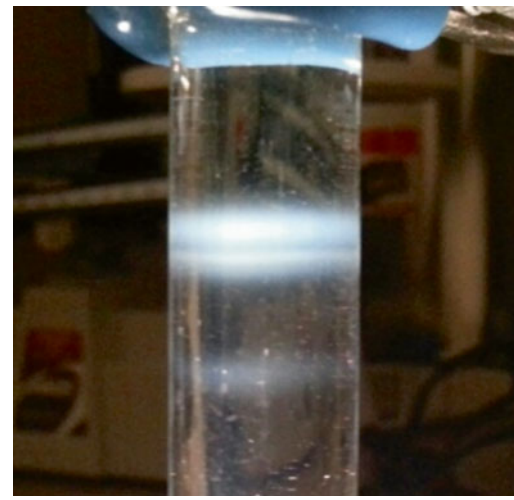

Fig. 3 CsCl gradient bands. The opaque, second band from the top of the gradient (bluish tint) is collected by syringe for dialysis

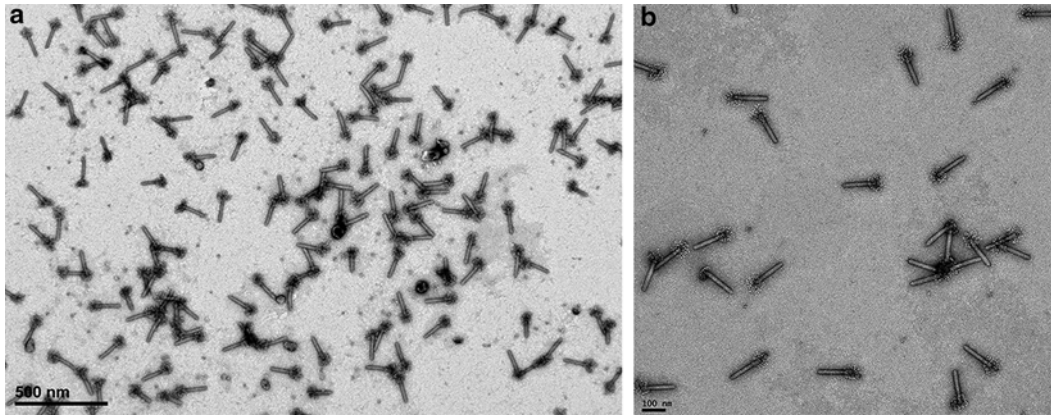

Fig. 4 Transmission electron microscopy of negatively stained phage tail-like particles

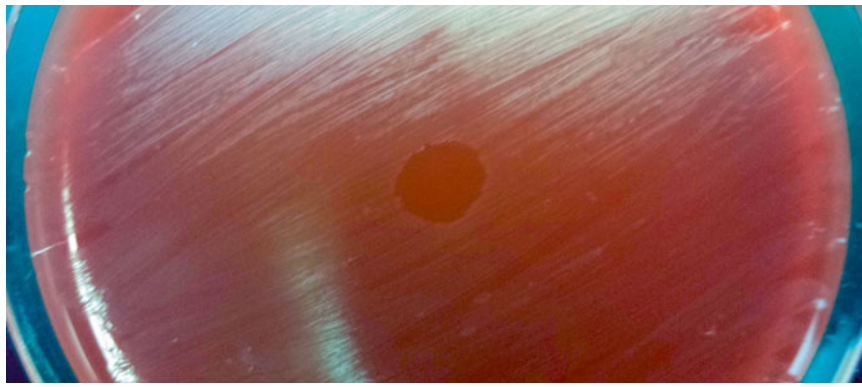

Fig. 5 Plate spot sensitivity testing. Clear zone of cell death is visible following spotting 10 μl PTLP for strain HMC114 onto ribotype 027 *C. difficile* bacterial lawn and anaerobic incubation for 16 h

1–5 min. Draw off excess sample from the edge of the grid with filter paper.

2. For UA stain:
   (a) Wash grid briefly by quickly placing grid surface atop two successive droplets of ultra-pure water.
   (b) Stain grid with 5 μl of 1.0% uranyl acetate for 15 s. Draw off excess stain from the grid edge using filter paper.
3. For UF stain:
   (a) Wash with three drops of ultra-pure water, blotting grid from the side between each drop.
   (b) Stain with 6 μl 0.75% uranyl formate for 10 s. Blot off excess stain from the grid edge using filter paper.
4. Air-dry stained covered grids, and then place in grid storage box.

5. Visualize grids using a JEOL JEM1400 transmission electron microscope at 120 kV.
6. In brief for cryo-EM: Glow discharge holey carbon Quantifoil electron microscopy grids for approximately 1 min. Place 3.5 μl aliquot of sample onto the carbon side of the glow-discharged grid. Blot and vitrify by plunging into a mixture of liquid ethane cooled by liquid nitrogen using a Gatan CP3 plunge-freezing robot operating at 90% humidity.

4 Notes

1. HMC114 is a ribotype 078 *C. difficile* strain isolated from human stool by our laboratory and identified as consistently producing PTLPs in significant quantities following norfloxacin or mitomycin C induction.
2. Our *C. difficile* induction protocols use broth dosages of 6 μg/ml norfloxacin, 3 μg/ml mitomycin C, or 100 J/m^2 UV light (G30T8, 30 W germicidal tube; with an incident dose rate of 20 ergs/mm^2/s for 22 s from 53 cm distance in open Petri dishes filled to depth of 3 mm with log-phage *C. difficile*). Our experience aligns with observations [14] that the induction efficacy for any given agent varies among individual isolates, likely due to sequence differences in regulatory regions flanking the PTLP clusters.
3. In our hands, induction of broth cultures produced disappointing PTLP yields with excessive cellular debris. We found that induction of cells grown on solid-media blood plates swabbed with 50× norfloxacin solution often yielded much better preparations.
4. For PTLP induction, swab each plate *immediately* before swabbing with bacterial culture.
5. When placed into the rotor, mark the outer side of each tube to assist in location of the pellet target area.
6. Suspend the precipitated PTLP pellet by repeat pipetting of buffer gently over the marked side wall of the tube. Using a plate-based induction, we observed very clean, barely visible, U-shaped pellets.
7. Use a glass pipette to transfer chloroform.
8. Rinsing removes trace manufacturing amounts of glycerin from the Omega membranes.
9. Low-speed centrifugation through the filters in a refrigerated centrifuge avoided PTLP receptor aggregation.
10. Glow-discharge carbon-coated and Quantifoil grids for 15 s and 1 min, respectively, using room oxygen at 20 mA in a plasma cleaner.

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