Isolation, Purification, and Characterization of Leptophages

Olivier Schiettekatte, Pascale Bourhy

Isolation, Purification and Characterization of Leptophages

Olivier Schiettekatte[1,2], Pascale Bourhy[1]
[1]Institut Pasteur, Biology of Spirochetes unit, Paris, France.
[2]Université Paris Diderot, Ecole doctorale BioSPC, Paris, France.

Running head: *Leptophage isolation and characterization*

Abstract

To date, only three bacteriophages of leptospires—leptophages—are known. Nonetheless, numerous prophages have been found in the genus, especially in the genomes of pathogenic species. Thus, some laboratories attempt to isolate leptophage particles from environmental samples or following mitomycin C induction of bacterial cultures. Here, we propose multiple procedures to isolate, purify, and characterize bacteriophages, based on protocols used for LE3 and LE4 characterization.

Keywords: Leptophages, Polyethylene glycol precipitation, Cesium chloride purification, TEM characterization, One-step growth kinetics

1. Introduction

Three bacteriophages of leptospires have been isolated as of yet, and are named "leptophages" (1). They have been sequenced (2, 3) and morphologically characterized (1, 3). In the last thirty years, no other leptophage has been isolated, but numerous prophages have been found in the genus, especially in the genomes of pathogenic species (3, 4), suggesting a role in pathogenicity acquisition (5). Thus, some laboratories have attempted to isolate additional phage particles by mitomycin C inductions (6), or from environmental samples, using the method of St Girons et al (1).

We propose, herein, a combination of methods for the extraction of leptophages from environmental samples and induced cultures as well as their isolation, purification, and phenotypic characterization. These protocols are based on classical bacteriophage purification methods—*i.e.* polyethylene glycol precipitation, plating, and TEM observations—considering *Leptospira* spp. specific medium requirements and slow growth.

2. Materials

2.1 Prophage inductions

1. Incubator.
2. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium: dissolve 2.3 g of Difco Leptospira Medium Base EMJH (Becton Dickinson) into 900 mL of distilled water, sterilize by autoclaving, and add 100 mL of Difco Leptospira Enrichment EMJH (Becton Dickinson).
3. 400 mL flasks.
4. Fresh *Leptospira* cultures.
5. Spectrophotometer.
6. Spectrophotometry cuvettes.
7. Mitomycin C: 1 mg/mL solution in sterile water.

2.2 Bacteriophage purification

1. Polyethylene glycol 8000.
2. Sodium chloride.
3. Cesium chloride.
4. TN buffer: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl.
5. Chloroform.
6. Centrifuge.
7. Ultracentrifuge.
8. Beckman Coulter rotor SW41.
9. 13 mL thin-walled polypropylene ultracentrifuge Coulter tubes.
10. Peristaltic pump and flexible plastic tubing.
11. Ultrafiltration cassette (100 kDa).
12. 3.5 kDa cutoff dialysis cassettes.
13. 0.22 µm dead end filters.
14. Syringes (1-5 mL) with 18-gauge needles.

2.3 Double agar overlay plaque lysis assay

1. EMJH medium supplemented with 0.6% and 1.2% agar.
2. Water bath (43 °C).
3. Petri dishes (90×15 mm).
4. Polypropylene Falcon tubes (15 mL).

2.4 Leptophage observation

1. Electron microscope.
2. Microscopy carbon-coated copper grids.
3. Uranyl acetate: 4% solution in water.
4. Glutaraldehyde: 25% solution in water (stock solution) and 2% solution (working solution).
5. PHEM buffer (2×): 60 mM PIPES, 25 mM HEPES, pH 7.0, 10 mM EGTA, 4 mM $MgSO_4$.
6. Filter paper.

2.5 Leptophage kinetics

1. Incubator.
2. EMJH medium.
3. 50 mL flasks.
4. Fresh *Leptospira* cultures.
5. Petroff-Hauser counting chamber.
6. TN buffer.
7. Centrifuge.
8. 0.22 µm dead-end filter.

3. Methods

3.1 Prophage inductions in *Leptospira spp.*

1. Grow the *Leptospira* sp. bacterial host in EMJH flasks at 30 °C (we suggest 400 mL).
2. Measure the optical density of the culture daily at a wavelength of 420 nm.
3. When the optical density of the culture reaches 0.1 (3 to 7 days depending on the species), divide the culture in two identical volumes. Induce one culture with 0.05 µg/mL final concentration mitomycin C.
4. Measure the optical density of the culture daily at a wavelength of 420 nm. If a significant difference (>50%) in optical density is observed between the control and the induced culture, this is potentially due to prophage expression.
5. Bacteriophages or induced particles are stored at 4 °C.

3.2 Bacteriophage extraction and purification

Two protocols exist for leptophage extraction. Tangential flow filtration is available to extract viruses from huge volumes of environmental water (*see* **Note 1**), but, due to high density of EMJH, it is not practical for the extraction of viruses from leptospiral lysates. In this case, phage recovery is facilitated by polyethylene glycol precipitation (*see* **Note 2**). If working with bacterial lysate, the supernatant should be recovered after pelleting cells and other debris at 6,000 × *g* for 45 min.

3.2.1 Tangential flow filtration

1. Filter up to 2 liters of environmental water with a 0.22 µm dead-end filter, recover the filtrate.
2. Concentrate this sample up to 400 times with a 100 kDa tangential flow filtration cassette (*see* **Fig 1** for assembly).
3. Add 10% final volume chloroform to eliminate remnant cell contaminants and mix gently.
4. Centrifuge at 1,700 × *g* for 30 min at 4 °C to pellet chloroform-insoluble material.
5. Filter the supernatant with a 0.22 µm dead-end filter.

3.2.2 Polyethylene glycol precipitation

1. Filter 200 mL of leptospiral lysate (*see* section **3.1**) with a 0.22 µm dead-end filter.
2. Add 8% polyethylene glycol 8000 and 0.3 M NaCl (final concentrations).
3. Mix thoroughly to dissolve polyethylene glycol.
4. Incubate overnight at 4 °C.
5. Centrifuge at 24,000 × *g* for 45 min at 4 °C.
6. Solubilize the pellet in a minimal volume of TN buffer for higher yields, we recommend 5 mL.

3.2.3 Phage amplification

1. Grow *Leptospira* sp. bacterial host in EMJH flasks at 30 °C (we suggest 400 mL).
2. When the optical density of the culture reaches 0.2, divide the culture in two identical volumes. Induce one culture with 5 mL of previously extracted phage (*see* section **3.2.1** or **3.2.2**), and the other culture with the same volume of TN buffer (control culture).
3. Measure the optical density of the culture daily at a wavelength of 420 nm. If a significant difference (>50%) in optical density is observed between the control and the induced culture, this is potentially due to phage amplification.
4. Repeat the PEG extraction and / or isolate phages with double agar overlay plaque lysis assay (*see* section **3.3**).

3.2.4 Cesium chloride gradient ultracentrifugation

A purification step by cesium chloride gradient ultracentrifugation is recommended for particle characterisation (TEM or mass spectrometry) but should be avoided prior to infection of bacteria because cesium chloride is toxic to leptospires.

1. Add 45% final concentration cesium chloride to sample.
2. Ultracentrifuge between 110,000 to 260,000 × g for 24 h at 10 °C in an ultracentrifuge tube.
3. Recover formed bands with a syringe (*see* **Note 3**).
4. Dialyze the sample with TN buffer with a 3.5 kDa cassette.

3.3 Double agar overlay plaque lysis assay

3.3.1 Phage isolation and counting

1. Prepare Petri dishes containing 20 mL EMJH with 1.2% agar and wait for complete solidification.
2. Prepare an equivalent number of Falcon tubes containing 5 mL EMJH with 0.6% agar, stored in 43 °C water bath to prevent solidification before plating.

3. Incubate 500 µL of exponential phase *Leptospira* sp. culture with 10 µL of different dilutions of an extract of leptophages, at 30 °C for 15 min.
4. Mix these co-cultures with 5 mL EMJH with 0.6% agar and pour immediately over 1.2% agar EMJH plates. Work efficiently, as the overlays harden quickly.
5. Incubate at 30 °C and check every 2 days for the appearance of lysis plaques (*see* **Fig 2**). The number of plaques formed is proportional to the bacteriophage concentration in the extract, considering the dilution used.

3.3.2 Fast dropping

1. Prepare Petri dishes containing 20 mL EMJH with 1.2% agar and allow for complete solidification.
2. Add 5 mL EMJH with 0.6% agar as an overlay and wait for complete solidification. Store dishes at 4 °C up to one month.
3. Spread 1 mL of exponential phase *Leptospira sp.* culture on a room temperature overlay medium.
4. Incubate at 30 °C for 2 h.
5. Remove any remaining liquid with a micropipette.
6. Leave the dish open under a sterile biosafety cabinet up to 15 min to allow the small volume of remnant liquid to dry.
7. A – For screening: Drop 10 µL of each purified phage sample to test for clearing.
   B – For raw counting: Drop 10 µL of serial dilutions of the bacteriophage extract to titer.
8. Incubate at 30 °C and check every 2 days for the appearance of lysis plaques. The number of plaques is proportional to the concentration of bacteriophages in the extract, considering the dilution used (*see* **Note 5**).

3.4 Leptophage transmission electron microscopy

3.4.1 Induced particles morphological characterization

1. Deposit a carbon-coated copper grid on a 15 µL drop of phage sample for 15 min.
2. Deposit the grid on a 30 µL drop of 4% uranyl acetate solution for 3 seconds for washing and staining.
3. Deposit the grid on another 30 µL drop of 4% uranyl acetate solution for 8 seconds for staining.
4. Dry the grid with a filter paper; take care to remove any remnant liquid.
5. Observe the grid with a transmission electron microscope at a magnification of 23,000 for screening, and 49,000 for characterization (*see* **Fig 3A**).

3.4.2 Infected bacteria observation

1. Deposit a carbon-coated copper grid on a 15 µL drop of phage-infected *Leptospira* culture (we suggest a MOI of 10) at the desired infection time, for 15 min.

2. Deposit the grid on a 15 µL drop of 2% glutaraldehyde in PHEM buffer for 10 min.
3. Deposit the grid successively on four 30 µL drops of distilled water for washing.
4. Deposit the grid on a 30 µL drop of 4% uranyl acetate solution for 3 seconds for washing and staining.
5. Deposit the grid on another 30 µL drop of 4% uranyl acetate solution for 8 seconds for staining.
6. Dry the grid with a filter paper; take care to remove any remnant liquid.
7. Observe the grid with a transmission electron microscope at a magnification of 23,000 for screening, and 49,000 for characterization (*see* **Fig 3B**).

3.5 Leptophage kinetics

3.5.1 Phage adsorption assays

1. Grow *Leptospira* sp. bacterial host in EMJH flask at 30 °C.
2. When the culture reaches exponential phase, dilute the bacteria to 10^8/mL in EMJH.
3. Infect this culture with a final concentration of 10^4 bacteriophages per mL (MOI of 0.0001), or with the same volume of buffer (control culture).
4. Prepare a control dilution of the bacteriophage at 10^4 bacteriophages per mL (without bacteria).
5. Sample 1 mL of the co-culture every 30 min for up to 6 hours and filter immediately with a 0.22 µm dead-end filter. Sample 1 mL of control culture and control dilution at times 0 and 6 hours and filter with a 0.22 µm dead-end filter.
6. Count the lytic bacteriophages from this sample with the double agar overlay plaque lysis assay (*see* **3.3**).
7. The number of phages found in the non-infected control culture should stay at 0, and the concentration of phages in the control dilution should remain close to 10^4 /mL and corresponds to the percent lost in the filter.
8. In the experimental co-culture, the number of recovered phages should decrease until the first burst. This decrease corresponds to the proportion of phages adsorbed on host cells (and retained in the 0.22 µm filter).

3.5.2 One-step growth kinetics

1. Grow *Leptospira* sp. bacterial host in EMJH flask at 30 °C.
2. When the culture reaches exponential phase, standardize dilute with EMJH to 10^8 bacteria/mL in a final volume of 10 mL.
3. Infect this culture with a final concentration of 10^7 bacteriophages per mL (MOI of 0.1).
4. Incubate at 30 °C for 30 min.
5. Centrifuge the mixture at 6 000 × *g* for 30 min to remove non-adsorbed phages.
6. Resuspend the supernatant in 100 mL EMJH.

7. Each 30 min, sample 1 mL of each culture or control and filter immediately with a 0.22 µm dead-end filter.
8. Count the lytic bacteriophages with the double agar overlay plaque lysis assays.
9. The number of phages should remain approximately 10^6 /mL (considering the dilution at step 6) as long as the latency period lasts. The burst is observed when this number grows, and the percent increase corresponds to the number of phages produced per infected cell (burst size).

4. Notes

1. To date, leptophages have only been isolated from water sources contaminated with *Leptospira* species (1). But soils (7) and organs of infected animals should also be considered.
2. For environmental samples, ultrafiltration can handle larger volumes than PEG purification with higher yields
3. If no band is found, or if bacteriophages are not recovered from the bands, a systematic sampling and TEM observation of different layers of the gradient may be necessary.
4. The double agar overlay plaque lysis assay is required to isolate phage clones, but samples can be pre-tested in a fresh liquid culture as a preliminary assay.
5. Fast dropping is a quick counting or screening method, especially when testing numerous samples, but can only be used to elucidate the order of magnitude of the virus concentration.

5. References

1. Saint Girons, I., D. Margarita, P. Amouriaux, et G. Baranton. 1990. « First isolation of bacteriophages for a spirochaete: Potential genetic tools for Leptospira ». *Research in Microbiology* 141 (9): 1131‑ 38. https://doi.org/10.1016/0923-2508(90)90086-6.
2. Bourhy, P., L. Frangeul, E. Couve, P. Glaser, I. Saint Girons, et M. Picardeau. 2005. « Complete Nucleotide Sequence of the LE1 Prophage from the Spirochete Leptospira Biflexa and Characterization of Its Replication and Partition Functions ». *Journal of Bacteriology* 187 (12): 3931‑ 40. https://doi.org/10.1128/JB.187.12.3931-3940.2005.
3. Schiettekatte, Olivier, Antony T. Vincent, Christian Malosse, Pierre Lechat, Julia Chamot-Rooke, Frédéric J. Veyrier, Mathieu Picardeau, et Pascale Bourhy. 2018. « Characterization of LE3 and LE4, the Only Lytic Phages Known to Infect the Spirochete Leptospira ». *Scientific Reports* 8 (1): 11781. https://doi.org/10.1038/s41598-018-29983-6.
4. Fouts, Derrick E., Michael A. Matthias, Haritha Adhikarla, Ben Adler, Luciane Amorim-Santos, Douglas E. Berg, Dieter Bulach, et al. 2016. « What makes a bacterial species pathogenic?: Comparative genomic analysis of the genus Leptospira ». *PLoS Negl Trop Dis* 10 (2): e0004403.

5. Picardeau, Mathieu. 2017. « Virulence of the zoonotic agent of leptospirosis: still terra incognita? » *Nature Reviews Microbiology*, mars. https://doi.org/10.1038/nrmicro.2017.5.
6. Zhu, Wei-Nan, Li-Li Huang, Ling-Bing Zeng, Xu-Ran Zhuang, Chun-Yan Chen, Yan-Zhuo Wang, Jin-Hong Qin, Yong-Zhang Zhu, et Xiao-Kui Guo. 2014. « Isolation and Characterization of Two Novel Plasmids from Pathogenic Leptospira interrogans Serogroup Canicola Serovar Canicola Strain Gui44 ». *PLoS Neglected Tropical Diseases* 8 (8). https://doi.org/10.1371/journal.pntd.0003103.
7. Thibeaux, Roman, Sophie Geroult, Claire Benezech, Stéphane Chabaud, Marie-Estelle Soupé-Gilbert, Dominique Girault, Emilie Bierque, et Cyrille Goarant. 2017. « Seeking the Environmental Source of Leptospirosis Reveals Durable Bacterial Viability in River Soils ». Édité par Claudia Munoz-Zanzi. *PLOS Neglected Tropical Diseases* 11 (2): e0005414. https://doi.org/10.1371/journal.pntd.0005414.

6. Acknowledgments

This work was part of the PhD thesis of O. S. who received financial support from “Université Paris Diderot” and “Sorbonne Paris Cité”. We thank Soizick Lucas-Staat and Nicolas Dufour for technical assistance during leptophages extractions, Olivier Gorgette for technical assistance during the TEM observations and Robert A. Gaultney for proofreading the manuscript.

Figure legends

Figure 1. Tangential flow filtration assembly.
Figure 2. LE4 leptophage plaque lysis on *Leptospira biflexa* overlay.
Figure 3. LE4 bacteriophages (A) in a lysate and (B) with the bacterial host.